Bundesanstalt für Züchtungsforschung an Kulturpflanzen
BAZ

Züchtungsforschung

Berichte aus der Bundesanstalt für Züchtungsforschung an Kulturpflanzen

International Symposium

75 YEARS

OF PHYTOPATHOLOGICAL AND

RESISTANCE RESEARCH

AT ASCHERSLEBEN

12 - 16 June, 1995
Aschersleben
Germany

PROCEEDINGS

LIST OF AUTHORS

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75 Years of Phytopathological and Resistance Research at Aschersleben

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Grußworte und Retrospektive

Dir.u. Prof. Dr. Th. Kühne
Leiter des Institutes für Resistenzforschung

Eröffnung des wissenschaftlichen Symposiums „75 Jahre Phytopathologie und Resistenzforschung in Aschersleben“

Guten Tag, meine sehr verehrten Damen und Herren,

es ist mir eine große Freude und eine Ehre, Sie im Namen des Organisationskomitees des Internationalen Symposiums „75 Jahre Phytopathologie und Resistenzforschung in Aschersleben“ hier im Saal des Volkshauses willkommen heißen zu dürfen.

Dear Ladies and Gentlemen, dear colleagues,
it is a great pleasure and a honour for me to welcome you as participants of the International symposium „75 Years of Phytopathological and Resistance Research at Aschersleben“.
As you know from our announcements this conference will be held in English but I do ask for your permission that we have chosen German language for the opening ceremony this afternoon.
As you can see from the programme one message of greetings will probably given in English and professor Naumann, the main speaker today, has prepared an English version of his lecture that you should have received in the conference bureau.
So please allow me to switch to German again. Thank you for your understanding.
Meine Damen und Herren, für die Mitarbeiter der Aschersleber Institute ist heute ein großer Tag, und ich freue mich und danke Ihnen, daß Sie so zahlreich der Einladung zu dieser festlichen Eröffnung unseres Symposiums gefolgt sind.
Dieser Nachmittag bietet eine gute Gelegenheit, aus unterschiedlichen Positionen heraus einen Blick zurück auf die nunmehr 75jährige Geschichte des Standortes zu werfen. Morgen vormittag beginnt das wissenschaftliche Programm, das mit 45 Vorträgen und ca. 60 Posterpräsentationen dicht gedrängt sein wird.
Ihnen ist sicherlich nicht entgangen, daß wir uns bei der thematischen Gliederung bewußt an das Profil unseres Forschungsstandortes in der überwiegenden Zeit seiner Existenz gehalten haben. Die vier Themenkomplexe Viruskrankheiten, Bakterienkrankheiten, Pilzkrankheiten sowie tierische Schaderreger und Vektoren sind zugegebenermaßen sehr breit angelegt und damit auch nicht ganz unproblematisch.
Aus Anlaß eines solchen Jubiläums sei uns dies jedoch erlaubt.
Sehr geehrte Gäste,
fast auf den Tag genau vor 35 Jahren fand die letzte Veranstaltung dieser Art hier in Aschersleben statt. Am 23. und 24. Juni 1960 wurde in Würdigung des 40jährigen Bestehens der Forschungsanstalt ebenfall ein wissenschaftliches Kolloquium durchgeführt. 35 Jahre, das sind eine lange Zeit, da darf man - so glaube ich - mit Fug und Recht von einem historischen Zeitabschnitt sprechen.
Mit dem notwendigen Abstand zum Tagesgeschehen erschließt sich diese Periode zunehmend den Analysen und Bewertungen. Hierüber werden wir am heutigen Nachmittag sicher etwas hören, ebenso wie zur Stetigkeit und Veränderlichkeit des Standortes über die siebeneinhalb Jahrzehnte seiner Existenz. Ich meine damit einerseits die bemerkenswerte Kontinuität in den wissenschaftlichen Hauptzielen, die immer auf die gesunde Pflanze ausgerichtet

waren, und andererseits meine ich damit die mehrfach vollzogenen tiefgreifenden Veränderungen in den gesellschaftspolitischen, aber auch in den wissenschaftsorganisatorischen Rahmenbedingungen.
Auch auf diese Aspekte werden Zeitzeugen im folgenden kurz eingehen, und ich bin sicher, daß der Werdegang des Forschungsstandortes Aschersleben mit seinen Höhen und Tiefen sowie das Wirken seiner Mitarbeiter über die Jahre auch den Inhalt einer Reihe von Gesprächen am heutigen Abend mitbestimmen werden.
Zu dem gemütlichen Beisammensein, das im Anschluß an unsere Festveranstaltung stattfindet, darf ich Sie schon jetzt recht herzlich einladen!

Meine sehr verehrten Damen und Herren,
bitte, erlauben Sie mir an dieser Stelle, daß ich Ihnen einige unserer Gäste namentlich vorstelle und mich für Ihr Kommen und die Bereitschaft, ein Grußwort an die Teilnehmer des Symposiums zu richten, herzlich bedanke.
Vom Bundesministerium für Ernährung, Landwirtschaft und Forsten, ohne dessen Unterstützung die Tagung in dieser Form nicht möglich wäre, begrüße ich sehr herzlich (gerade ist er eingetroffen) Herrn Ministerialdirektor
Dr. **Padberg.** Dr. Padberg ist Leiter der Abteilung Agrarische Erzeugung und Veterinärmedizin. Seinem Verantwortungsbereich sind alle Institute der uns seit 1992 so vertrauten Bundesanstalt für Züchtungsforschung an Kulturpflanzen zugeordnet.
Ich begrüße ferner den Leiter der Abteilung Landwirtschaft, Umweltpolitik, Ernährung und Berufsausbildung beim Ministerium für Ernährung, Landwirtschaft und Forsten des Landes Sachsen-Anhalt, Herrn Ministerialdirektor
Dr. **Aeikens**.
Als Präsident des Senates der Bundesforschungsanstalten beim BML und Präsident der Biologischen Bundesanstalt für Land- und Forstwirtschaft, eine Einrichtung, mit der uns nicht nur die gemeinsamen Wurzeln, sondern eine Vielzahl sehr nützlicher wissenschaftlicher Kontakte über die Jahrzehnte verbindet, begrüße ich herzlich Herrn Prof. Dr. **Klingauf**. Das Grußwort wird in Abstimmung mit Herrn Klingauf Herr Prof. Dr. **Casper** übernehmen, der dem Senat der Bundesforschungsanstalten in der Wendezeit vorstand und lange Jahre das BBA-Institut für Biochemie und Pflanzenvirologie in Braunschweig leitete.
Ein Forschungsstandort, meine Damen und Herren, mag er auch zum Geschäftsbereich eines überregionalen Ministeriums gehören, ist natürlich in eine regionale und eine kommunale Struktur eingebettet und hat dort seinen bestimmten Platz und seine Bedeutung. Es ist deshalb für mich sehr angenehm, daß ich die obersten Repräsentanten unseres Landkreises und unserer Kreisstadt, hier im Volkshaus begrüßen darf. Herzlich willkommen, Herr Landrat **Leimbach** und Herr Oberbürgermeister **Michelmann**!
Weiterhin freue ich mich auch, einen unserer Herren Bundestagsabgeordneten, Herrn Dr. **Brecht** begrüßen zu dürfen und darf Ihnen in diesem Zusammenhang zur Kenntnis geben, daß Herr Krziskewitz, ebenfalls Mitglied des Deutschen Bundestages auch gern an dieser Veranstaltung teilgenommen hätte, aber auf Grund einer Delegationsreise des Bundestages leider verhindert ist.
Last but not least, möchte ich aus der großen Zahl der Gäste und Teilnehmer noch die ehemaligen wissenschaftlichen Mitarbeiter des Standortes hier namentlich oder als Gruppe willkommen heißen. Einigen war ich noch Kollege, andere haben die Geschicke des Institutes schon zu einer Zeit bestimmt als ich noch zur Schule ging.
Zwei von Ihnen, Herr Prof. **Shukla** und Herr Prof. **Hoffmann** werden heute Nachmittag stellvertretend auch für andere zu Worte kommen. Ihnen und allen anderen Gästen, die ich namentlich in der Kürze der Zeit nicht nennen konnte, gilt noch einmal mein Dank für ihr Kommen.
Abschließend sei mir, gewissermaßen außer der Reihe, noch ein Willkommensgruß gestattet.

Die Mitglieder des wissenschaftlichen Vorbereitungskomitees für dieses Symposium, in deren Namen ich spreche, habe ich nicht gesondert begrüßt. Eine Ausnahme möchte ich dennoch machen: Es ist Herr Prof. Dr. **Alleweldt**, der erste Leiter unserer Bundesanstalt für Züchtungsforschung an Kulturpflanzen. Herzlich willkommen!
Unsere Jubiläumstagung „75 Jahre Phytopathologie und Resistenzforschung in Aschersleben" ist hiermit eröffnet, und ich wünsche allen Teilnehmern einen angenehmen Aufenthalt hier in dieser mehr als 1200jährigen Stadt, interessante und informationsreiche Tage mit den Fachkollegen und viele zündende Ideen für die weitere Arbeit zum Wohle der Landwirtschaft in unseren Ländern.

Vielen Dank für Ihre Aufmerksamkeit!

MinDir Dr. Padberg
Bundesministerium für Ernährung, Landwirtschaft und Forsten
Leiter der Abteilung Agrarische Erzeugung, Veterinärwesen

Grußwort
anläßlich der Festveranstaltung zur Eröffnung des internationalen Symposiums
„75 Jahre phytopathologische Forschung in Aschersleben" am 12. Juni 1995

Sehr geehrter Herr Professor Kühne,
meine sehr verehrten Damen und Herren,

Herr Bundesminister Borchert hat mich gebeten, Ihnen seine herzlichen Glückwünsche und Grüße zum 75jährigen Bestehen der phytopathologischen Forschung in Aschersleben zu überbringen. Er wünscht den Forschungseinrichtungen und den Wissenschaftlern in Aschersleben alles Gute zu diesem Festtag und ein erfolgreiches Gelingen des Symposiums. Darüber hinaus wünscht er für die Zukunft eine gute Hand bei der Gestaltung der Forschungsvorhaben und der Erarbeitung von zukunftsorientierten Ergebnissen, die die deutsche Landwirtschaft im Hinblick auf eine umweltgerechte und an den Wünschen der Verbraucher orientierte Produktion weiter voranbringen. Diesen Wünschen schließe ich mich als der für die agrarische Erzeugung im Bundesministerium zuständige Abteilungsleiter gerne an. Die zurückliegenden 75 Jahre sind eine lange und an Ereignissen reiche Zeitspanne. Am 1. April 1920 wurde hier in Aschersleben - in dieser Region bedeutenden Acker- und Pflanzenbaus - eine Zweigstelle der Biologischen Reichsanstalt eingerichtet.
Die Aufgabenstellung war damals so aktuell wie sie es heute unter anderen Umständen noch ist: Schäden und Verluste an Kulturpflanzen vermeiden, indem man ihre Ursachen erforscht und zielgerichtete Maßnahmen einer frühzeitigen Vorbeuge und gegebenenfalls Bekämpfung entwickelt. Diese Konzepte des Pflanzenschutzes sind für die land- und forstwirtschaftliche Praxis anwendbar zu gestalten, um hohe und qualitativ wertvolle Pflanzenbestände und Ernten zu sichern. Als ein ebenso wichtiges Ziel bei der Entwicklung und Anwendung von Pflanzenschutzmaßnahmen ist der Schutz der natürlichen Lebensgrundlagen, also Boden, Wasser, Luft und Artenreichtum an Pflanzen und Tieren, hinzugetreten. Unsere Landwirtschaft braucht einen wirkungsvollen Pflanzenschutz, in dem Fruchtfolge und Bodenbearbeitung, standortgerechte Kulturarten- und Sortenwahl, biologische und nicht zuletzt chemische Pflanzenschutzmittel zur Anwendung kommen.

Ich will hier nicht im einzelnen auf die Arbeiten und Ergebnisse der phytopathologischen Forschung in Aschersleben in den vergangenen 75 Jahren eingehen. Das wird nachher aus berufenem Munde durch Herrn Professor Naumann geschehen. Aber ich will doch den Dank und die Anerkennung des Bundesministeriums für Ernährung, Landwirtschaft und Forsten zum Ausdruck bringen.

Bald ist es fünf Jahre her, daß die Grenzen zwischen beiden deutschen Staaten gefallen sind. Wenn auch noch nicht alle Schwierigkeiten ausgeräumt sind, die mit der Vereinigung zweier so unterschiedlicher Systeme zusammenhängen, so können wir uns alle doch glücklich schätzen, daß die Geschichte diesen Weg eingeschlagen hat. In dem vereinigten Deutschland entwickelte sich eine neue Forschungslandschaft. Nach Begutachtung durch den Wissenschaftsrat wurde insbesondere im Osten Deutschlands eine neue, an die Erfordernisse der Zukunft

angepaßte Forschungslandschaft aufgebaut. Auch Aschersleben konnte dem Wissenschaftsrat durch seine hohen Leistungen beweisen, daß dieser Standort für die zukünftige Forschung in Deutschland unverzichtbar ist.

Die Bundesanstalt für Züchtungsforschung an Kulturpflanzen - kurz BAZ -, zu der Aschersleben gehört, kann noch nicht auf eine lange Tradition zurückblicken, schließlich wurde sie erst 1992 gegründet. Ihre Forschungsstandorte besitzen jedoch eine lange Tradition und hohen nationalen und internationalen Bekanntheitsgrad. In Aschersleben wird schon seit 1920 Forschung zum Pflanzenschutz betrieben. Bereits damals wurden Grundlagen für den heutigen „integrierten Pflanzenschutz" geschaffen. Das zeigt der 1934 veröffentlichte Satz von HANS BREMER „Der billigste Weg, Schaden von Pflanzenkrankheiten zu verhüten, ist die Verwendung von Sorten, die gegen Krankheiten widerstandsfähig sind". Dieser Satz ist heute wie damals Grundlage für die phytopathologische Forschung in Aschersleben. Dabei fügt es sich besonders glücklich, daß von hier aus ein fruchtbarer wissenschaftlicher Dialog mit dem benachbarten Institut für Pflanzengenetik und Kulturpflanzenforschung in Gatersleben geführt werden kann.

Pflanzenschutz soll dem Konzept der „gesunden Pflanze" folgen. Die gegen Krankheiten, Schädlinge und abiotische Einflüsse widerstandsfähige Pflanze ist nach wie vor eine geeignete Grundlage für einen effektiven, integrierten Pflanzenschutz. Derjenige, der nach den Grundsätzen des integrierten Pflanzenschutzes produziert- also die Resistenz von Pflanzen in sein Pflanzenschutzkonzept einbezieht -, wählt einen umweltverträglichen Weg, qualitativ hochwertige Nahrungsmittel oder industriell nutzbare Rohstoffe zu angemessenen Preisen zu produzieren.

Eine gegenüber Schadorganismen widerstandsfähige Pflanze kommt mit weniger Chemie aus. Das heißt jedoch noch nicht, daß der Landwirt ganz auf chemische Pflanzenschutzmittel verzichten kann, wenn er resistente Sorten zur Verfügung hat. Viele Schadorganismen sind trotz aller nichtchemischen Verfahren nach wie vor nur mit Hilfe chemischer Pflanzenschutzmittel unter der jeweiligen Schadensschwelle zu halten.

Die BAZ mit ihrem Hauptsitz in Quedlinburg, und einer Reihe von Außenstandorten, zu denen auch Aschersleben gehört, ist nach der Forschungsanstalt für Landwirtschaft und der Biologischen Bundesanstalt für Land- und Forstwirtschaft die drittgrößte der 10 Ressortforschungseinrichtungen des Bundesministeriums für Ernährung, Landwirtschaft und Forsten. Sie erkennen daraus, daß der Züchtung damit in Deutschland ein besonderer Stellenwert eingeräumt wird. Ich sage dies aus dem Bewußtsein heraus, daß die Zwänge zur Einsparung von Mitteln im Bundeshaushalt auch an diesem Standort nicht vorbeigehen werden.

Wir werden in den nächsten Jahren noch enger zusammenrücken müssen, um noch zielgerichteter den Notwendigkeiten der Praxis Rechnung tragen zu können. Dazu gehört auch, daß im Rahmen des eigenen Verantwortungsbereiches gezielt Drittmittel eingeworben werden, um bestimmte Themen vertiefen zu können. Resistenzforschung und Resistenzzüchtung bleiben dabei wichtige Schwerpunkte in der Ressortforschung des Bundesministeriums für Ernährung, Landwirtschaft und Forsten.

Die Aufgaben der Züchtungsforschung sind breit gefächert und an den Ansprüchen von Wirtschaft, Verbrauchern und Umwelt orientiert. Einige dieser Aufgaben seien schlagwortartig genannt:

- Erforschung der Toleranz- und Resistenzursachen gegen Schadorganismen und abiotische Streßfaktoren
- Evaluierung pflanzengenetischer Ressourcen
- Entwicklung von neuen Zuchtstrategien und Züchtungsmethoden zur Verbesserung und Beschleunigung der Pflanzenzüchtung
- Weiterentwicklung und Anwendung biotechnologischer und gentechnischer Methoden
- Entwicklung von Methoden zur Züchtung von low-input-Sorten
- Evaluierung und Entwicklung von Pflanzen zur Nutzung als industrielle Rohstoffe.

Große wissenschaftliche Herausforderungen kommen auf uns sicher in den Bereichen Biotechnologie und Gentechnik zu. Das bedeutet aber nicht, daß Fragen der klassischen Züchtung nicht mehr von Bedeutung sind. Im Gegenteil, erst die sinnvolle Kombination der verschiedenen Techniken wird zur Lösung der anstehenden Probleme führen.

Das Symposium greift eine Reihe schwieriger Fragen der Phytopathologie durch fundierte wissenschaftliche Beiträge auf. Beim Studium der Kurzfassungen hat mich besonders gefreut, daß es sich offensichtlich lohnt, neben wichtigen Themen bei großen Kulturen auch Projekte an kleinen Kulturen zu bearbeiten. Gezielte Resistenzzüchtung muß in Zukunft zunehmend zur Schließung von Lückenindikationen beitragen. Unter Lückenindikationen versteht man fehlende Pflanzenschutzmittel oder Pflanzenschutzverfahren für bestimmte Anwendungsgebiete z. B. bei Obst, Gemüse oder Heil- und Gewürzpflanzen. Daß Sie sich auch dieser Problematik annehmen, stimmt mich im Hinblick auf praxisreife Lösungen in diesem Bereich für die Zukunft hoffnungsvoll.

Es erfüllt mich als verantwortlichem Abteilungsleiter im Bundesministerium für Ernährung, Landwirtschaft und Forsten auch mit gewissem Stolz, daß es gelungen ist, so viele Wissenschaftler aus mehr als 25 Ländern und fünf Kontinenten hier in Aschersleben zu einem mehrtägigen Symposium zusammenzurufen. Diese Beteiligung zeigt, welch guten Ruf dieser renommierte, traditionsreiche Forschungsstandort in einer neuen Bundesanstalt genießt.

Ihnen, meine sehr verehrten Gäste aus dem Ausland, sage ich ein besonders herzliches Willkommen. Zugleich hoffe ich, daß Sie hier einen angenehmen Aufenthalt haben.
Dem Symposium wünsche ich einen guten Verlauf und hoffe, daß Sie interessante Vorträge hören werden, daß Sie aber auch Zeit finden für persönliche Gespräche, um alte Kontakte vertiefen und neue Kontakte knüpfen zu können.

MinDir Dr. E. Aeikens
Ministerium für Ernährung, Landwirtschaft und Forsten Magdeburg
Leiter der Abteilung Landwirtschaft, Marktpolitik, Ernährung, Berufsausbildung

Sehr geehrter Herr Prof. Kühne,
meine sehr verehrten Damen und Herren!

Hiermit möchte ich Ihnen im Namen des Ministers für Ernährung, Landwirtschaft und Forsten, Herrn Dr. Rehhahn, herzliche Grüße anläßlich des 75jährigen Jubiläums der Forschungseinrichtung in Aschersleben überbringen.

Die Landesregierung ist dankbar, daß mit der Einrichtung der Bundesanstalt für Züchtungsforschung an Kulturpflanzen am 1. Januar 1992 mit Hauptsitz in Quedlinburg und weiteren Standorten, wie hier in Aschersleben, an eine langjährige, erfolgreiche Tradition der Agrarforschung in Deutschland angeknüpft werden konnte. Es gelang auf diese Weise, wesentliche Bestandteile der Agrarforschung in der ehemaligen DDR in die Gesamtheit der deutschen landwirtschaftlichen Forschungsinstitutionen zu integrieren und die Basis der pflanzlichen Forschung entscheidend zu erweitern. Der weit über die Grenzen hinaus bekannte Forschungsstandort Aschersleben war von Anfang an durch eine gelungene Kombination von gezielter Grundlagenforschung und praxisnaher Anwendungsforschung gekennzeichnet.

Jahrzehntelange Arbeiten auf dem Gebiet der Resistenzforschung haben erheblich zur Realisierung des Konzeptes eines integrierten Pflanzenschutzes und damit zur Verbesserung der Umweltverträglichkeit pflanzlicher Erzeugung beigetragen.

Eine Vielzahl von Methoden und Verfahren, die heute zum festen Bestandteil der täglichen Arbeit im amtlichen Pflanzenschutzdienst oder in der Züchtungsforschung geworden sind, wurden durch die hiesigen Forschungseinrichtungen entwickelt.

Danken möchte ich an dieser Stelle für die gute Zusammenarbeit zwischen den hier in Aschersleben angesiedelten Instituten der Bundesanstalt und der Agrarverwaltung Sachsen-Anhalts. So haben sie z. B. wertvolle Unterstützung bei der Einarbeitung von technischen Mitarbeitern des amtlichen Pflanzenschutzdienstes in moderne Diagnoseverfahren geleistet.

Eng verbunden mit der phytopathologischen Forschung in Aschersleben sind auch die langjährigen Arbeiten zum Feuerbrand bei Obstgehölzen. In abgestimmter Gemeinschaftsarbeit mit dem amtlichen Pflanzenschutzdienst wird gegenwärtig versucht, Warndienst und Prognose noch sicherer zu gestalten, um Schäden durch diesen gefährlichen Krankheitserreger besser als bisher zu vermeiden.

Ein anderes Beispiel: Der Aufbau virusfreier Reiser-Mutterbestände durch das Aschersleben Institut von 1956 bis 1991 versetzt das Land Sachsen-Anhalt heute in die Lage, den Baumschulen des Landes und darüber hinaus weiteren Interessenten jährlich von 166 Obstsorten Veredlungsreiser in hoher Qualität aus dem Reiser-Muttergarten Magdeburg zur Verfügung zu stellen.

Meine Damen und Herren, diese Beispiele zeigen nur einen kleinen, jedoch für Sachsen-Anhalt wichtigen Ausschnitt der vielfältigen Aufgaben und Aktivitäten Ihrer Institute. Die Möglichkeiten zur Züchtung resistenter Pflanzensorten sind bei weitem noch nicht ausgeschöpft. Die Nutzbarmachung vorhandener

Resistenzpotentiale in Pflanzen setzt die Intensivierung der Forschung und die Weiterentwicklung bereits vorhandener Methoden, insbesondere auf den Gebieten der Biotechnologie und Gentechnik, voraus.

Die Bedingungen zur Bewältigung dieser Aufgaben sind gerade in Sachsen-Anhalt auf Grund der räumlichen Nähe von universitärer Forschung. Grundlagen- und Ressortforschung in Halle, Gatersleben und Quedlinburg/Aschersleben besonders günstig.

Ich bin davon überzeugt, daß die hervorragend ausgebildeten und motivierten Mitarbeiter in Aschersleben die Herausforderung auf den Gebieten der Phytopathologie und der Züchtungsforschung mit Erfolg bewältigen und somit die große Tradition des Standortes Aschersleben in gebührender Weise fortsetzen werden.

Die Teilnehmerliste Ihres Symposiums ist Indikator für die internationale Akzeptanz Ihrer Einrichtung.

Den Gästen ein herzliches Willkommen in Sachsen-Anhalt, in dem Land mit den besten Böden in der Mitte Deutschlands.

Wir wünschen Ihnen einen angenehmen Aufenthalt bei uns.

Dem Symposium möge ein erfolgreicher Verlauf beschieden werden!

Der Aschersleben Agrarforschung viel Erfolg und damit weiterhin nützliche Ergebnisse für die Landwirtschaft!

Danke!

Herr Th. Leimbach
Landrat des Landkreises Aschersleben-Staßurt

Sehr verehrter Prof. Dr. Kühne,
meine Damen und Herren,

die Teilnehmer am wissenschaftlichen Symposium aus Anlaß der 75jährigen Geschichte der Züchtungsforschung an Kulturpflanzen, insbesondere der Resistenzforschung in Aschersleben, darf ich im Landkreis Aschersleben-Staßfurt auf das herzlichste willkommen heißen.
Ihre zahlreiche Teilnahme am Symposium darf ich als ein Indiz dafür werten, welche Bedeutung Sie dieser wissenschaftlichen Tagung beimessen.
Im Vorfeld des heute beginnenden Meinungs- und Erfahrungsaustausches auf den von Ihnen vertretenen speziellen Forschungsgebieten habe ich mich über die wechselvolle, jedoch gleichzeitig stets kontinuierliche intensive Forschungstätigkeit am Institut Aschersleben informieren können.
Dabei ist mir aufgefallen, daß in 75 Jahren wissenschaftlicher Tätigkeit in Aschersleben, neben den vielfältigen wissenschaftlichen Ergebnissen, die Identifikation der Wissenschaftler und Mitarbeiter mit **„Ihrem Institut“** besonders deutlich hervortritt.
Die Bürger selbst - und dies kann ich mit Fug und Recht behaupten - sprechen kurz und bündig vom „Institut“ , und jeder weiß, was damit gemeint ist. Dies mag u.a. auch der Grund dafür gewesen sein, daß breite Teile der Bevölkerung nach der politischen Wende den Fortbestand des Ascherslebener Institutes mit besonderer Aufmerksamkeit und Freude verfolgt haben.
Dies ist Vergangenheit, und ich gehe angesichts des heute beginnenden wissenschaftlichen Symposiums davon aus, daß inzwischen die Institute in Aschersleben einen unverzichtbaren Platz als Forschungseinrichtungen der Bundesanstalt für Züchtungsforschung an Kulturpflanzen einnehmen. Dies gibt mir Gelegenheit, allen wissenschaftlich Tätigen am Institut eine erfolgreiche Arbeit zu wünschen.
Dem heute beginnenden Symposium darf ich einen guten Verlauf wünschen. Dem wissenschaftlichen Gedankenaustausch sollte in Aschersleben ein fruchtbarer Boden bereitet sein.
Allen Teilnehmern der Tagung wünsche ich einen erlebnisreichen Aufenthalt im Landkreis Aschersleben-Staßfurt.
Den Gastgebern des Symposiums darf ich zum erfolgreichen Gelingen der Tagung viel Glück wünschen.

Herr A. Michelmann
Oberbürgermeister der Stadt Aschersleben

Sehr geehrter Herr MinDir Dr. Padberg,
Sehr geehrter Herr MinDir Dr. Aeikens,
Sehr geehrter Herr Landrat,
Sehr geehrter Herr Prof. Dr. Kühne,
Sehr geehrte Damen und Herren!

Es ist mir eine große Freude, Sie hier im Saal des Volkshauses - wohl bald des Bestehornhauses - zur Festveranstaltung „75 Jahre Phytopathologie und Resistenzforschung in Aschersleben" begrüßen zu können. Gleichzeitig möchte ich den Mitarbeiterinnen und Mitarbeitern der hiesigen Institute der Bundesanstalt für Züchtungsforschung die herzlichsten Glückwünsche im Namen von Rat und Verwaltung der Stadt Aschersleben aussprechen.
Ein solcher Tag gibt auch Anlaß, Rückschau auf Erreichtes zu halten, Höhen und Tiefen in Forschung und Organisation zu analysieren und einen Blick in die Zukunft zu werfen.
Gestatten Sie mir bitte an dieser Stelle einige Gedanken zum Zusammenleben von Forschungseinrichtung und Kommune:
Eine relativ kleine Stadt wie Aschersleben kann sich glücklich schätzen, wissenschaftliche Forschungsstätten wie die Institute der Bundesanstalt für Züchtungsforschung beherbergen zu dürfen. Gerade von den Forschungseinrichtungen und ihren Mitarbeiterinnen und Mitarbeitern gehen kaum zu unterschätzende Impulse für die Stadt und ihr Umland aus. Dies gilt nicht nur für die ökonomische Entwicklung, sondern ganz besonders für die Bereicherung des geistigen, kulturellen und politischen Lebens der Stadt.
Wohl wissend um die Vorteile, bemühten sich schon im Jahre 1918 die Stadtväter nachhaltig um die Ansiedlung einer Zweigstelle der damaligen Biologischen Reichsanstalt für Land- und Forstwirtschaft. Besonderer Dank gilt dabei dem damaligen Bürgermeister, Dr. Bunde, der nicht nur bei der Beschaffung eines geeigneten Domizils und eines Versuchsgeländes behilflich war, sondern auch Widerstände gegen die Ansiedlung der Reichsanstalt überwandt und sich für finanzielle Unterstützung einsetzte.
Heute ist die Stadt stolz auf die Leistungen, die von den Wissenschaftlern in den 75 Jahren hier an diesem Standort erbracht wurden. Eine weltweit anerkannte Traditionslinie in der pflanzenvirologischen Forschung, sowohl in Einzeldisziplinen als auch in der Breite des Wissenschaftsgebietes, wurde aufgebaut. Im besonderen Maße ist dabei dem Wissenschaftler und Institutsleiter Maximilian Klinkowski Dank zu zollen, den man mit Fug und Recht als eine der wichtigsten geistigen Persönlichkeiten, die in unserer Stadt gewirkt haben, ansehen darf.
Die wissenschaftlichen Erfolge der Phytopathologie und Resistenzforschung haben den Namen der Stadt Aschersleben ins Land und - wie auch diese Veranstaltung zeigt - weit über die Landesgrenzen hinausgetragen.

Ich brauche an dieser Stelle nicht ausdrücklich zu betonen, daß ich diesem Standortfaktor eine wichtige Bedeutung für die perspektivische Entwicklung der Stadt Aschersleben beimesse, zumal es in jüngster Zeit positive Anzeichen gibt, auch auf anderen Gebieten Forschungs- und Entwicklungspotential in der Stadt anzusiedeln.

Die Stadt Aschersleben wird mit ihren bescheidenen Möglichkeiten alles tun, das für wissenschaftliche Forschung erforderliche Umfeld zu schaffen. Dies reicht von so scheinbaren Kleinigkeiten wie einem Hinweisschild auf den Standort der Bundesanstalt bis hin zu einem vom Stadtrat am 08. 12. 1994 mit eindeutiger Mehrheit gefaßten Beschluß zum Beitritt der Stadt zur Gemeinschaft der Förderer und Freunde der Bundesanstalt für Züchtungsforschung e.V.

In diesem Zusammenhang sei es jedoch erlaubt, meine Besorgnis darüber zum Ausdruck zu bringen, daß aus einem großen, leistungsfähigen Institut drei kleine, nicht weniger leistungsstarke, aber durch die Eingrenzung der Forschungsgegenstände in der Außenwirkung beschränkte Institute entstanden sind.

Ich denke, ein Land wie die Bundesrepublik Deutschland kann es sich nicht leisten, in der wissenschaftlichen Grundlagenforschung in den Schatten anderer Länder zu treten, dies gilt auch, aber nicht nur, für den Standort Aschersleben.

Ich jedenfalls gehe davon aus, daß die Wissenschaftlerinnen und Wissenschaftler der Institute am Standort Aschersleben auch in Zukunft den vor ihnen stehenden Aufgaben gewachsen sein werden. Sofern eine Unterstützung der Stadt Aschersleben möglich ist, sei diese Ihnen von dieser Stelle noch einmal ausdrücklich zugesagt.

Ich wünsche Ihnen allen wissenschaftliche Erfolge und persönliches Wohlergehen und diesem Symposium einen angenehmen und anregenden Verlauf.

Prof. Dr. R. Casper
Biologische Bundesanstalt für Land- und Forstwirtschaften Braunschweig

Meine Damen und Herren,
liebe Kolleginnen und Kollegen!

Zum 75. bringt man eigentlich Blumen und Rotwein für beschauliche Stunden, aber hier haben wir einen jungen alten Jubilar, dem wir auch von der Biologischen Bundesanstalt herzlich gratulieren wollen und viel jugendlichen Schwung wünschen.
Wenn ich heute hier als Vertreter der Biologischen Bundesanstalt sprechen darf, dann hat dies einen besonderen Grund, und ich danke Herrn Präsident Klingauf für diesen ehrenvollen Auftrag.
Die Virologen in Braunschweig haben immer ein besonderes Verhältnis zur Virologie in Aschersleben gehabt. Wir waren einmal Teil eines Ganzen und die Wiedervereinigung hat uns, paradoxer Weise, keine Wiedervereinigung gebracht.
Schade, einfach schade.
Dreimal habe ich an diesem Institut Vorträge gehalten; 1966 - als noch der große Klinkowski das Institut leitete. Damals haben wir abends im „Braunen Hirsch" zusammengesessen und politische Witze ausgetauscht und herrlich gelacht, und der Kollege Wolfgang wußte die besten.
Dann wurde das Verhältnis zwischen uns offiziell immer verkrampfter, aber inoffiziell liefen noch einige Kontakte.
1988/89 wurde ich hierher zum Vortrag eingeladen, und das war vielleicht schon ein Beginn der Wende. Aber vom Betreten des Geländes bis zur Abreise wurde ich so hautnah betreut, ich empfand es als eine geradezu lähmende Kontrolle, daß ich nicht ein unkontrolliertes Wort mit den Kollegen sprechen konnte. Für mich wurden ein erschreckender wirtschaftlicher Niedergang und eine niedergedrückte Spannung in der Bevölkerung deutlich. Damals bekam ich einen Brief.
Ich zitiere: „ ... übersende ich Ihnen für die Einreise in die DDR den Berechtigungsschein zum Empfang eines Visums zu einem von Ihnen gewählten Grenzübergang sowie Zählkarte und Zollerklärung .."
Heute können wir zum Kaffeetrinken von Braunschweig herüber kommen. Wir können Hin- und Herfahren. Wir sollten dafür sehr, sehr dankbar sein.
Am 29./30. Januar 1990 konnte ich mit Hilfe der Deutschen Forschungsgemeinschaft die Aschersleber nach Braunschweig einladen. Wir rechneten mit 20. - 60 kamen zu unserer großen Freude, und es wurde nach einer anfänglichen Befangenheit ein sehr lockeres und sehr schönes Treffen.
Die Zukunft wird uns zwingen, sehr eng zusammenzuarbeiten. Die Forschungsthemen müssen wir noch exakter abstimmen und die Vorhaben genauer einhalten. Wir wissen alle, daß keine besonders leichten Zeiten in Zukunft auf uns zukommen. Die jahrzehntelange, ungleiche Konkurrenz - möchte ich es einmal nennen - hat zu einem Egoismus geführt, der noch nicht völlig überwunden ist; aber unter einer Zuckerdecke brodelt es immer etwas. - Entschuldigen Sie diese deutlichen Worte. Aber: Nur in kooperativer Gemeinsamkeit ist unsere Zukunft zu sichern, und vielleicht werden wir eines Tages doch noch zusammengelegt.

Prof. Dr. G. Proeseler
komm. Leiter der Bundesanstalt für Züchtungsforschung an Kulturpflanzen
Leiter des Instituts für Epidemiologie und Resistenz

Sehr geehrter Herr MinDir Dr. Padberg,
sehr geehrter Herr MinDir Dr. Aeikens,
sehr geehrte Herren Abgeordnete und Mandatsträger,
verehrte Gäste,
liebe ehemalige Mitarbeiterinnen und Mitarbeiter,
meine Damen und Herren!

Dieses Grußwort habe ich als gegenwärtiger kommissarischer Leiter der Bundesanstalt für Züchtungsforschung an Kulturpflanzen besonders gern übernommen, da ich in Aschersleben geboren bin und sich fast meine gesamte berufliche Entwicklung - besonders geprägt durch meinen verehrten Lehrer und Doktorvater Maximilian Klinkowski - am Forschungsstandort Aschersleben vollzogen hat. Ich habe erstmalig bereits im Sommer 1954 einige Wochen im Institut für Phytopathologie gearbeitet und konnte ab November 1961 kontinuierlich die Entwicklung dieser wissenschaftlichen Einrichtung miterleben und mitgestalten.

Während Herr Kollege Naumann heute in seinem Vortrag die gesamte Entwicklung im Verlauf der 75 Jahre analysieren wird, möchte ich etwas ausführlicher auf die letzten fünf Jahre eingehen.

Ganz entscheidend für den Fortbestand war die Frage, wie sich die Aschersleben Einrichtung nach der Wiedervereinigung Deutschlands in die gesamtdeutsche Forschungslandschaft einbringen konnte.

Wie mein Vorredner, Herr Kollege Casper, bereits zum Ausdruck brachte, erwarteten wir auf Grund der historischen Entwicklung, angefangen als Zweigstelle der Biologischen Reichsanstalt für Land- und Forstwirtschaft, gemeinsam mit dem Institut für Pflanzenschutzforschung Kleinmachnow eine Angliederung an die Biologische Bundesanstalt für Land- und Forstwirtschaft. Es fanden deshalb verschiedene gemeinsame wissenschaftliche Veranstaltungen in Braunschweig und Aschersleben sowie zahlreiche Beratungen statt, wobei das persönliche Engagement der Herren Priew und Petzold aus dem Bundesministerium für Ernährung, Landwirtschaft und Forsten, Klingauf, Crüger, Casper, Bartels und Huber von Seiten der BBA besonders zu würdigen ist.

In Aschersleben engagierte sich an meiner Seite - neben den Abteilungsleitern, Wissenschaftlern und allen betroffenen Mitarbeitern, besonders intensiv Herr Fritzsche für den Erhalt des Standortes Aschersleben.

Während im Oktober 1990 für Kleinmachnow bereits die ersten Stellen durch das BML bewilligt wurden, fielen die Entscheidungen für Aschersleben erst fast ein Jahr später.

Dazwischen lagen bange Monate, die um die Jahreswende mit dem Gerücht verbunden waren, daß man die Absicht hatte, den Forschungsstandort Aschersleben ganz zu schließen. Die Evaluierung durch die Kommission des Wissenschaftsrates im Februar 1991 ließ neue Hoffnungen aufkommen. Professor Neuweiler verkündete nach langer Beratung die Entscheidung: Aschersleben muß mit erheblichem Personalabbau rechnen, bleibt aber Forschungsstätte.

Für viele von uns war diese Entscheidung mit einer bisher nicht gekannten, bitteren Enttäuschung verbunden, bedingt durch den Verlust des vielfach seit Jahrzehnten innegehabten Arbeitsplatzes. Andererseits war die positive Seite dieser Entscheidung, nämlich der Fortbestand der Forschungsstätte, das Ergebnis des engagierten

positive Seite dieser Entscheidung, nämlich der Fortbestand der Forschungsstätte, das Ergebnis des engagierten Wirkens aller im Verlauf der langen Entwicklung Tätigen; auch der in die Ungewißheit Entlassenen - und dafür danke ich allen!

In der zweiten Hälfte des Jahres 1991 erhielten wir verbindliche Informationen über die zukünftige organisatorische Zugehörigkeit, Struktur und den Personalumfang. Der Wissenschaftsrat hatte empfohlen, daß das solide Potential der Züchtungsforschung an Kulturpflanzen in den neuen Bundesländern zu erhalten ist. Das BML entschloß sich dankenswerterweise daraufhin, eine neue Bundesanstalt, die zehnte in seinem Bereich der Ressortforschung, zu gründen. Der entsprechende Erlaß durch Herrn Bundesminister Kiechle ist auf den 27. November 1991 datiert. Der Anstalt wurden 330 Personalstellen, davon 82 für Wissenschaftler, zugewiesen. Dem Gründungsausschuß, bestehend aus den Herren Alleweldt, Fischbeck, Franck, Habben, Heitefuß, Priew, Schmalz, Wenzel und Wricke, war die fundamentale Bedeutung der Resistenz von Kulturpflanzen gegenüber den Schaderregern bewußt, weshalb er sich entschloß, Aschersleben dieser neuen Bundesanstalt für Züchtungsforschung an Kulturpflanzen anzugliedern.

Mit dem 1. Januar 1992, dem Gründungsdatum der BAZ, begann eine neue Etappe für die Forschungsanstalt mit den Standorten Quedlinburg als Hauptsitz, Aschersleben, Groß Lüsewitz und Dresden-Pillnitz. Unter der erfahrenen Anleitung von Herrn Alleweldt als Anstaltsleiter formierten sich 10 Institute.

In Aschersleben waren dies die drei Institute für Resistenzforschung, Pathogendiagnostik sowie Epidemiologie und Resistenz. Wie an den anderen drei Standorten, begannen die Mitarbeiter voller Elan mit der Einrichtung der Institute, formulierten ihre zukünftige Forschungskonzeption und leiteten davon ihre neuen Forschungsprojekte ab. Es war notwendig, vom Hauptarbeitsgebiet „Phytopathologie" zur „Züchtungsforschung" mit dem Schwerpunkt „Resistenz gegen biotische Schaderreger" zu wechseln. Diese Umstellung wurde errfolgreich vollzogen. Es waren intensive Abstimmungen zwischen den Instituten innerhalb der BAZ, mit anderen Forschungsanstalten, den in der Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung vereinten Pflanzenzüchtern und den Einrichtungen der Universitäten erforderlich. Von Anfang an betrachteten sich die Wissenschaftler aller drei Institute in Aschersleben als Kooperationspartner der fruchtartenspezifischen Institute an den anderen drei Standorten in der BAZ. Dieses Prinzip wurde auch nach Angliederung der drei Standorte Ahrensburg, Grünbach und Siebeldingen weiterverfolgt.

Da in Aschersleben erfahrene Virologen, Bakteriologen, Mykologen und Entomologen wirken und die Resistenz der Kulturpflanzen gegenüber den verschiedensten Schaderregergruppen als wichtigste Aufgabe der BAZ zu betrachten ist, ergibt sich daraus im Zusammenwirken mit den Züchtungsforschern an den anderen Standorten ein umfangreiches Betätigungsfeld. Die Evaluierung der Genbankmuster des IPK Gatersleben, der FAL und anderer Einrichtungen stellt ebenfalls eine wichtige Aufgabe dar, wobei die territoriale Nähe und die jahrzehntelange gute Zusammenarbeit zwischen Gatersleben und Aschersleben sowie Quedlinburg hervorzuheben sind.

Wenn in Aschersleben auch zukünftig Fragen der Diagnose und Epidemiologie bearbeitet werden, kann nur der Uneingeweihte dadurch Forschungsüberschneidungen oder Doppelarbeit bezogen auf die Biologische Bundesanstalt bzw. andere Forschungseinrichtungen vermuten. Die Untersuchungen zur Diagnose und Epidemiologie von Schaderregern konzentrieren sich ausschließlich auf Objekte, die in der Resistenzzüchtung bedeutungsvoll sind und die Selbständigkeit einer Bundesanstalt gewährleisten.

Insgesamt werden an dieser Bundesanstalt alle Kulturpflanzen der gemäßigten Klimaregion, vom Getreide bis zur Weinrebe - mit Ausnahme der Forstgehölze - bearbeitet. Die Forschungsaufgaben konzentrieren sich auf folgende Schwerpunkte:

- Verbesserung der Widerstandsfähigkeit der Kulturpflanzen gegen Schaderreger, um den Einsatz von Pflanzenschutzmitteln zu reduzieren und eine „gesunde Pflanze" wachsen zu lassen.
- Verbesserung der Toleranz der Kulturpflanzen gegen klimabedingte Streßfaktoren, wie Trockenheit und Frost, um wirtschaftliche Ausfälle zu minimieren.
- Erhöhung der Nährstoffeffizienz der Kulturpflanzen, um den Aufwand an Düngemitteln herabzusetzen.
- Steigerung der Produktqualität, um bessere Nahrungs- und Industriepflanzen bereitzustellen.
- Verbesserung von Züchtungsmethoden zur Intensivierung des Zuchtprozesses.
- Erweiterung des Kulturartenspektrums, z. B. nachwachsende Rohstoffe, um die Fruchtfolgeprobleme zu vermeiden oder die Uniformität der Landschaft aufzulockern.

Zur Erfüllung dieser hier aufgeführten Aufgaben werden von den 112 Wissenschaftlern gegenwärtig insgesamt 230 Forschungsprojekte bearbeitet.
Die Reihenfolge der Schwerpunkte läßt erkennen, daß die Resistenz gegen biotische Schaderreger im Vordergrund steht. Diesem Themenkomplex sind mehr als 50 % der Forschungsprojekte der BAZ gewidmet.
Den Resistenzforschern in Aschersleben eröffnet dieser Themenkomplex ein sehr umfangreiches und anspruchsvolles Betätigungsfeld, das die Möglichkeit zur nationalen und internationalen Zusammenarbeit bietet.
Das Symposium der kommenden Tage ist eine ausgezeichnete Gelegenheit, wissenschaftliche Erfahrungen auszutauschen, neue Kooperationsbeziehungen anzubahnen und nicht zuletzt sich persönlich kennenzulernen.

Diese Festveranstaltung sollte auch genutzt werden, dem Bundesministerium, den fördernden Gemeinschaften - und damit dem Steuerzahler - für die kontinuierliche Finanzierung unserer angewandten Forschung, den zahlreichen Partnern im In- und Ausland für die erfolgreiche Kooperation sowie den ehemaligen und den jetzigen Mitarbeiterinnen und Mitarbeitern für ihr Engagement, ihre Treue und Verbundenheit aufrichtig zu danken.

Ich wünsche der Tagung einen erfolgreichen Verlauf und dem Forschungsstandort Aschersleben eine gesicherte Perspektive im kommenden Jahrhundert.

Prof. Dr. D.D. Shukla
CSIRO, Division of Biomolecular Engineering

Professor Kühne, distinguished guests and fellow scientists!

First and foremost, I wish to express my profound gratitude to the organizers of this symposium, Prof. Kühne in particular for inviting me to take part and give a welcome speech in this historic International Symposium to celebrate the 75 years of resistance research at Aschersleben. I am indeed humbled by your trust in me for this very unique occassion. Today's familiar surroundings, the friendly atmosphere and the prevailing environment throw my memories back to 1968 when, as a young, unimaginative country boy from India, I arrived at this Institute on a German Govern-ment Exchange scholarship to pursue my studies toward a Ph.D. degree in Plant Virology. I have used the word imagination in the sense that when I came here in 1968, I was not sure in my mind whether viruses really exist.

The sense of skepticism with which my mind was preoccupied prior to, and my arrival here, soon transformed into a sense of belonging, to fulfil a call of duty, and to some extent my conviction to match my concealed childhood dream that I should excel in all my achievements. After arriving at the Institute I was first introduced to Professor Maximilian Klinkowski, the then director of the Institute. My first contact and my later dealings with him, left a lasting impression on me. He was a man of vision, extreme talents and had excellent leadership qualities. He was a person who believed in perfection. I owe a very deep sense of gratitude to Professor Klinkowski for shaping my career as a plant virologist.

The other person responsible in shaping my career as a plant virologist was the late Dr. Klaus Schmelzer, an extremely intelligent and brilliant scientist. He became my mentor, my guide and my powerhouse of strength to propel my entire energy into learning plant virology . He encouraged me at every step, and led me all the way and even pushed me at every step of the ladder of success to the end. The foundation of my achievements in plant Virology is certainly of Dr. Schmelzer's making, which I have adequately acknowledged on the dedication page of my book „The Potyviridae", published last year by the C.A.B. International, Wallingford, U.K.

I also owe a deep sense of gratitude to my other former colleagues of this institute, a number of them are here in the audience today. I have many cherished and memorable moments treasured in my heart and mind of this majestic surroundings. A great deal of my aspiration and outlook in life was moulded in various corridores and laboratories of this great Institute for which I will be eternally greatful. Because of my training here, I was able to receive several awards recently. In November last year I was awarded the most prestigious CSIRO award, the Chairman's Medal for my work on potyviruses.

This award honours the very best in CSIRO research and consists of a gold Medal and $ 25,000 in cash. This year I have been awarded the Australian medal of Agricultural Science, and have been elected a fellow of the American Phytopathological Society. The credit to my achievements go to Dr. Schmelzer and the Institut für Phytopathologie Aschersleben, because my initial training in plant virology happened at this Institute. Although I do not work on plant viruses any more, the knowledge I acquired here is helping me in my current research area. At present I head the Human Hepatitis Viruses Programme at the Biomolecular Research Institute in Melbourne, where my main aims are

to develop rationally designed drugs against hepatitis B and hepatitis C viruses based on the three-dimensioned structure of their virion and regulatory proteins. For this project I have received a grant of $ 40 million over five years. I would again like to emphasize that this would not have happened to me if I have not received my training in plant virology at the Institut für Phytopathologie Aschersleben.

Besides the good qualities and knowledge I acquired at this Institute, I also learnt some bad habits here. Before coming to Aschersleben, I was a devout Hindu, a strict vegetarian and a non-drinker. At Aschersleben, I learnt to drink beer, which has become my most favourite drink ever since, and eat every kind of meat including beef which is prohibited in Hindu religion. However, I do not regret a bit learning these bad habits because they bring joyful moments in my life.

With these words I would like to wish every success for this historic symposium to celebrate the 75 years of resistance research at Aschersleben. I trust that our meetings in the days ahead would be memorable and would strengthen our bond of fellowship with each other, and our resolve to serve the world and its future well-being, which will be enhanced through more gatherings of this nature. I am privileged and honoured to participate in this symposium and wish that the Institute will keep on producing excellent results as it has done in the past, and will maintain its name in the list of great Insitutes of the world involved in phytopathological research in the future.

Universitäts-Prof. Dr. Dr. habil. G.M. Hoffmann
Technische Universität München

Hochverehrte Festversammlung,
meine sehr geehrten Damen und Herren!

Die wechselhafte Geschichte unseres Volkes hat es zugelassen, daß Aschersleben als Standort wissenschaftlicher Arbeiten auf dem Gebiet der Phytomedizin, der Entomologie, Mikrobiologie und Virologie siebeneinhalb Jahrzehnte Bestand hatte und dieser auch für die Zukunft gesichert erscheint. Das ist nach bewegter Zeit Anlaß zu großer Freude, Genugtuung und Dankbarkeit. Wie könnte man dies besser feiern als durch ein Symposium - wir wollen das nicht altgriechisch als Trinkgelage nach üppiger Mahlzeit interpretieren - sondern im Sinne Platons '*Symposion*' als eine Zusammenkunft zu ernsthaftem Gespräch, beziehungsweise in der heutigen Auslegung als Tagung von Fachgelehrten, zu deren Eröffnung ich Ihnen einen aufrichtigen Gruß entbieten möchte.
75 Jahre ununterbrochene Forschung beinhalten die Tätigkeit von 3 bis 4 Generationen von Wissenschaftlern, fast lückenlos ineinander verzahnt und im großen Rahmen vereint durch die Zielsetzung, der Suche nach der Wahrheit, dem Unbekannten, dem Machbaren und dem Fortschritt auf einem Wissensgebiet, welches uns Einblicke in die vielfältigen Regelkreise des Naturhaushaltes auf der Grundlage von antagonistischen und synergistischen Beziehungen zwischen Lebewesen aus dem Tier- und Pflanzenbereich ermöglicht; ein Wissensgebiet, welches uns zeigt, daß Parasitismus in allen Bereichen des Lebens anzutreffen ist, das heißt, daß das Vorkommen von Krankheitserregern und Schadtieren bei Kulturpflanzen ein allgemeines biologisches Phänomen darstellt und der Mensch gut beraten ist, wenn er nach Wegen und Methoden sucht, um die daraus erwachsenden Nachteile für seine Existenz so niedrig wie möglich zu halten.
„Alles wissenschaftliche Arbeiten", so hat Wilhelm v. Humboldt 1933 formuliert, „ist nichts anderes, als immer neuen Stoff in allgemeine Gesetze zu bringen". Aus der langjährigen eigenen Erfahrung kann ich auch die Ansicht von Justus v. Liebig nur bestätigen: „Die Wissenschaft fängt eigentlich erst da an interessant zu werden, wo man aufhören muß".

Dieser Wissenschaft hat sich die Institution in Aschersleben immer verpflichtet gefühlt, und sie ist gepflegt worden; daher verdienen die Bemühungen ihrer zahleichen, über Generationen verteilten Mitarbeiter, den Aufgaben gerecht zu werden, uneingeschränkten Respekt und hohe Anerkennung. Es kann kein Dissens darüber bestehen, daß die wissenschaftliche Leistung einzelner Persönlichkeiten aus Aschersleben, aber auch die Gesamtheit der Arbeiten, internationale Akzeptierung gefunden haben; diese über siebeneinhalb Jahrzehnte laufenden Bestrebungen nach wissenschaftlichen Erkenntnissen haben, nach meiner Einschätzung, letztlich auch dazu geführt, daß die Aschersleben Institution in einem neuen Verbund erhalten bleibt. Zu dieser Entscheidung kann ich den beteiligten staatlichen Stellen nur gratulieren - möchte aber gleichzeitig all denjenigen den Dank aussprechen, die sich in den vielen Jahrzehnten in der Weise verdient gemacht haben, daß diese Neuorganisation eine sinnvolle Lösung darstellt.

Wenn mich diese Entwicklung persönlich sehr erfreut, dann nicht zuletzt aus dem Grund, weil Aschersleben unter Maximilian Klinkowski mich als Student und Graduierten aufnahm und mir die Möglichkeiten zur Promotion und Habilitation an der Universität Halle gab, eine entscheidende Phase im Leben eines jungen Wissenschaftlers - und so wie ich in Aschersleben gelernt und reichliche fachliche und persönliche Erfahrungen gesammelt habe - sind viele andere junge Menschen an dieser Institution geprägt worden - wofür ich im Namen vieler in diesen Kreis eingeschlossener sog. Ehemaliger Dank aussprechen möchte. Wir haben uns bei unserer Weiterentwicklung bewußt oder unbeweuß bemüht, dem lateinischen Spruch zu entsprechen: „Gaudeamus eo, quod dabitur, redda musque id cum reposcemur" (Freuen wir uns über das, was wir bekommen, und geben wir es zurück, wenn es uns abverlangt wird).

Die 75 Jahre phytomedizinische Forschung, die Anlaß zu diesem Symposium geben, fielen in eine Zeit, in der sich politische Regime und Ideologien abwechselten, bis sich für einen Restteil des alten Reiches früher, für einen anderen leider erst spät eine demokratische Gesellschaftsordnung zu entwickeln begann. Diese Konstellationen haben sich auf die Mitarbeiter der Aschersleben er Institution in unterschiedlicher Weise auswirken müssen; es gab in den 30er Jahren Emigrationen wie in den 50ern, und Einzelschicksale sind durch Belastungen, Entbehrungen und Beschränkungen über eine mehr oder weniger lange Zeit gekennzeichnet. Vielleicht kann abschwächend ein Wort Seneccas etwas helfen: „Nirgends hat es die Natur besser mit uns gemeint: da sie wußte, in welchem Leid wir geboren sind, erfand sie zur Linderung die Gewohnheit und die Toleranz."

Diese Zeiten haben uns wach werden lassen - „Nihil ergo magis praestandum est" (Vor nichts sollten wir uns mehr in acht nehmen) - als einer Herde zu folgen, anstatt unseren eigen Weg zu gehen. Lassen Sie mich zum Abschluß daher auch an alle diejenigen denken, welche schwierige Zeiten in der Aschersleben er Institution überstehen konnten, und ich bitte um Ehrlichkeit, Gerechtigkeit und Toleranz bei der Bewertung vergangener Jahrzehnte. „Wir wissen, daß alles zwei Seiten hat, aber erst wenn man erkennt, daß es drei sind, erfaßt man eine Sache", hat Lichtenberg formuliert.

Das Entscheidende steht bevor, nämlich die Zukunft der Institute, zu der ich wünschen und bitten möchte, daß es mit großzügiger Unterstützung gelingen möge, den internationalen wissenschaftlichen Anschluß zu finden und ein anerkanntes Mitglied der Gemeinschaft der Wissenschaften in einer demokratischen Ordnung zu sein. „Die Zukunft hat viele Namen", schrieb Victor Hugo, „für die Schwachen ist sie das Unerreichbare, für die Furchtsamen ist sie das Unbekannte, für die Tapferen ist sie die Chance"

In diesem Sinne:

Ad multos annos !

G. M. Hoffmann (aus der Schule von Maximilian Klinkowski, 1950-1958)

Dr. H. Wilhelm *)
1. Vorsitzender der Deutschen Phytomedizinischen Gesellschaft e. V.

Hochansehnliche Festversammlung!
Meine sehr verehrten Damen und Herren!

Zu meinem großen Bedauern kann ich wegen unaufschiebbarer Verpflichtungen nicht persönlich an dieser Festveranstaltung teilnehmen. Ich habe daher den Landessprecher der DPG für Sachsen-Anhalt, Herrn Prof. Naumenn, gebeten, Ihnen an meiner Stelle die Grüße unserer Gesellschaft zu überbringen.

Die Deutsche Phytomedizinische Gesellschaft beglückwünscht im Namen ihrer mehr als 1700 Mitglieder alle Beschäftigten am Standort zu diesem bedeutsamen Jubiläum. Sie würdigt das jahrzehntelange Wirken der in Aschersleben beschäftigt gewesenen Wissenschaftler und aller Mitarbeiter der jetzigen BAZ - Institute. Die hier durchgeführten Arbeiten und deren Ergebnisse haben von Anfang an und bis auf den heutigen Tag einen bedeutenden Beitrag zum Ansehen der deutschen phytomedizinischen Forschung geleistet.

Eine größere Zahl der heute hier tätigen Pflanzenpathologen, Molekularbiologen und Chemiker ist Mitglied der DPG und vielfach schon seit Jahren in unserer wissenschaftlichen Gesellschaft, besonders im Rahmen der Arbeitskreise und der Deutschen Pflanzenschutztagung, aktiv.

Die Deutsche Phytomedizinische Gesellschaft möchte an dieser Stelle die Erwartung aussprechen, daß der Forschungsstandort Aschersleben im Rahmen der BAZ auch in Zukunft mit seiner Tätigkeit auf dem Gebiet der Resistenzforschung einen sehr wesentlichen Beitrag zum Erkenntnisfortschritt in der Phytomedizin und zum Nutzen der gesamten Landwirtschaft leisten möge.

Dazu wünscht die Gesellschaft allen Mitarbeitern eine erfolgreiche Arbeit und persönliches Wohlergehen!

*) vorgetragen von Prof. Dr. Naumann

Prof. Dr. K. Naumann *)
Leiter des Instituts für Pathogendiagnostik Aschersleben

75 Jahre phytopathologische Forschung in Aschersleben

Einleitung

Mit diesem Beitrag soll der Versuch gemacht werden, die Anfänge, die Entwicklung, die Höhen und die Tiefen der wissenschaftlichen Tätigkeit in den letzten 75 Jahren am Forschungsstandort Aschersleben nachzuzeichnen.
Die Entwicklung der phytopathologischen Forschung in den Mauern dieser Stadt wird nur verständlich vor dem Hintergrund der deutschen Geschichte dieses Jahrhunderts auf der einen und der Entwicklung der biologischen Wissenschaften auf der anderen Seite.
In diesem Rückblick soll daher sowohl von den wissenschaftlichen Aktivitäten als auch von den besonderen Bedingungen hier am Standort die Rede sein.

Natürlich ist es nicht möglich, in diesem Beitrag alle in den zurückliegenden Jahren bearbeiteten Forschungsthemen zu nennen oder alle am Standort je tätig gewesenen Wissenschaftler namentlich zu erwähnen, geschweige denn ihre Arbeiten zu würdigen. Sind doch im Laufe der Jahre weit über 100 Wissenschaftler hier für kürzere oder längere Zeit beschäftigt gewesen!
Dennoch wird von einer Reihe ehemaliger Mitarbeiter die Rede sein, deren Wirken für bestimmte Arbeitsrichtungen prägend waren oder die durch besondere wissenschaftliche Ergebnisse hervorgetreten sind.

Die Geschichte der phytopathologischen Forschung in Aschersleben läßt sich vereinfacht in sechs Phasen einteilen:

- I - Aufbau der Zweigstelle bis zur Mitte der 30er Jahre (1920 - 1935)
- II - Vorkriegszeit bis zum Kriegsende (1936 - 1945)
- III - Wiederaufbauphase nach dem Krieg (1945 - 1951)
- IV - Übernahme in die Deutsche Akademie der Landwirtschaftswissenschaften und Etablierung des Instituts für Phytopathologie (1952 - 1970)
- V - Die Zeit nach M. KLINKOWSKI bis zur Abwicklung des Instituts lt. Einigungsvertrag (1971-1991)
- VI - Gründung und Aufbau der Bundesanstalt für Züchtungsforschung an Kulturpflanzen (BAZ) (ab 1992)

2. Die Gründung einer Zweigstelle der BRA in Aschersleben

Die Geschichte der phytopathologischen Forschung in Aschersleben beginnt mit der Errichtung einer Zweigstelle der Biologische Reichsanstalt am 1. April 1920.
Für dieses Ereignis waren im wesentlichen vier Gründe maßgebend:

1. Die Erzeugung landwirtschaftlicher Produkte war gerade in der Zeit nach dem Ersten Weltkrieg ein bedeutender Zweig der Volkswirtschaft und von existentieller Bedeutung für die Ernährung der Bevölkerung im allgemeinen und für die bäuerlichen Betriebe im besonderen.
2. Man erkannte immer mehr, daß Ernteverluste durch Krankheiten und Schädlinge sich einschränken oder ganz vermeiden lassen, wenn man die Ursachen solcher Schäden rechtzeitig ermittelt und alles tut, um sie auszuschalten bzw. ihre Auswirkungen zu mildern.
   Zur wissenschaftlichen Bearbeitung derartiger Probleme war kurz vor der Jahrhundertwende in Deutschland die Biologische Reichsanstalt für Land- und Forstwirtschaft gegründet worden, die gehalten war, in allen Bereichen der Landwirtschaft wirksam zu werden.
3. Ein bedeutendes Zentrum der landwirtschaftlichen Produktion in Deutschland war schon von alters her die Region zwischen Harz und Magdeburg, weil hier sehr günstige edaphische (tiefgründige Böden) und klimatische Bedingungen gegeben sind und bei vielen Kulturen besonders gute Erträge erzielt werden können.
   Das Gebiet am nordöstlichen Rand des Harzes mit den Städten Quedlinburg, Aschersleben und Bernburg ist zudem auf Grund seiner relativ geringen Jahresniederschlagswerte (um 500 mm) besonders begünstigt. Dadurch hatte sich dieser Raum schon seit dem vorigen Jahrhundert zu einem

*) In Zusammenarbeit mit Prof. Dr. J. Richter, Aschersleben

bedeutenden Anbaugebiet für Gemüse- und Blumensämereien, Heil- und Gewürzpflanzen, Zuckerrüben und Ölsaaten entwickelt.

4. Die Anbauer in diesem Gebiet hatten immer wieder den dringenden Wunsch nach einer sachkundigen wissenschaftlichen Beratung im Gemüsesamenanbau durch eine staatliche Institution zum Ausdruck gebracht.

Welche hohe Wertschätzung der Pflanzenschutz seinerzeit in der hiesigen Gegend genoß, kann man aus der Tatsache ersehen, daß in den 20er Jahren in Aschersleben Notgeld mit Pflanzenschutzmotiven gedruckt worden ist (KLINKOWSKI 1961).
Diese hier kurz skizzierten Erwägungen führten zu dem Ersuchen, eine Zweigstelle der BRA im hiesigen Raum zu errichten. Der Rat der Stadt Aschersleben machte in Verhandlungen, die sich einige Zeit hinzogen, ein wirtschaftlich günstiges Angebot, das zunächst gute Aussichten für den künftigen Ausbau der neuen Außenstation eröffneten. Das 1. Dokument, was wir darüber besitzen, ist ein Brief des damaligen Oberbürgermeisters Dr. BUNDE an den Direktor der BRA vom 2. August 1918 (!). Die Leitung der BRA unter ihrem damaligen Direktor Johannes BEHRENS und seinem Stellvertreter Otto APPEL entschloß sich daher Anfang 1920, die geplante Zweigstelle hier am Ort einzurichten. Es gibt darüber ein Besprechungsprotokoll vom 11. Juli 1919 und eine Aktennotiz vom April 1920.

3. Aufbauphase (1920 - 1935)

Der Aufbau der hiesigen Zweigstelle vollzog sich allerdings zunächst nur in einem sehr bescheidenen Rahmen. Untergebracht war sie in den Anfangsjahren in vier Räumen des Städtischen Schlachthofs in der Hecklinger Straße. Dort richtete man auch eine Dienstwohnung für den Zweigstellenleiter ein. Aus dieser Zeit gibt es ein Bilddokument von dem betreffenden Gebäude (Abb. 1). Die an die Treppe angelehnten Fahrräder dienten den Mitarbeitern zur Fahrt auf das Versuchsfeld, das sich anfangs in ca. 2 km Entfernung am südöstlichen Stadtrand (hinter dem Friedhof bzw. am Ausgang der Lindenstraße) befand.

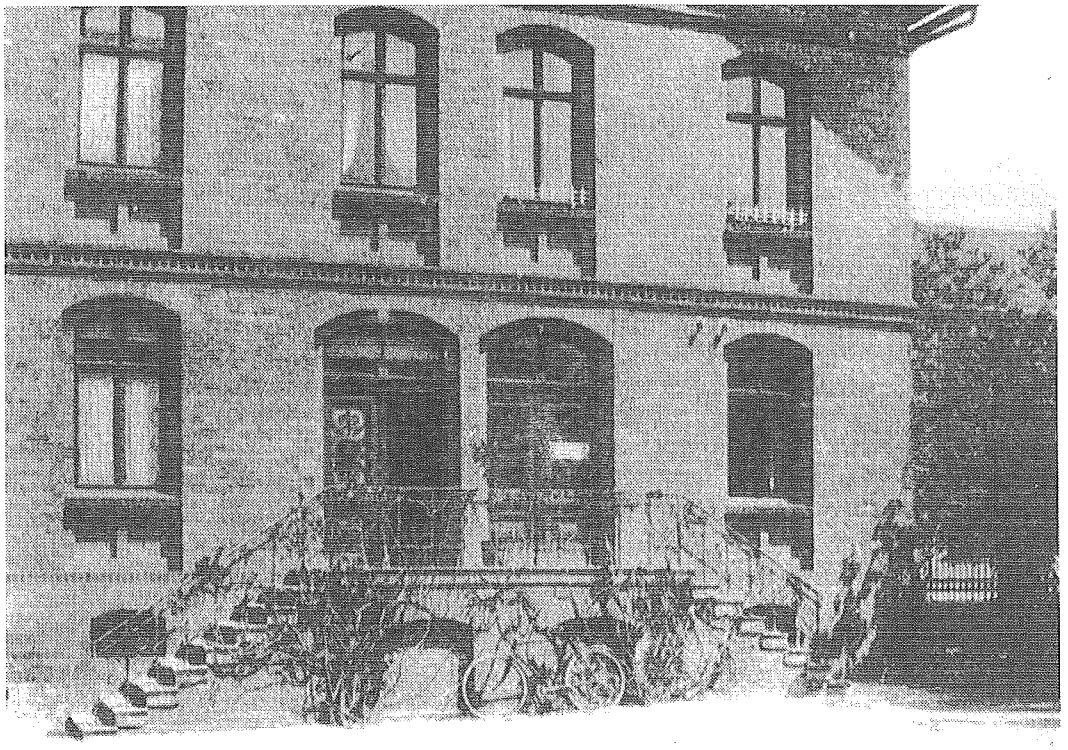

Abb. 1
1. Domizil der Zweigstelle Aschersleben im Städtischen Schlachthof

In den ersten Jahren arbeiteten hier lediglich ein Botaniker und ein Zoologe mit insgesamt fünf technischen Mitarbeitern. Als erstem Leiter oblag Herrn Dr. Paul RABBAS die Verantwortung für den Aufbau (Abb. 2). Ihm zur Seite stand anfangs Werner EXT, später kam Johannes WILLE als Entomologe hinzu. RABBAS knüpfte sehr rasch Kontakte zu interessierten landwirtschaftlichen und gärtnerischen Betrieben, beriet sie bei der Bekämpfung von Krankheiten und Schädlingen und bemühte sich sehr, die Arbeit der Zweigstelle in der Region bekannt zu machen.

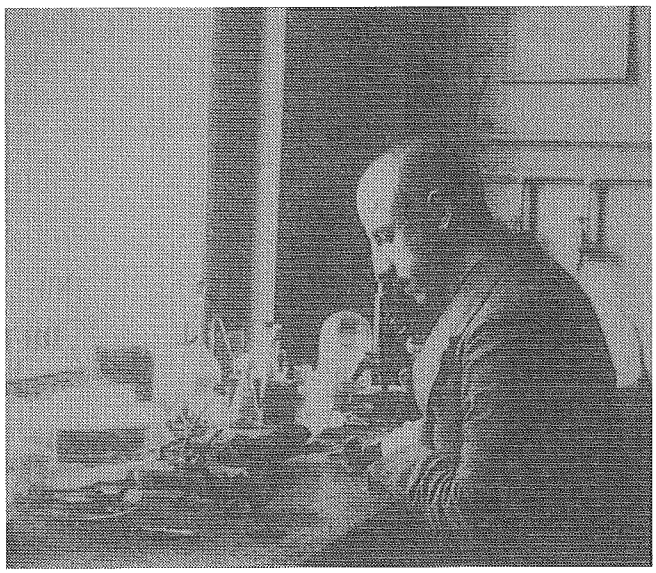

Abb. 2
Paul RABBAS
Zweigstellenleiter 1920 - 1923

Die Konzeption der neuen Zweigstelle sah insbesondere die Erforschung von Krankheiten und Schädlingen im Gemüsesamenbau vor. Diese Aufgabenstellung schloß die Prüfung von Bekämpfungsmitteln, die Erarbeitung von Vorbeugungsmaßnahmen, die Untersuchung von Boden-, Witterungs- und Düngungsfaktoren sowie die Bewertung des Resistenzverhaltens von Sorten gegenüber bestimmten Krankheitserregern ein. Hinzu kam eine rege Auskunftstätigkeit, die von Anbeginn an eine enge Verbindung zur Praxis schuf.
Die ersten Publikationen aus der Zweigstelle erschienen bereits 1921.

Unter den Aschersleber Arbeiten aus den 20er Jahren ragen die Untersuchungen über die Rübenblattwanze und die durch sie verursachte Kräuselkrankheit der Zuckerrübe hervor, die - von EXT und DYCKERHOFF begonnen - vor allem mit dem Namen WILLE verbunden sind. In seiner 1929 erschienenen Monographie beschrieb WILLE die Kräuselkrankheit als eine Virose, die von der Wanze *Piesma quadratum* übertragen wird.

Doch der Anfang war sehr schwer. Das Provisorium in der Hecklinger Straße dauerte immerhin sieben Jahre! Die Situation spitzte sich auch dadurch zu, daß Angebote von den Städten Quedlinburg und Erfurt auf Verlegung der Zweigstelle eingingen, die u. a. auch bessere Arbeitsbedingungen in Aussicht stellten. Die endgültige Entscheidung, die Zweigstelle doch in Aschersleben zu belassen, fiel nach langwierigen Verhandlungen erst in der 2. Hälfte der 20er Jahre. Ausschlaggebend dafür mögen die großzügige finanzielle Unterstützung durch die Stadt, die vorhandenen guten Versuchsflächen und die Zusicherung gewesen sein, daß

in Kürze ein geeignetes Gebäude zur Verfügung gestellt würde. Außerdem dürften die relativ guten Eisenbahnverbindungen nach Berlin eine Rolle gespielt haben, wo sich seit 1898 die Zentrale der Biologischen Reichsanstalt befand.

Eine entscheidende Verbesserung, die vor allem den aufopferungsvollen Bemühungen von Regierungsrat Dr. Leo PETERS, dem Nachfolger von Dr. RABBAS als Zweigstellenleiter, zu danken war, trat dann Ende 1928 ein. Zu diesem Zeitpunkt endlich konnte die ehemalige Westphal'sche Villa in der Ermslebener Straße 52 erworben, ausgebaut und bezogen werden (Abb. 3). Vielen älteren Aschersleber Bürgern ist dieses Gebäude noch als die "Bio" ein Begriff.
Der Bau eines bis dahin fehlenden Gewächshauses auf einem neuen, in nächster Nähe gelegenen Versuchsfeld wirkte sich zusätzlich günstig aus.

Das Jahr 1928 brachte auch insofern eine wichtige Veränderung mit sich, als gegen Jahresende Regierungsrat Dr. Hans BREMER zum Zweigstellenleiter ernannt wurde (Abb. 4). Damit war der Aufbau der Zweigstelle auch formell endgültig abgeschlossen. Unter der Leitung BREMERs nahm die wissenschaftliche Tätigkeit einen raschen Aufschwung. Er selbst - obwohl von Haus aus Botaniker - bearbeitete sowohl entomologische als auch mikrobiologische Probleme. So stammen von ihm und seinen Mitarbeitern grundlegende Untersuchungen zur Biologie und Bekämpfung der Kohl- und der Zwiebelfliege, die von hoher wissenschaftlicher Qualität waren und zur Sicherung des bedeutenden Kohl- und Zwiebelanbaus in der hiesigen Region entscheidend beitrugen.

Abb. 3
Gebäude der Zweigstelle in der Ermslebener Straße 52

Pionierarbeit hat BREMER auch bei der Erkennung, Verhütung und Bekämpfung von Samenkrankheiten bei verschiedenen Gemüsearten geleistet. Bereits 1936 wurde die von ihm entwickelte Inkrustierungsmethode zur

Bekämpfung des Zwiebelbrandes eingesetzt. Er konnte auch schon wahrscheinlich machen, daß die Gelbstreifigkeit der Zwiebel virösen Ursprungs ist. Seine Ergebnisse und Empfehlungen brachten den Gemüsesamenbau in Deutschland entscheidend voran und hatten über Jahrzehnte Bestand (KLINKOWSKI 1961). Grundlegende Erkenntnisse wurden zur damaligen Zeit auch von seinen Mitarbeitern - unter denen hier insbesondere BRANDENBURG, HÄHNE, HEILING, LANGENBUCH und ORTH genannt werden sollen - bei der Bearbeitung von Krankheiten der Tomate und von Schädlingen des Rapses und der Erbse gewonnen.

Abb. 4
Hans BREMER
Zweigstellenleiter 1928 - 1935

BREMER mußte auf Grund der Rassengesetzgebung der Nationalsozialisten (das berüchtigte Reichsbürgergesetz) Ende 1935 aus dem Staatsdienst ausscheiden. Er konnte Deutschland noch rechtzeitig verlassen und war jahrelang in der Türkei beim Aufbau des dortigen Pflanzenschutzdienstes tätig, ehe er nach dem Krieg zurückkehrte und ein Institut der Biologischen Bundesanstalt in Neuß übernahm. Er war später auch noch mehrfach in Aschersleben zu Besuch und hat auch 1960 an der 40-Jahrfeier des Instituts hier in diesem Saal teilgenommen. BREMER erhielt im Jahre 1964 die Ernst-Appel-Gedenkmedaille, bei deren Verleihung in Wiesbaden er tragischerweise verstarb.

4. Vorkriegsjahre und Kriegszeit (1936 - 1945)

Nach der Ära BREMER, die einen ersten Höhepunkt in der Geschichte der phytopathologischen Forschung in Aschersleben darstellte, wurde der Zoologe R. LANGENBUCH (seit 1929 in Aschersleben), der nach der Schilderung von Zeitzeugen ein strammer Parteigenosse, aber auch ein guter Entomologe war, 1937 zum neuen Zweigstellenleiter ernannt. Außer ihm waren in jenen Jahren die schon erwähnten Mitarbeiter HÄHNE (seit

1930) und HEILING sowie der Zoologe KÖRTING als Wissenschaftler in Aschersleben tätig. Wie einschlägigen Zeitungsbeiträgen zu entnehmen ist, sahen sich die Mitarbeiter seinerzeit in die sog. "Erzeugungsschlacht" einbezogen, bei der es um die Sicherung der Ernährung für die Bevölkerung aus heimischen Produkten ging.

Mit dem Beginn des Zweiten Weltkriegs nahm der Niedergang der Aschersleber Zweigstelle, der mit dem Ausscheiden BREMERs begonnen hatte, seinen Fortgang. Während des Krieges verringerte sich die Zahl der Wissenschaftler durch Einberufungen zum Wehrdienst immer mehr, so daß zuletzt nur noch der Zweigstellenleiter verblieben war. Ganz kurz vor Kriegsende traf K. O. MÜLLER, der bis zu diesem Zeitpunkt die ausgelagerte Dienststelle für Vererbungslehre und Angewandte Züchtungsforschung in Eichhof / Pommern geleitet hatte, in Aschersleben ein. In Verbindung mit der Evakuierung von Dienststellen aus Berlin-Dahlem wurde Anfang 1945 auch L. BEHR, der später als Ordinarius für Phytopathologie an der MLU Halle wirkte, nach Aschersleben versetzt.

Nachdem am 18. April 1945 amerikanische Truppen die Stadt besetzt hatten, übernahm K. O. MÜLLER auf Anordnung der Militärbehörden die Leitung der inzwischen verwaisten Zweigstelle. Doch schon nach wenigen Wochen hat die nunmehr zuständige sowjetische Militärverwaltung diesen zu den bedeutendsten Wissenschaftlern der BRA gehörenden Mykologen zum Vorsitzenden des Verwaltungsrates der DSG nach Halle /S. berufen, wo er auch den neu gegründeten Lehrstuhl für Phytopathologie der Universität Halle erhielt. An seiner Stelle wurde mit Wirkung vom 1. August 1945 Maximilian KLINKOWSKI, einer seiner früheren Mitarbeiter, der sich zu dieser Zeit in Stecklenberg befand, neuer Leiter der Zweigstelle (Abb. 5).

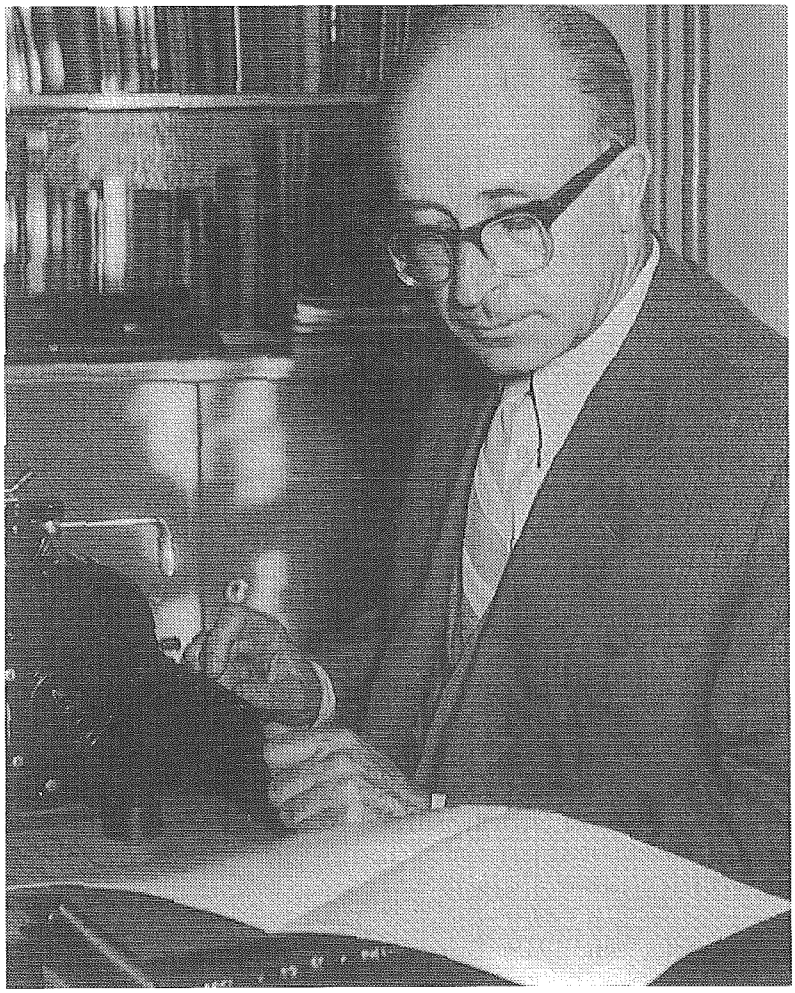

Abb. 5
Maximilian KLINKOWSKI
Zweigstellenleiter und Direktor 1945 - 1970

5. Zeit des Wiederaufbaus (1945 - 1951)

KLINKOWSKI gelang es, die kleine Zweigstelle über die schwierigen Nachkriegsjahre zu retten und mit neuen Mitarbeitern (STOLL, NOLTE, EICHLER) wieder aufzubauen. Er hat gern erzählt, daß es in der ersten Zeit nicht nur um die Wiederaufnahme der wissenschaftlichen Arbeit ging, sondern oftmals ganz handfest die Zahlung der Gehälter und die Sicherung der Ernährung für die Mitarbeiter organisiert werden mußte. Ganz selbstverständlich wurden damals Feldfrüchte und Gemüse zur Versorgung der eigenen kleinen Institutsküche und den Eigenbedarf angebaut, wobei jeder Mitarbeiter mit Hand anlegen mußte.

Am 1. Januar 1946 erfolgte die Zuordnung der Zweigstelle Aschersleben zur Biologischen Zentralanstalt für Land- und Forstwirtschaft (BZA) in Berlin-Dahlem als Nachfolgeinstitution für die BRA. Die BZA gehörte zur neu geschaffenen "Deutschen Verwaltung für Land- und Forstwirtschaft" (DVLF), die ihrerseits im März 1948 als "Hauptverwaltung Land- und Forstwirtschaft" in die "Deutsche Wirtschaftskommission" übernommen wurde.

Die Ereignisse im Zusammenhang mit der Teilung Berlins im Jahre 1949 brachten es mit sich, daß es zu einer Spaltung der BZA kam, deren damaliger Präsident SCHLUMBERGER mit einem Teil der Einrichtungen und einigen Mitarbeitern nach Kleinmachnow übersiedelte.
Die Entwicklung der Zweigstelle Aschersleben wurde durch diese bedauerlichen Geschehnisse jedoch nicht entscheidend beeinträchtigt. Auch die fachlichen Kontakte mit den Kollegen in Dahlem, Braunschweig, Kiel und anderswo blieben noch über viele Jahre erhalten. Daß für die Zweigstelle keine negativen Auswirkungen eintraten, war nicht zuletzt ein Verdienst des neuen Leiters. Er erreichte es auch, daß sich die Zahl der Mitarbeiter allmählich erhöhte und Anfang der 50er Jahre bereits wieder auf den Vorkriegsstand angestiegen war. Ein neues kleines Laborgebäude konnte schon 1950 gebaut und im darauffolgenden Jahr bezogen werden.

6. Ausbau des Instituts zu einer zentralen Forschungseinrichtung (1952 - 1970)

Am 1. Juli 1952 wurden die in den Ländern Brandenburg und Sachsen-Anhalt gelegenen Zweigstellen der BZA, darunter auch Aschersleben, in die neu gegründete Deutsche Akademie der Landwirtschaftswissenschaften (DAL) übernommen. Die Zweigstelle bekam gleichzeitig den Rang eines selbständigen Instituts und erhielt den Namen "Institut für Phytopathologie Aschersleben". Die Akademie der Landwirtschaftswissenschaften wurde seinerzeit nach sowjetischen Vorbild als kleine Schwester der Deutschen Akademie der Wissenschaften zu Berlin geschaffen und bildete das Rückgrat der Agrarforschung in der DDR.

Mit der Bildung des "Instituts für Phytopathologie Aschersleben" erfolgte schrittweise eine Erweiterung des Aufgabengebiets und der Forschungskapazität. Dieser zielstrebige Ausbau des Forschungsstandorts Aschersleben ist in einem hohen Maße dem wissenschaftlichen Ruf, der Weitsicht und den organisatorischen Fähigkeiten von M. KLINKOWSKI zu danken. Seine vielseitigen wissenschaftlichen Interessen und seine fachliche Kompetenz haben entscheidend dazu beigetragen, daß Aschersleben allmählich zu einem Schwerpunkt der phytopathologischen Forschung in der DDR wurde. Ganz wesentlichen Einfluß hat darauf auch der langjährige Präsident der DAL, Hans STUBBE, genommen, der zugleich Direktor des benachbarten Instituts für Genetik und Kulturpflanzenforschung Gatersleben war.

Der Ausbau der Agrarforschungskapazität in Aschersleben, wie auch an anderen Standorten in der DDR, war eine Konsequenz aus der erklärten Partei- und Regierungspolitik jener Jahre, die einerseits die Organisationsstrukturen der Sowjetunion zum Vorbild hatte und andererseits eine von äußeren Einflüssen möglichst unabhängige Forschung für den spezifischen Bedarf der hiesigen Landwirtschaft installieren wollte. Auf dem Gebiet des Pflanzenschutzes gab es von Anfang an eine klare Aufgabenteilung zwischen dem damals ebenfalls geschaffenen "Institut für Pflanzenschutzforschung" in Kleinmachnow und dem hiesigen Institut. In Kleinmachnow lag weiterhin das Schwergewicht auf Fragen des unmittelbaren Pflanzenschutzes und den damit verbundenen hoheitlichen Aufgaben (Mittelanerkennung etc.), während in Aschersleben mehr grundlagenorientierte Untersuchungen zur Diagnose, Übertragung und Entstehung von Pflanzenkrankheiten und Schädlingsbefall betrieben wurden, wobei allerdings Aspekte der Verhütung und Bekämpfung der bearbeiteten Krankheiten und Schädlinge nicht ausgeklammert blieben.

Während zuvor vornehmlich Virus-, Bakterien- und Pilzkrankheiten sowie Schädlinge an Gemüsekulturen bearbeitet wurden, kamen nach und nach Getreide, Zuckerrüben, Kartoffeln, bestimmte Obstarten sowie verschiedene andere Kulturen als neue Wirtspflanzen hinzu. Im Zuge der Kapazitätserweiterungen entwickelte sich parallel dazu die pflanzliche Virologie immer mehr zu einem Forschungsschwerpunkt, ohne daß allerdings die mikrobiologischen und zoologischen Forschungsarbeiten eine Einschränkung erfahren hätten.

Dies ging einher mit einer systematischen Erweiterung der personellen Kapazitäten und dem Neubau von Labor- und Wirtschaftsgebäuden sowie Gewächshäusern. So entstanden in den Jahren 1954 und 1957 zwei große neue Laborgebäude (Abb. 6). Allein in der Zeit von 1949 bis 1969 wurden in Aschersleben Investitionen in Höhe von 6 Mill. Mark getätigt!

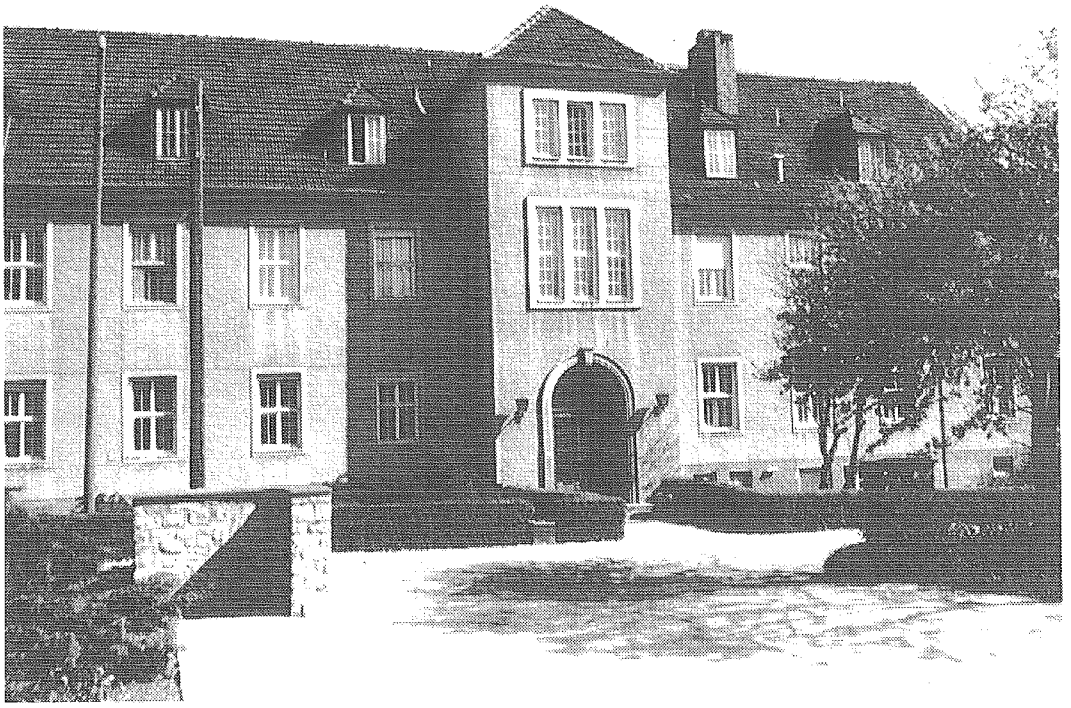

Abb. 6
Hauptgebäude im Theoder-Roemer-Weg

In diesen Zeitabschnitt, den wir jetzt betrachten, fällt allerdings auch das Jahr 1961 mit dem Mauerbau und der rigorosen Abgrenzungspolitik. Der Kalte Krieg erreichte damals seinen Höhepunkt und für viele von uns war von da an die Fortführung oder gar der Aufbau von fachlichen Kontakten zu Kollegen in der "alten" Bundesrepublik und Westberlin oder im westlichen Ausland nicht mehr möglich. Konnten bis zu diesem Zeitpunkt noch wechselseitige Instituts- oder Tagungsbesuche stattfinden, waren die bestehenden Verbindungen danach zunächst völlig unterbrochen. Lediglich an internationalen Tagungen konnten noch einzelne Wissenschaftler teilnehmen. Dagegen wurden die Beziehungen zu den Kollegen in den sog. sozialistischen Ländern gepflegt und ausgebaut. Auch hier waren zweifellos die guten persönlichen Beziehungen von M. KLINKOWSKI zu den dortigen Kollegen sehr hilfreich. Mit ihnen wurden auch regelmäßige Konsultationen, gemeinsame Tagungen, Studienaufenthalte usw. organisiert. Diese wissenschaftlichen Kontakte waren für beide Seiten sehr nützlich, konnten aber natürlich das Fehlen von Verbindungen zu westlichen Forschungsgruppen nicht wettmachen.
Eine Reihe von profilierten Mitarbeitern haben aus diesen und anderen Gründen für sich die Konsequenz gezogen und sind - solange es noch möglich war und meist unter Zurücklassung all ihrer Habe - nach Westdeutschland gegangen, um sich dort eine neue Existenz aufzubauen. Wir freuen uns sehr, daß einige von ihnen an dieser Jubiläumsveranstaltung teilnehmen können.

Im Rahmen der Institutserweiterung vollzog sich nach 1952 auch eine Aufgliederung der Fachabteilungen. Bestanden zunächst - wie vor dem Krieg - nur zwei, später drei Abteilungen, nämlich Entomologie, Mikrobiologie und Virusforschung, wurden nach der Gründung des selbständigen Instituts auch einige neue geschaffen.
Von 1970 an bestand das Institut für Phytopathologie aus sieben Abteilungen:

Virusforschung	Mikrobiologie
Obstvirosenforschung	Entomologie *
Elektronenmikroskopie *	Biochemie *
Serologie *	

Die mit einem * markierten Abteilungen beschäftigten sich ebenfalls fast ausschließlich mit Untersuchungen an Pflanzenviren.

Aus den 50er und 60er Jahren sollen an dieser Stelle eine Reihe von Arbeiten und Ergebnissen Aschersleber Wissenschaftlern erwähnen werden, die teils grundlegende wissenschaftliche Bedeutung hatten, teils über längere Zeit für die landwirtschaftliche Praxis von unmittelbarem Nutzen waren.

Betrachten wir zunächst das Fachgebiet der Pflanzlichen Virologie.

Zu Beginn der 50er Jahre haben KLINKOWSKI und SEDLAG erstmals für das Gebiet systematische Erhebungen zum Problem der Virösen Rübenvergilbung durchgeführt. Im Rahmen der Untersuchungen wurde auch die Bedeutung der Blattlauspopulationsdichte für die Befallsstärke erkannt und eine gezielte Bekämpfung durch die räumliche Trennung von Produktionsflächen und Saatgutvermehrung (durch Stecklingsanbau in isolierten Lagen) empfohlen. Auch haben die Bearbeiter schon damals auf die Bedeutung der Resistenzzüchtung hingewiesen.
Von praktischer Auswirkung für den gesamten europäischen Raum waren Untersuchungen von KLINKOWSKI und SCHMELZER über das Virus der Tabakrippenbräune, das sich als Stamm des Kartoffel-Y-Virus erwies. Erstmals konnte das Vorkommen dieses Virusstamms in mitteleuropäischen Kartoffelbeständen nachgewiesen werden.

Im größeren Umfang wurden seit 1953 Viruskrankheiten der Obstbäume bearbeitet. Am Beispiel des Sauerkirschenverfalls konnten G. BAUMANN und KLINKOWSKI mit Hilfe von Pfropfübertragungen den virösen Charakter dieser auch als "Stecklenberger Krankheit" bekannt gewordenen Erscheinung nachweisen. Neben Steinobstviren wurden auch Viren bei Kernobst bearbeitet und praktische Schritte zur Bekämpfung von Obstviren eingeleitet (KEGLER). So wurde frühzeitig erkannt, daß die Auslese und Vermehrung virusfreier Mutterpflanzen die wirksamste Form der Bekämpfung von Obstvirosen darstellen. Bis zur Mitte der 60er Jahre konnten bereits von 64 Obstsorten und -unterlagen virusfreie Pflanzen für die gesunde Aufzucht in Baumschulen selektiert werden.

In den 60er Jahren hat SCHMELZER gründliche Untersuchungen über die Virosen der Zier-, Forst- und Wildgehölze durchgeführt. Es konnten dabei auch eine Vielzahl von Virosen ermittelt werden, die bisher unbekannt bzw. nicht als virusbedingte Krankheiten erkannt worden waren. Einen weiteren Schwerpunktobjekt der virologischen Forschung stellte über viele Jahre der Hopfen dar (H. E. SCHMIDT). Diese Untersuchungen führten schließlich zur Ausarbeitung einer Bekämpfungsmethode, die aus der Kombination einer Wärmetherapie von Fechsern mit der in vitro-Kultur von Spitzenmeristemen bestand.

In die 60er Jahre fallen auch die Anfänge der später erheblich erweiterten Bearbeitung von Grundlagen für die Virusresistenzzüchtung bei Gemüsekulturen durch eine enge Zusammenarbeit mit den Pflanzenzüchtern. So gelang SKIEBE und SCHMELZER beim Spinat die Züchtung einer gegenüber dem Gurkenmosaik-Virus resistenten Hybridlinie.

Elektronenmikroskopische Untersuchungen zur Analyse von Viruskrankheiten wurden Mitte der 50er Jahre von PAWLITSCHEK aufgenommen und später von H. B. SCHMIDT fortgesetzt. Zu den Viren, die erstmals abgebildet und vermessen werden konnten, gehörten neben einigen bekannten Hopfen- und Apfelviren auch das Scharkavirus der Pflaume (plum pox virus).
Nachdem Ultrazentrifugen mit Schwenkbecherrotoren zur Verfügung standen, wurden auch bei der Reindarstellung isometrischer Viren wichtige Fortschritte mit Hilfe der Dichtegradienten-Zentrifugation erreicht (PROLL).

Mit Arbeiten zur Serologie von Pflanzenviren konnte erst in den Jahren 1962/63 begonnen werden. Die Untersuchungen konzentrierten sich zunächst auf den Nachweis von Viren der Obst- und Ziergehölze (NEPO- und ILAR-Viren). Durch Virusreinigung über Dichtegradienten-Zentrifugation wurden durch J. RICHTER und Mitarbeiter bald deutliche Fortschritte bei der Herstellung diagnostischer Antiseren erzielt.

Mikrobiologie

In der Abteilung für Mikrobiologie standen in den frühen 50er Jahren u.a. Fußkrankheiten der Erbse, insbesondere *Ascochyta pinodella*, im Mittelpunkt. STOLL gelang es, eine Infektionsmethode für Erbsengenotypen zu entwickeln, die für Feldprüfungen geeignet und daher für die Resistenzzüchtung von großer Bedeutung war. Einen weiteren Schwerpunkt bildete der Kartoffelschorf und seine Bekämpfung (G. M. HOFFMANN). Durch umfangreiche Vergleichsuntersuchungen konnte festgestellt werden, daß lediglich Vertreter von *Streptomyces scabies* die vielgestaltigen Erscheinungsformen des Kartoffel- und Rübenschorfs auszulösen vermögen. Im Rahmen dieser Arbeiten wurde auch eine neue Resistenzprüfmethode entwickelt, die für die Kartoffelzüchtung große Bedeutung gewann.

Grundlegende Arbeiten zur Ätiologie, Übertragung, wirtschaftlichen Bedeutung und Bekämpfung des Parasitären Halmbruchs bei Weizen und anderen Getreidearten wurden in den Jahren bis 1966 von LANGE-de la CAMP durchgeführt. Diese Arbeiten haben in wissenschaftlicher und praktischer Hinsicht grundlegende Bedeutung erlangt und sind teilweise noch heute hochaktuell.

Eine gründliche Bearbeitung des Luzerneklappenschorfes, *Pseudopeziza medicaginis*, der in Mitteldeutschland eine beachtliche Bedeutung hatte, ist SCHMIEDEKNECHT zu danken. Hierbei nahmen epidemiologische Fragen, Anfälligkeitsprüfungen an Luzernesorten und verwandten Leguminosenarten sowie Untersuchungen zur Resistenzvererbung einen breiten Raum ein.

Einen weiteren Schwerpunkt bildete eine heute sehr moderne Problematik, nämlich die Analyse von Pflanzenschutzmittelnebeneffekten auf das Gleichgewicht und die Leistungsparameter von Bodenmikroorganismen (NAUMANN). Aus den Arbeiten wurde deutlich, daß bei Anwendung normaler Aufwandmengen keine nachhaltige Beeinträchtigung der mikrobiellen Stoffumsetzung im Boden eintritt.

Aus den 60er Jahren sind ferner Untersuchungen zur Rolle von Unkrautpflanzen für die Übertragung von bodenbürtigen Krankheitserregern (*Verticillium*-Arten; *Rhizoctonia solani*) (SKADOW und E. GRIESBACH) sowie Arbeiten zur Ökologie, Pathologie und Bekämpfung von *Typhula incarnata* an Wintergetreide (H. LEHMANN) zu nennen.

Bakterielle Pflanzenkrankheiten wurden zunächst nur in geringem Umfang bearbeitet. Es gab lediglich zu Beginn der 60er Jahre Befunde über das Auftreten von *Pseudomonas syringae* pv. *coronafaciens* und pv. *striafaciens* an Hafer (MÜLLER) und Untersuchungen zur Ausbreitung und Bekämpfung der "Eckigen Blattflecken"-Krankheit der Gurke, die seinerzeit beachtliche Schäden im Vermehrungsanbau hervorrief (NAUMANN).

Eine interessante Episode in der Geschichte der mikrobiologischen Forschung in Aschersleben stellte die Bearbeitung von antimikrobiellen Hemmstoffen (Antibiotika) und ihre Anwendung zur Bekämpfung von pflanzlichen Infektionskrankheiten dar. Ausgehend vom erfolgreichen Einsatz von Antibiotika in der Human- und der Veterinärmedizin sollte die Wirkung dieser Substanzen im Pflanzenschutz erprobt werden. Hierfür wurde 1956 unter Hedwig KÖHLER eine eigene Abteilung, später Arbeitsgruppe, geschaffen, die nicht nur mit kommerziellen Antibiotika arbeitete, sondern insbesondere auch natürliche Antagonisten von Schaderregern - vor allem aus der Gruppe der Streptomyzeten - und deren Stoffwechselprodukte zur direkten biologischen Bekämpfung einsetzte. Die Hemmstoffproduktion sollte sogar halbtechnisch erfolgen. Diese auch heute wieder sehr aktuelle Bekämpfungsstrategie wurde gegen die verschiedensten Krankheitserreger im Getreide-, Gemüse-, Zierpflanzen- und Obstbau erprobt. Trotz teilweise recht erfolgversprechender Ergebnisse wurde diese Forschungsrichtung nur bis 1968 betrieben.

Entomologie

Von den entomologischen Arbeiten dieser Zeit sollen vor allem die langjährigen Untersuchungen über Rapsschädlinge Erwähnung finden, die in den 50er Jahren komplex durch NOLTE und FRITZSCHE bearbeitet wurden. Das Prinzip der Bestandesüberwachung zur Bestimmung der Flugtermine von Schädlingen und der Signalisierung der Bekämpfungstermine auf Grund biologisch-ökologischer Daten für Wirtspflanzen und Schaderreger ist im Falle von Rapsglanzkäfer, Großen Kohltriebrüßler, Kohlschotenrüßler und Kohlschotenmücke auch heute noch gültig.

Pionierarbeit, die vor allem mit dem Namen NOLTE verbunden ist, wurde auch bei der Einführung der Inkrustierungstechnik für Zwiebelsaatgut geleistet, wodurch eine effektive Bekämpfung der Zwiebelfliege mit einem Minimum an Pflanzenchutzmittelaufwand erreicht werden konnte. Schon damals (um 1960) wurde die Rückstandsproblematik bei Anwendung von Kontaktinsektiziden intensiv diskutiert.

Ende der 50er Jahre begann FRITZSCHE umfangreiche Untersuchungen zum Spinnmilbenauftreten in Obst- und Gemüsekulturen, wobei die sorgfältige Analyse der Wechselbeziehungen zwischen Wirtspflanze und Schädling sowie die Akarizidresistenz im Mittelpunkt standen.

Im Verlauf der 60er Jahre veränderte sich das Profil der Abteilung Entomologie insofern wesentlich, als sich von dieser Zeit an die Forschung ganz auf die komplexe Bearbeitung tierischer Vektoren von Viruskrankheiten konzentrierten. Die Untersuchungen erstreckten sich auf Nematoden, Blattläuse, Zikaden, Wanzen und Gallmilben. So wurde für eine Reihe von Aphidenarten erstmals der Nachweis erbracht, daß sie als Vektoren für Viren mit nichtpersistentem Übertragungsmodus fungieren können (KARL). Im gleichen Zeitraum bearbeitete W. LEHMANN die Gruppe der Zikaden als Überträger der Blütenvergrünung, einem Krankheitstyp, den man in den folgenden Jahren als Mykoplasma-bedingt erkannte. Weiterhin wurden zur Übertragung von Pflanzenviren befähigten Gallmilben bearbeitet (PROESELER).

Biochemie

Ebenfalls in den 50er Jahren wurde eine Abteilung für Biochemie aufgebaut, deren Aufgabe darin bestand, die in kranken Pflanzen ablaufenden Stoffwechselvorgänge bis hin zur Symptombildung zu analysieren. Im Vordergrund des Interesses standen dabei zunächst die pathologischen Prozesse in viruskranken Pflanzen. Die mit den Namen von WOLFFGANG, OPEL, HERZMANN und HOFFEREK verbundenen Arbeiten erstreckten sich vornehmlich auf den Kohlenhydrat- und den Proteinstoffwechsel der Wirtspflanzen und dabei vor allem auf die Rolle der beteiligten Enzyme. Einen weiteren Problemkreis bildeten Untersuchungen über natürliche Virushemmstoffe in der Pflanze, die letzten Endes zu einer erheblichen Verbesserung der Methodik zur Virusinokulation führten (OPEL).

Besondere wissenschaftliche Höhepunkte am Standort Aschersleben in jenem Zeitraum waren

- die internationale Tagung aus Anlaß des 40. Jahrestags der Institutsgründung 1960 mit prominenten Teilnehmern aus Ost und West
- das internationale Symposium zur "Hemmung und Förderung phytopathogener Mikroorganismen im Boden" 1961
- das Symposium "Biochemische Grundlagen der Pflanzenpathologie" 1964 mit einer großen Zahl weltbekannter Fachleute aus Europa und Übersee und
- das 6. Europäische Obstvirosensymposium 1968.

Diese wohl organisierten Tagungen waren Meilensteine der wissenschaftlichen Arbeit des Instituts in jenen Jahren.

7. 1971 - 1991

1970 trat KLINKOWSKI in den Ruhestand. Ungeachtet dessen, blieb er - bis zu seinem allzufrühen Tod 1971 - weiterhin wissenschaftlich aktiv und für seine ehemaligen Mitarbeiter jederzeit ansprechbar.

Unter seinen Nachfolgern D. SPAAR (1970 - 1972), H. J. MÜLLER (1972 - 1976) und H. KLEINHEMPEL (1976 - 1990) setzte sich in den 70er und 80er Jahren der systematische Ausbau des Forschungsstandorts Aschersleben fort. Es wurden zwei neue Laborgebäude, ein großer Bibliotheksanbau sowie ein kombiniertes

Klima- und Gewächshaus errichtet. Zuletzt waren fast 300 Mitarbeiter im Institut tätig, die freilich unter immer schwieriger werdenden Bedingungen phytopathologische Forschung betrieben haben. Vor allem wirkten sich die weitgehende Unterbindung von wissenschaftlichen Kontakten zu Kollegen und Instituten in allen westlichen Ländern, die extreme Sicherheitshysterie als Folge des Kalten Krieges und die zunehmende ideologische Indoktrinierung bis hin zur Allgegenwart des Staatssicherheitsdienstes sehr nachteilig aus. Zusätzliche Probleme verursachten die wachsenden Schwierigkeiten bei der Beschaffung moderner Forschungsmittel (Geräte, Chemikalien).

Dennoch wurden auch zu dieser Zeit international beachtete wissenschaftliche Leistungen erbracht und ganz neue Forschungsrichtungen (Molekularbiologie, Anlage von pflanzlichen in vitro-Kulturen, Herstellung von Präparaten zur biologischen Bekämpfung von Pflanzenschädlingen, Gewinnung hochspezifischer monoklonaler Antikörper zum Nachweis von Krankheitserregern u.a.m.) aufgegriffen.

Besondere Erwähnung verdient die Tatsache, daß in dieser Zeit eine regelmäßige Tagungsreihe unter dem Titel
"Recent Results on Virus Research"
ins Leben gerufen wurde, die in dreijährigem Turnus Phytovirologen aus den RGW-Ländern zusammenführte. Daneben wurden mehrere kleinere Workshops und Konferenzen zu Fragen der Resistenz gegen Pilzkrankheiten und zu serologischen Fragen mit Teilnehmern aus den osteuropäischen Ländern durchgeführt.

Zum 50jährigen Institutsjubiläum im Jahre 1970 fand in diesen Räumen hier eine Tagung statt, an der neben vielen osteuropäischen Kollegen auch einige Gäste aus dem westlichen Ausland und der Bundesrepublik teilnehmen konnten.
1976 wurde vom Institut eine internationale Konferenz zur Problematik bakterieller Naßfäulen der Kartoffeln mit Vertretern aus den meisten osteuropäischen Ländern einschließlich Jugoslawiens ausgerichtet.
Aus Anlaß des 30jährigen Jubiläums der Namensgebung für das Institut für Phytopathologie Aschersleben fand 1982 mit Kollegen aus den RGW-Ländern eine größere Tagung zur
"Resistenz gegen Schaderreger"
in Halle statt.

Die auf den meisten dieser Tagungen gehaltenen Beiträge wurden in den
"Tagungsberichten der Akademie der Landwirtschaftswissenschaften der DDR"
publiziert und damit der Öffentlichkeit zugänglich gemacht.

Im folgenden soll auf einige wesentliche wissenschaftliche Ergebnisse aus dem Zeitraum von 1971 bis 1990 eingegangen werden:

Pflanzliche Virologie

Auf dem Gebiet der Pflanzlichen Virologie wurden in diesem Zeitraum verstärkt Untersuchungen zur Virusresistenz verschiedener Kulturpflanzengruppen aufgenommen. So entwickelten H. E. SCHMIDT bei Leguminosen und später L. SCHUBERT, I. WEBER und K. GRAICHEN bei Gemüsekulturen, PROESELER und A. HABEKUSS bei Getreide sowie KEGLER und SCHIMANSKI bei Baumobst und GRAICHEN bei Beerenobst gleichzeitig mit der Selektion virusresistenten Zuchtmaterials neue Verfahren zur Virusresistenzprüfung. Diese Arbeiten waren die Voraussetzung dafür, daß bis 1990 in der ehemaligen DDR etwa 40 Sorten landwirtschaftlicher und gärtnerischer Kulturpflanzenarten mit Virusresistenz zugelassen werden konnten. An einer Reihe von Modellobjekten wurden seinerzeit auch Grundlagen der quantitativen Virusresistenz bearbeitet (KEGLER, I. WEBER, H. E. SCHMIDT und U. MEYER).
Besondere Erwähnung verdienen auch die durch den Einsatz moderner Elektronenmikroskope und Präparationsverfahren möglich gewordenen Untersuchungen an Champignon-Viren (H. B. SCHMIDT) und der verschiedenen Gramineenviren sowie des Milden Rübenvergilbungsvirus (STANARIUS). Mit dem Gurkenblattflecken-Virus konnten I. WEBER und STANARIUS ein bisher an Gurken unbekanntes Virus isolieren und beschreiben.

Neben der klassischen experimentellen Forschung an krankem Pflanzenmaterial und zur Virusmorphologie wurden auch zunehmend Untersuchungen zu den immunologischen und physiko-chemischen Eigenschaften von Viruspartikeln durchgeführt, insbesondere an Bromo-, Carla-, Diantho- und Cryptoviren (PROLL, KÜHNE).

In den 80er Jahren wurden die serologischen Arbeiten - parallel mit der Einrichtung eines Tierstalls - erheblich intensiviert. Neben diagnostischen Aspekten konzentrierten sie sich vor allem auf die Stamm- und

Isolatecharakterisierung sowie die Analyse und Lokalisierung von Epitopen auf dem Virushüllprotein. Als Nachweisverfahren gewann seit 1980 der enzyme-linked immunosorbent assay (ELISA) mit seinen Varianten immer mehr an Bedeutung. Speziell für die Amtliche Pflanzkartoffelprüfung wurde eine miniaturisierte Variante mit gebrauchsfertigen Testkits entwickelt. Insgesamt konnten in dieser Zeit Testsysteme für 50 verschiedene Pflanzenviren entwickelt werden (J. RICHTER, EISENBRANDT, REICHENBÄCHER, I. HAACK u. a.).

In die Zeit der 80er Jahre fällt auch die Einführung der Hybridomtechnik zur Herstellung monoklonaler Antikörper (RABENSTEIN).

Parallel zur Erzeugung der ersten Hybridzellinien konnten in Aschersleben auch erstmals virale Nukleinsäuren kloniert und auf ihre Eignung als DNS- bzw. RNS-Sonden zum Virusnachweis geprüft werden (LEISER, SCHUBERT).
Mit ersten molekularbiologisch-gentechnologischen Experimenten wurde 1982 begonnen. Diese Arbeiten kamen zunächst wegen der schon erwähnten schwierigen Versorgungssituation bei Geräten und Biochemikalien nur langsam voran. Im Laufe der Zeit wurde aber nach und nach die RNS verschiedener Viren erfolgreich extrahiert und kloniert (SCHUBERT, KÜHNE) . Bei einigen ausgewählten Potyviren konnten durch Sequenzanalyse die Hüllproteingene identifiziert, rekloniert und in *Escherichia coli* exprimiert werden. Es gelang auch, verschiedene Methoden zur Einschleusung fremder DNS in pflanzliches Material zu etablieren und so die Voraussetzungen für die Erzeugung transgener Pflanzen mit erhöhter Virusresistenz zu schaffen.

Phytobakteriologie

Das Aufgabengebiet der Abteilung Mikrobiologie änderte sich ab Ende 1968 grundlegend. Damals wurden die mykologischen und bodenmikrobiologischen Arbeiten zugunsten bakteriologischer Themen eingestellt. Hierbei bildeten sich in der Folge zwei Schwerpunkte heraus, nämlich die Knollennaßfäulen der Kartoffel und die bakteriellen Obstkrankheiten.

Anlaß zur forcierten Bearbeitung der Kartoffelnaßfäulen waren große Verluste in der Kartoffelwirtschaft durch den massiven Einsatz von Vollerntemaschinen. Diese Arbeiten drehten sich vor allem um das Spektrum der beteiligten Erreger, ihre Übertragungswege, die Belastung der Pflanzkartoffeln, den Infektionsvorgang und die Einflüsse äußerer Faktoren auf die Krankheitsentstehung (NAUMANN, ZIELKE, FICKE und PETER).

Ende der 70er Jahre rückte dann auch die Bakterienringfäule der Kartoffel ins Blickfeld. Es wurden sichere Nachweisverfahren für diesen Quarantäneerreger erarbeitet, eine umfassende Sortenanfälligkeitsprüfung durchgeführt und die Schadwirkung von Mehrfachinfektionen (Naß- und Ringfäuleerreger) untersucht (ZIELKE, PETER, NAUMANN).

Einen weiteren Schwerpunkt bildeten die bakteriellen Obstkrankheiten, vor allem die Diagnose, Übertragung und Bekämpfung des Feuerbrandes (G. WOLF, FICKE, BEYME und SCHAEFER). Deren Ergebnisse führten schließlich zur Aufstellung eines Computer-gestützten Überwachungssystems für Apfelanlagen (MÄURER und GUTSCHE). Wesentliche Fortschritte wurden auch bei der Feuerbrandbekämpfung (SCHAEFER, M. NACHTIGALL und K. RICHTER) und - in Zusammenarbeit mit Ch. FISCHER vom Institut für Obstforschung Dresden-Pillnitz - vor allem bei der Resistenzevaluierung und -züchtung erzielt.

In den 80er Jahren wurde mit der Bearbeitung von Bakteriosen an wichtigen Futter- und Gemüsepflanzen begonnen (NAUMANN, E. GRIESBACH und ZIELKE). Schwerpunkte waren von da an Bakteriosen der Ackerbohne, des Kohls, der Tomate und der Gemüsebohne. Insbesondere bei der Bakteriellen Tomatenwelke gelang es, die Basis für die erfolgreiche Züchtung resistenter Sorten zu schaffen, die durch KLEINHANNS in der Zuchtstation Eisleben erfolgte. Weitere spezielle Untersuchungsobjekte waren die Saatgutübertragung und die Ausbreitung der Bakteriellen Tomatenwelke in anfälligen und resistenten Sorten. Die Untersuchungen an Bohnen galten vor allem der Übertragung der Fettfleckenkrankheit durch das Saatgut und der Bekämpfung dieser im mitteldeutschen Anbaugebiet sehr verbreiteten Krankheit sowie dem Verhalten dieses Erregers in anfälligen und resistenten Pflanzen (NAUMANN und H. B. SCHMIDT).

Ende der 80er Jahre konnte in Aschersleben auch erstmals der Schwärzliche Bohnenbrand auf dem Gebiet der ehemaligen DDR nachgewiesen und die Anfälligkeit der wichtigsten Bohnensorten ermittelt werden.

Mykologie

Arbeiten auf dem Gebiet der Mykologie wurden nach längerer Pause in den 70er Jahren wieder aufgenommen (SCHMIEDEKNECHT, WOLFFGANG, HOFFEREK, SCHUBERT). Dabei standen u. a. die biochemisch-physiologischen Grundlagen der Resistenz von Pflanzen gegen Pilzkrankheiten im Mittelpunkt. Als Versuchsobjekt diente das Wirt/Pathogen-System Gerste/Gelbrost. Dabei konnte aus den Wurzeln infizierter resistenter Pflanzen ein spezifischer Resistenzfaktor isoliert werden.

In den Folgejahren wurde die mykologische Forschung wieder stärker anwendungsorientiert und in enger Kooperation mit der Züchtungsforschung, der praktischen Züchtung und der Genbank des Instituts für Genetik und Kulturpflanzenforschung (IPK) in Gatersleben betrieben. In diesem Zusammenhang konnten effektive Methoden zur Resistenzprüfung gegen Fußkrankheiten bei Erbsen sowie gegen *Phytophthora nicotianae* und *Fusarium oxysporum* f.sp. *lycopersici* bei Tomate (SKADOW) und gegen *Phomopsis sclerotioides* (HARTLEB) sowie zwei *Fusarium*-Krankheiten an Gurke (J. PELCZ) entwickelt werden.

Ab 1981 rückten wieder verstärkt pilzliche Getreidekrankheiten in den Mittelpunkt. Hauptziel war dabei die Erfassung der horizontalen Resistenzen am Beispiel des Weizengelb- und des Gerstenzwergrostes sowie später auch für das Pathosystem Sommergerste/Zwergrost. OPEL, HARTLEB und GERLACH erarbeiteten dafür quantifizierbare Kriterien (Auslösung von Miniepidemien in Klimakammern, Messung der gebildeten Sporenmenge).

Die Bearbeitung der Wirt/Pathogen-Kombination Gerste/Gerstenmehltau führte zur großflächigen Anwendung von Sortenmischungen bei Sommergerste in der Praxis. Dadurch ließ sich die Fungizidanwendung ganz erheblich reduzieren (SKADOW, HARTLEB u. a.).
Ein weiteres Versuchsobjekt war die Netzfleckenkrankheit der Gerste. Im Ergebnis dieser Arbeiten wurde schon damals Basismaterial für die Resistenzzüchtung bei Sommer- und Wintergerste bereitgestellt.

Mit dem Aufbau eines Zell- und Gewebekulturlabors Anfang der 80er Jahre konnten auch verschiedene in vitro-Systeme in die Untersuchungen zur Resistenz gegen Pilzkrankheiten, vorzugsweise die Kombinationen Kartoffel/Krautfäule und Gerste/Netzfleckenkrankheit, einbezogen werden (SCHLEGEL, R. KRÄMER und HUNOLD). Als Selektionsfaktoren dienten dabei die jeweiligen Erregertoxine.

Auf Grund der kritischen Situation in den damals vorhandenen großen Obstanlagen bildete die Analyse parasitärer Rindenkrankheiten für mehrere Jahre einen weiteren Schwerpunkt der mykologischen Forschung in Aschersleben. Dabei konnten 20 verschiedene Krankheitserreger - darunter einige Erstfunde - nachgewiesen und wirksame Gegenmaßnahmen ausgearbeitet werden (FICKE, U. KASTIRR, A. SENULA und SCHAEFER).

Zoologie

Die zoologischen Arbeiten in Aschersleben konzentrierten sich - wie schon erwähnt - im Laufe der Zeit immer stärker auf die Rolle der Insekten und anderer Tiergruppen als Virusüberträger. Unter den Nematoden wurden vor allem Arten der Gattungen *Xiphinema, Longidorus* und *Trichodorus* bearbeitet (FRITZSCHE).

Aufbauend auf den langjährigen Untersuchungen zur Virusübertragung durch Aphiden begannen FRITZSCHE, GEISSLER und R. KASTIRR in den 80er Jahren systematische Erhebungen über das Blattlausauftreten und die Virusbelastung der natürlichen Blattlauspopulationen. Später entwickelte sich die Blattlausbekämpfung in Zuckerrübenbeständen und deren Auswirkung auf das Auftreten der virösen Rübenvergilbung zu einem Forschungsschwerpunkt. Hierbei lieferten biologisch-ökologische Parameter (Flugaktivität, Populationsdynamik, Virusbelastung) die wesentliche Grundlage.

Einen breiten Raum nahmen auch Untersuchungen zur Resistenz von Möhren gegen Möhrenfliege, Zwiebeln gegen Nematoden, Zuckerrüben u. a. Wirtspflanzen gegen Blattläuse ein (W. LEHMANN, FRITZSCHE, KARL).

Neben angewandten Fragen wurden in Aschersleben durch FRITZSCHE, W. LEHMANN und KARL intensiv kausalanalytische Modelluntersuchungen zur Virusübertragung und zur Resistenz gegen tierische Vektoren betrieben.

In den letzten Jahren bis 1991 konzentrierten sich die entomologischen Arbeiten vor allem auf die Entwicklung moderner biologischer Verfahren zur Bekämpfung von Schadinsekten mit Hilfe von insektenpathogenen Kernpolyederviren (W. LEHMANN, SCHLIEPHAKE, LEISTNER). Auf der Basis von Larvenzuchten hergestellte Viruspräparate wurden bereits erfolgreich gegen Apfelwickler und Kohleule eingesetzt und bei der schwierigen Herstellung von tierischen Zellkulturen als Voraussetzung zur in vitro-Produktion derartiger Viren konnte ein beachtlicher Stand erreicht werden.

8. Publikationstätigkeit

Dieser Rückblick auf die wissenschaftliche Tätigkeit am Standort Aschersleben wäre unvollständig, würde nicht auch erwähnt, daß von Mitarbeitern des IfP im Laufe der Jahre eine Reihe von bedeutenden Fachbüchern erarbeitet und herausgegeben worden sind, die z. T. den Charakter von Standardwerken erlangt haben. Anführen möchte ich hier vor allem die folgenden Monographien:

KÖHLER & KLINKOWSKI: Viruskrankheiten im SORAUER. 1954
SCHICK & KLINKOWSKI: Die Kartoffel (2 Bände). 1961/62
KLINKOWSKI, MÜHLE, REINMUTH: Phytopathologie und Pflanzenschutz (4 Bände). 1965 ff.
KLINKOWSKI et al.: Pflanzliche Virologie (4 Bände). 1967/68
FRITZSCHE, GEILER & SEDLAG: Angewandte Entomologie. 1968
SPAAR, FRITZSCHE & KLEINHEMPEL: Diagnose von Krankheiten und Schädlingen (8 Bände). 1985 ff.
KEGLER & KLEINHEMPEL: Virusresistenz der Pflanzen. 1987
SPAAR & KLEINHEMPEL: Bekämpfung von Viruskrankheiten der Kulturpflanzen. 2. Aufl. 1987
FRITZSCHE, KLEINHEMPEL & PROESELER: Die Viröse Vergilbung der Beta Rübe. 1988
FRITZSCHE, DECKER, LEHMANN, KARL und GEISSLER: Resistenz von Kulturpflanzen gegen tierische Schädlinge. 1988
KLEINHEMPEL, NAUMANN & SPAAR et al.: Bakterielle Erkrankungen der Kulturpflanzen. 1989.

Nicht unerwähnt bleiben soll, daß seit den 50er Jahren durch die Wissenschaftler des Standorts zwischen 50 und 90 wiss. Arbeiten pro Jahr in diversen Zeitschriften publiziert worden sind.

9. Umstrukturierung der Forschung am Institut lt. Einigungsvertrag und Neuaufbau der Bundesanstalt für Züchtungsforschung an Kulturpflanzen ab 1992

Der letzte Abschnitt in der langen Geschichte des Forschungsstandorts Aschersleben begann im Jahre 1990. Die Wende in der ehemaligen DDR hat auch im Institut für Phytopathologie Aschersleben zu großen Veränderungen geführt. Im April 1990 kam es anläßlich einer Wissenschaftlervollversammlung zu einem Wechsel in der Institutsleitung. Herr Kollege PROESELER wurde bei diesem Anlaß zum neuen Institutsleiter gewählt. Er hat damals diese schwierige Aufgabe übernommen, wofür wir ihm zu besonderem Dank verpflichtet sind.

Alsbald knüpfte das Institut auch an eine alte Tradition an und schloß sich mit dem Institut für Pflanzenschutzforschung Kleinmachnow zur "Biologischen Zentralanstalt Berlin" zusammen.

Die lt. Einigungsvertrag vorgesehene Evaluierung des Instituts für Phytopathologie durch den Wissenschaftsrat der Bundesregierung fand im Februar 1991 statt. Sie führte dazu, daß nach dem Auslaufen der Übergangsregelungen für die Akademie-Institute am 31. 12. 1991 ab 1. Januar 1992 in Aschersleben anstelle des bisherigen Instituts für Phytopathologie drei Institute der neu gegründeten Bundesanstalt für Züchtungsforschung an Kulturpflanzen (BAZ) eingerichtet wurden.

Dieser Neuanfang war allerdings mit einer sehr schmerzlichen Reduzierung der Mitarbeiterzahl verbunden. Von den ursprünglich fast 300 Mitarbeitern des IfP (Stand Ende 1989) waren zum Jahresende 1991 bereits 100 ausgeschieden. Von den noch verbliebenen 200 Mitarbeitern konnten von der neuen Bundesanstalt nur etwa 70 auf feste Stellen übernommen werden; hinzu kamen noch einige Mitarbeiter aus dem ehemaligen Institut für Getreideforschung Bernburg/Hadmersleben.

Die Züchtung und der Anbau von Kulturpflanzen mit erhöhter Resistenz gegen pflanzenpathogene Viren, Bakterien und Pilze sowie gegen tierische Schädlinge stellt einen entscheidenden Beitrag zur Verwirklichung eines integrierten Landbaus dar. Sie trägt damit wesentlich dazu bei, hochwertige Nahrungsmittel und Rohstoffe kostengünstig zu erzeugen, ohne umweltbelastende und teure Pflanzenschutzmittel anwenden zu müssen. Die Bereitstellung von gesundem Pflanzenmaterial durch zielgerichtete Förderung und Intensivierung der Resistenzzüchtung ist daher ein zentrales Anliegen der neuen Bundesanstalt. Handelt es sich doch dabei um eine seit langem bewährte Methode, um Verluste durch Schaderreger einzuschränken oder ganz zu vermeiden. Ich darf in diesem Zusammenhang an eine Äußerung von BREMER erinnern, die als Motto für diese Tagung gewählt wurde:

"Der billigste Weg, Schaden durch Pflanzenkrankheiten zu verhüten, ist die Verwendung von Sorten, die gegen Krankheiten widerstandsfähig sind".

Die Konzeption der Bundesanstalt für Züchtungsforschung sieht vor, daß die drei in Aschersleben gebildeten Institute im Rahmen der Züchtungsforschung speziell Fragen der Krankheitsresistenz in ihren verschiedenen Aspekten bearbeiten. Mit der Entscheidung, in Aschersleben ein Zentrum der Resistenzforschung aufzubauen, wurde erreicht, daß die hier seit Jahrzehnten bestehenden Erfahrungen auf diesem Gebiet genutzt sowie die vorhandenen technischen und personellen Kapazitäten optimal wirksam werden können. Die Gründung der Bundesanstalt bietet somit die einmalige Gelegenheit, eine besondere und bewährte Tradition zum Nutzen der deutschen Landwirtschaft hier am Standort fortzusetzen.

Für die Lösung der Aufgabe, in Aschersleben Ressortforschung auf dem Gebiet der Krankheitsresistenz und ihre Anwendung zu betreiben, bieten die räumliche Nähe und die seit langem bestehenden engen Verbindungen zum Forschungsstandort Quedlinburg mit seinen jetzigen BAZ-Instituten und zum Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK) in Gatersleben hervorragende Voraussetzungen.

Die damit umrissene Forschungskonzeption für den Standort Aschersleben bedeutet eine neue Schwerpunktsetzung und eine großartige Perspektive zugleich. Mit der Gründung der neuen Bundesanstalt wurden in Aschersleben die technischen Vorbedingungen dafür geschaffen, um neben den klassischen Verfahren auch moderne Forschungsmethoden und -richtungen in die Resistenzforschung einzuführen. Um die für die Züchtungsforschung in Deutschland derzeit besonders bedeutsamen Probleme aufzugreifen, fanden schon im ersten Halbjahr 1992 intensive Diskussionen mit der Biologischen Bundesanstalt und den verschiedenen Abteilungen der Gemeinschaft der privaten Deutschen Pflanzenzüchtung (GFP) statt. Dabei hat die BAZ als Ressortforschungseinrichtung des Bundes von Anfang an streng darauf geachtet, daß alle Aktivitäten mit der Biologischen Bundesanstalt und den an Resistenzfragen interessierten Hochschulinstituten abgestimmt werden. Das Ziel war dabei stets, die in der Bundesrepublik für die Resistenzforschung verfügbaren Kapazitäten zu bündeln und in ihrer Gesamtheit nutzbar zu machen. Auf diese Weise sind Forschungsprojekte formuliert und in Angriff genommen worden, an denen Mitarbeiter der BAZ und der BBA beteiligt sind, und werden Drittmittelprojekte gemeinsam von Mitarbeitern der BAZ und verschiedener Hochschulen bearbeitet.

Ein Teil der in den letzten 3 1/2 Jahren erarbeiteten wissenschaftlichen Ergebnisse werden in den nächsten Tagen während der wissenschaftlichen Konferenz vorgestellt werden. An dieser Stelle soll daher auf diesen allerjüngsten Abschnitt unserer wechselvollen Geschichte und den dabei erreichten Stand nicht näher eingegangen werden.

10. Zur Zukunft des Forschungsstandorts Aschersleben

Abschließend sei es erlaubt, noch einige Gedanken zu den künftigen Perspektiven der phytopathologischen Forschung in Aschersleben zu äußern:
Der Pflanzenzüchtung kommt bei der Versorgung der Wirtschaft mit qualitativ hochwertigen Nahrungsgütern sowie nachwachsenden Rohstoffen für die Industrie und im Hinblick auf die Stellung der deutschen Landwirtschaft in der EU und auf der Welt eine besondere Bedeutung zu. Der Schlüssel zum Erfolg ist dabei

eine effektive Züchtungsforschung, denn - so hat K. EIGEN neulich formuliert - "die Züchtungsforschung war niemals so dringend wie heute!"

Eine Schlüsselstellung nimmt dabei die Resistenzzüchtung ein. Deren Aufgabengebiet wird sich in der Zukunft noch wesentlich erweitern, wenn nach und nach neue Resistenzquellen erschlossen und neue methodische Fortschritte auf züchterischem und molekularbiologischem Gebiet gemacht werden. Man braucht kein Prophet zu sein, um sagen zu können, daß sie sich in Zukunft immer mehr zu einer tragenden Säule eines modernen integrierten Landbaus entwickeln wird. Der Standort Aschersleben als Teil der BA f. Züchtungsforschung kann dazu in den kommenden Jahrzehnten - so ist zu hoffen - einen wichtigen Beitrag leisten. Die für die nächsten Jahre geplanten Investitionen im Technischen Bereich werden dafür günstige Voraussetzungen schaffen.
Die vielfältigen wissenschaftlichen Kooperationen mit in- und ausländischen Partnerinstituten, die engen Kontakte zur praktischen Pflanzenzüchtung und das Engagement der hiesigen Mitarbeiter sind die Gewähr dafür, daß auch künftig eine intensive Forschung zum Nutzen eines modernen umweltfreundlichen Landbaus betrieben wird. Durch die kontinuierliche Aufnahme von Gastforschern und Praktikanten aus der Dritten Welt, aber auch aus osteuropäischen Ländern, am Standort Aschersleben werden diese Aktivitäten im Sinne der Aufgaben der Ressortforschung auch künftig über Europa hinaus wirksam werden.

Die geschilderten Umstände bieten ausgezeichnete Voraussetzungen dafür, daß der Standort Aschersleben auch in den nächsten Jahrzehnten eine erfolgreiche Entwicklung nimmt. Die Mitarbeiter in Aschersleben werden das Ihre tun, um im Sinne von Hans BREMER und Maximilian KLINKOWSKI weiterzuwirken und den Ruf des Forschungsstandorts Aschersleben zu erhalten und auszubauen!
Auf die nächsten 25 Jahre im Dienste der Forschung!
Der Forschungsstandort Aschersleben - VIVAT, CRESCAT, FLOREAT - er blühe, wachse und gedeihe!

11. Literatur

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Virus diseases of plants

BEET YELLOWS CLOSTEROVIRUS: ADVANCES IN MAPPING FUNCTIONS ENCODED IN A LARGE RNA VIRUS GENOME

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Introduction

Closterovirus group, of which beet yellows virus (BYV) is the type member, combines several positive-strand RNA viruses with filamentous particles that cause economically important infections in plants (1,2). The genomes of BYV (3), citrus tristeza virus (4), and lettuce infectious yellows virus (5) have been completely sequenced. Closteroviruses are unique with respect to several molecular biological aspects. First, they have recordingly large genomes among plant RNA viruses and utilize the expression strategy of animal coronavirus type, with proteolytic processing, ribosomal frameshifting, and subgenomic mRNAs formation being the key mechanisms (3). Second, the closteroviruses contain a unique tandem of genes, one for the viral capsid protein, and another for its diverged duplicate (6); the latter protein forms a distinct 'tail' at one end of the BYV particles revealed by immunoelectron microscopy (7). The third hallmark of the closteroviruses is the putative 65-kDa protein (p65) they encode, which is evidently related to the HSP70 family of cell chaperones (8-10). Previous studies have shown that the p65 of BYV contains the eight regions conserved in all HSP70s (8). It has been suggested that the p65 gene resulted from RNA recombination between an ancestral closterovirus and a cell mRNA, and has diverged while being adapted to specific functions in virus infection (8).

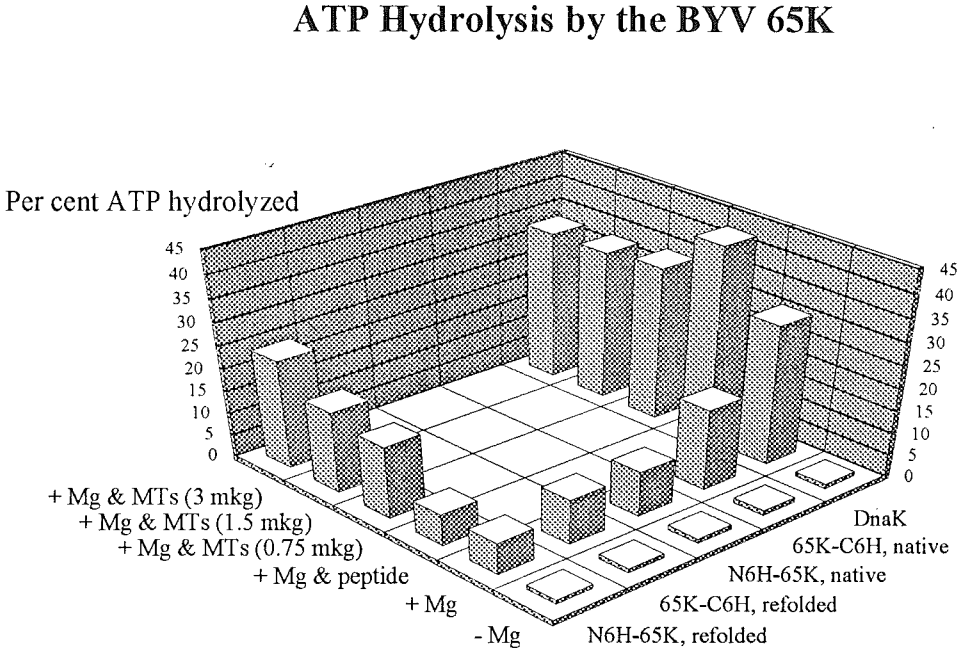
Materials and Methods

The BYV p65 gene sequence were cloned into pQE vectors (DIAGEN) under the control of T5 promoter and a ribosome binding site. The inserts for pQE-p65C6H and pQE-N6H52K were obtained by the PCR. Plasmids pQE-N6H40K and pQE-N6H35K were constructed by cloning the *Bam*HI - *Xho*I and *Bam*HI - *Sca*I fragments of the cloned p65 gene. The plasmids were used for transformation of the *E.coli* strains M15 or SG13009 containing the repressor pREP4 plasmid. Recombinant proteins were purified on Ni-NTA agarose (DIAGEN) in denaturing or native conditions according to the manufacturer's protocols.

ATPase activity in protein samples was assayed in 20 μl reactions containing 1 μCi of [γ-^{32}P]ATP. Some samples were supplemented with sequence-nonspecific peptide bacitracine (Serva) or purified bovine brain microtubules, as specified in the Fig. 1. After incubation, the samples were analysed by thin-layer chromatography. The spots were developed by autoradiography, excised, and counted in toluene scintillation cocktail.

Results

We produced the full-sized recombinant BYV p65 in two versions, N6H-p65 and p65-C6H, having the N- or C-terminal tag of six histidines (6xHis), respectively. The p65 fragments representing N-terminal 35K, 40K, and 52K fragments, all had the N-terminal 6xHis. The proteins were expressed in IPTG-induced *E.coli* cells bearing the respective plasmids and purified by the affinity chromatography on Ni-NTA agarose.



ATPase activity in protein samples was determined *in vitro* by the release of inorganic ^{32}P from [γ-^{32}P]ATP. The results shown in Fig. 1 indicate that the full-sized BYV p65 indeed has a magnesium-stimulated ATPase activity which is typical of the HSP70 chaperones (10). The activity of N6H-p65 and p65-C6H obtained in native conditions was comparable to that of DnaK, the bacterial HSP70 taken as a

positive control, whereas that of the refolded p65 preparations was much lower, perhaps due to incomplete restoration of the proper p65 conformation after gradient dialysis. The p65-N6H exerted a lower ATPase activity than the p65-C6H, indicating that the sequence of six histidines placed at the N-terminus, *versus* the C-terminus of the recombinant protein, impaired either folding or the activity of the p65. Hence, the 6xHis tag may not necessarily have neutral effect on biochemical properties of proteins, and more respect should be paid to its location on the molecule when engineering recombinant proteins. The ATPase activity was fully retained by the N-terminal 40-kDa and 52-kDa fragments of the p65, but not by its 35-kDa (not shown), thus confirming the mapping of the ATPase domain to the N-terminal part of the viral protein (8).

The ability of cell HSP70s to interact with sequence-nonspecific peptides and denatured proteins in ATP-dependent fashion has been demonstrated for DnaK, a multifunctional chaperone encoded by a single *E.coli* gene and for several products of the eucaryotic hsp70 multigene family (reviewed in ref. 10). Unlike that of DnaK taken as positive control, the ATPase activity of the BYV p65 was not stimulated in the presence of a sequence-nonspecific peptide (Fig. 1). Furthermore, the purified p65 failed to bind to immobilized denatured ovalbumine in conditions when the related HSC70 readily bound to it and was then eluted with 1 mM ATP (not shown). The p65 ATPase was markedly stimulated by the purified bovine brain microtubules added to the *in vitro* system (Fig. 1). This effect was specific to the p65, as the ATP hydrolysis by DnaK did not change significantly in the presence of microtubules (Fig. 1).

Discussion

Recently, several functions encoded in the closterovirus genomes have been mapped by computer-assisted and experimental approaches, including the key replication domains, the leader papain-like proteinase domain, and the major and minor capsid proteins (3, 7).

In this work we demonstrate that the BYV p65, a homologue of cell HSP70 chaperones, possesses a Mg-dependent ATPase activity mapped to its N-terminal portion. The p65, unlike its cell homologues, was apparently unable to interact with random peptide in ATP-dependent manner, thus suggesting a function different from that of classical "heat shock proteins". The ability of the BYV p65 synthesized in rabbit reticulocyte lysates to bind to the purified bovine brain microtubules (MTs), was the first hint to this function (9). Consistent with these results obtained for nonpurified p65, we showed that the ATPase activity of the purified BYV p65 (but not of DnaK) is strongly stimulated by the MTs.

It has been suggested that the BYV p65 functions in the cell-to-cell transport of closterovirus infection (8) that may involve specific interactions with the cell cytoskeleton and translocation machinery (9). The results obtained in this work show that the p65 is a Mg- and MT-stimulated ATPase. However, elucidation of the p65 function in closterovirus infection awaits further experiments.

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PRODUCTION AND EPITOPE CHARACTERISTICS OF MONOCLONAL ANTIBODIES TO POTATO LEAFROLL VIRUS.

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Introduction

Potato leafroll virus (PLRV) is a member of the luteovirus group. It occurs in a low concentration in phloem tissue and is difficult to purify free from the host material [1]. The PLRV antisera preparating in rabbits often have relatively high levels of non-specific reaction and it make ELISA less than ideal for the detection of PLRV [2]. The monoclonal antibodies (MAbs) technology provides a mean to produce an unlimited supply with the uniform antibody preparation of a required specifity.

The objective of the research was the producing of hybridomas secreting PLRV- specific MAbs that will be useful for immunochemical analyses and for the detection of PLRV.

Materials and methods

Production and purification procedure of the PLRV isolate and anti-PLRV sera were described previously [3].

Production of MAbs. Two female BAIB/c mice were immunized by intraperitoneal injection with 30-50 mcg of purified PLRV-26V in 200 mcl of phosphate buffer (pH 7.4) and 200 mcl of Freunds complete adjuvant on day 0. Second injection with 30 mcg purified PLVR-26V emulsified with Freunds incomplete adjuvant was on day 21. After 1-2 weeks 20-30 mcg PLRV in phosphate buffer were given intravenously. The final booster was 2 week later. The mice were sacrificed and the spleens harvested in 4 days after the last injection.

Materials and methods used in preparation of the immune spleen cells, screening, cloning, antibody production in mice , purification of immunoglobulins and preparation of enzyme-labeled antibodies were the same as described [4].

Epitope characterization of MAbs were determinated in the competitive ELISA [5].

Results

Six hybridoma clones secreting PLRV-specific antibodies were obtained from two fusion experiments (Table 1).

All antibodies belong to Ig G class and have high antigen binding constant ($5*10^9$ - $6.4*10^{10}$ M^{-1}).

Table 1 **Properties of MAbs to PLRV**

Design	K_a*10^{10}, M^{-1}	Titer in I-ELISA		Results in competitive binding assay with		
		Cultural fluid	Ascite fluid	L 2.4*	L 4.3	L 1.3
L 1.3	0.5	$1*10^3$	$5*10^4$	-	+	+
L 2.4	1.2	$5*10^2$	$5*10^4$	+	+	-
L 3.2	1.9	$2*10^2$	$1*10^6$	-	-	-
L 4.3	0.5	$1*10^3$	$2*10^4$	-	+	+
L 1.5	0.2	$5*10^2$	$1*10^6$	-	-	-
L 4.4	6.4	$1*10^4$	$2*10^3$	+	+	-

* - Peroxidase labeled MAbs.

Epitope analysis of virus coat protein was carried out by competitive binding assay. Peroxidase marked MAbs were challenged for antigen binding with unlabeled MAbs which were titrated in serial dilution (Table 1).

MAbs L 3.2 and L 1.5 did not competitive with antibodies are indicating, that they were directed against distinct epitopes. The other four unlabeled MAbs L 2.4, L 1.3, L 4.3 and L 4.4 inhibited (20-60%) the binding of labeled MAbs. It appeared, that they have overlapping epitopes (Fig.1).

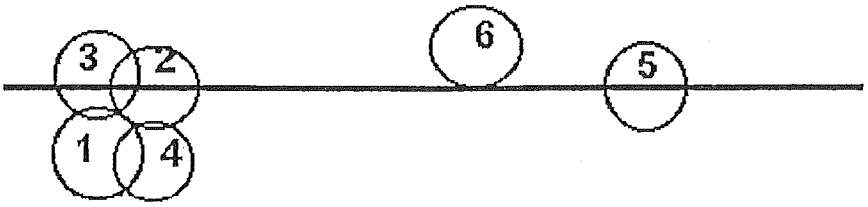

Fig 1. The topological relationships of the epitopes of the MAbs to PLRV L 4.4 (1), L 2.4 (2), L 1.3 (3), L 4.3 (4), L 1.5 (5), L 3.2 (6).

The specifity of the PLRV MAbs was tested with 5 different isolates of PLRV and another luteovirus - barley yellow dwarf virus (BYDV)- in TAS-ELISA (Table 2). All MAbs give a specific reaction with isolates of PLRV and not reacted with BYDV. These MAbs demonstrate rather virus specifity than group specifity.

Table 2 **Homologous and heterologous reactivity of MAbs generated to PLRV to a number of PLRV and BYDV isolates.**

Designation	A_{492} in TRS-ELISA					
	26 V	Nevski	Aschersleben	Prior	Ukama	BYDV
L 1.3	1.34	1.00	0.27	0.87	0.63	0.03
L 4.4	1.06	1.01	0.28	0.61	0.63	0.05
L 2.4	1.29	0.93	0.45	0.62	0.74	0.06
L 1.5	1.15	0.90	0.24	0.26	0.30	0.08
L 3.2	1.40	1.22	0.42	0.73	0.94	0.07
L 4.3	1.24	0.97	0.24	0.26	0.30	0.06

The stability of the epitopes in direct ELISA with different variants of antigenic immobilization was studied. Our studies indicate, that virions of PLRV were not stable in the alkaline conditions. During the coating procedure virions may undergo to degradation (Fig. 2). The using of polyclonal antibodies (PAbs) with more broad spectrum of specifity allowed to determine the virus in different variants of sorbtion.

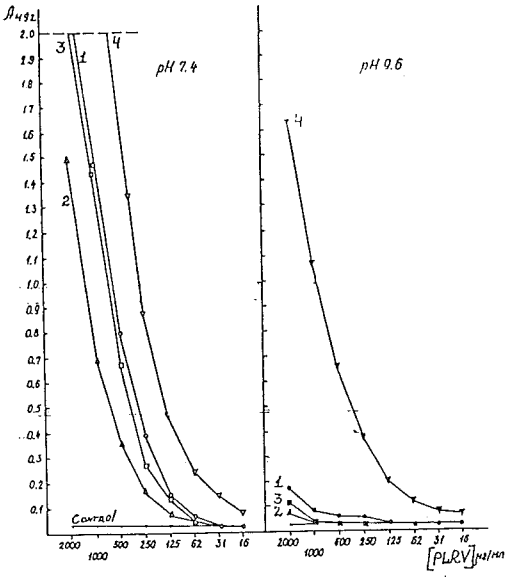

Fig. 2. The results of the different variants of antigen immobilization with MAbs reacting in direct ELISA.

pH 7.4 MAbs: L 2.4 (1); L 1.3 (2); L 4.3 (3); PAbs (4).
pH 9.6 Mabs: L 2.4 (1); L 1.3 (2); L 4.3 (3); PAbs (4)
Control: with Arabis mosaic virus

The monoclonal immunoenzyme test-system was developed. For MAbs L 2.4 the lowest concentration of virus determination in purified preparations was 10-15 ng/ml (Fig 3).

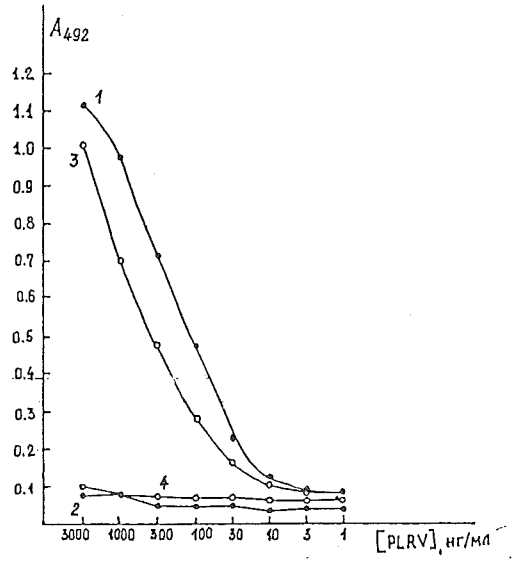

Fig. 3 Determination of PLRV in purified preparations of PLVR - 26V.

Immobilised antibodies: MAbs L 2.4 (1,2) and PAbs (3,4)
Detection by the conjugates of MAbs L 2.4-HRP (1,3); PAbs-HRP (2,4)
Control with Arabis mosaic virus.

Discussion

The obtained MAbs were differentiated by their testing with PLRV in the different ELISA formats: by competitive binding assay and by the analyzing of the reactions with different isolates of PLRV and BYDV. The results of MAbs binding with 5 isolates of PLRV allowed to conclude that they are detecting a common epitope on the PLRV coat protein. The positive reaction of these MAbs with all PLRV isolates support the Hypothesis that the PLRV coat protein is highly conserved [1]. Competitive experiments showed 3 regions of antibody's binding with antigen. Two MAbs L 3.2 and L 1.5 have different epitopes and other four have different but overlapping epitopes. The results of competitive binding assay can within certain limits supply with the information on the topological relationships of the epitopes (Fig.1). The MAb's L 2.4, L 1.3 and L 4.3 detecting epitopes are sensitive to alkaline degradation. Coating viruses in alkaline conditions may alter the antigenic structure due to the conformation changes of virion proteins, thus creating neotopes not present in intact virions. The epitopes of three antibodies probably are conformation dependent [5]. Nine MAbs reacted with conformation-dependent epitopes on the PLRV coat protein were determinated previously [6]. Our studies indicate that the ELISA formats used in screening of antibody's activities in culture fluids greatly influence the selection of these antibodies [6,7].

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MOLECULAR CLONING, SEQUENCING AND HOMOLOGY STUDIES OF POTATO POTYVIRUS A (PVA) HELPER COMPONENT GENE FROM APHID TRANSMISSIBLE (AT) AND NON-APHID TRANSMISSIBLE (NAT) ISOLATES

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Introduction.

Potyvirus helper-component (HC) is a multifunctional protein that has been characterized as a helper factor for acquisition and transmission of virus by aphids (N-terminus and central domain) (1, 2, 3, 4, 5); C-terminal one-third of HC functions as proteinase that catalyzes autoproteolytic cleavage on HC C-terminus (6, 7); HC also was identified as long-distance (leaf-to-leaf) movement factor that facilitates vascular-associated movement in tobacco (8). It is generally accepted that successful transmission of potyviruses by their aphid vectors depends upon the interaction of two viral-encoded proteins, the coat protein (CP) N-terminus and the HC (9, 10, 11, 12). Also, it was suggested that long-distance movement of potyviruses involves an interaction between HC and CP terminal regions, but the nature of the interaction required for both processes may differ (8).
In our previous studies we have shown that CP N-terminal domain sequence devides PVA isolates into AT and NAT subgroups. PVA NAT subgroup (isolates B11, Advira) have sequence DAS (Asp-Ala-Ser) within their N-terminus but PVA AT subgroup (isolates USA and Rouge) represents tripeptide DAG (Asp-Ala-Gly), which has been shown to be associated with aphid transmissibility of potyviruses (10, 11). PVA Juliniere also belongs to AT subgroup but do not transmitted by aphid vectors and has sequence DTG within CP N-terminus (13, 14). On the basis of amino acid conservation in PVA subgroups as well as their differences between subgroups we had supposed that the N-terminal part of PVA CP might have some additional biological functions aside aphid transmissibility (14).
In this paper we represent the results of molecular cloning, sequencing and comparison of HC genes from two AT and three NAT PVA isolates.

Methods.

Virus isolates. All analysed isolates were cultured in *Nicotiana clevelandii.*
Viral RNA purification. Total RNA was purified from infected tobacco leaf material as described by Verwoerd et al., (17). Total RNA was used as template for PCR reaction.
cDNA synthesis and cloning. First strand synthesis was primed with PVA specific d(GGGTACCTTATCCAACCCTGTAGTGCTTCATTTC) oligo, MMTV reverse transcriptase; then the cDNA fragment was amplified in PCR reaction with Taq DNA polymerase, using PVA specific oligo d(GGGATCCATGTATTACACAGGGGATGTTTTC TGG) as 5 primer. cDNA fragments were purified from agarose gel and cloned into PCR fragment cloning vector pUC57/T (Fermentas).
DNA sequencing. Subclones were generated by restriction endonuclease digestion. Double stranded plasmid DNA was sequenced directly in both directions by the didesoxynucleotide chain termination method (18).
Computer analysis. Nucleotide and amino acid sequence analyses were performed with the PCGENE software package (19).

Results.

We had previously detected that five PVA isolates such as NAT B11, Advira and Juliniere, and AT USA and Rouge differs in their CP N-terminus amino acid sequences from each other. To detect whether there are differences in their HC genes we have cloned and sequenced HC regions of five named above PVA isolates (full-lenght sequence of PVA-NAT B11 was taken from 15). Within the HC region there was 95,7% nucleotide sequence homologie . The derived amino acid sequences of five isolates are 458 amino acids long (Fig. 1). The highest sequence homology is between NAT isolates B11 and Advira (99.8%). The lowest sequence homology shared isolates NAT PVA Advira and AT PVA Rouge (97.6%). Comparison of five PVA isolates HC proteins revealed the highest sequence diversity of isolate Rouge which has five amino acid substitution at the conserved among four other isolates homological positions (Fig.1 pos. 39, 48, 86, 98, 108). Isolate Juliniere HC protein has three amino acid differences at positions 14, 231, 281 comparing to four other PVA isoaltes where amino acids in homological position are conserved. The lowest sequence variability show isolates Advira and USA. Both of them. have only one amino acid substitution at the positions 203 and 451 respectively (Fig.1).
In addition, there several amino acid differencies which are common to AT and NAT isolates respectively. Comparison of five PVA isolates HC proteins revealed amino acid differences between AT isolates (Rouge, USA) and NAT isoaltes (B11, Advira and Juliniere) at two positions. AT PVA isolates have Lys 88 and Ser 153, but NAT isolates represents Glu and Gly respectively. Furthermore three amino acid changes are present between AT Rouge, AT USA and intermediate isolate NAT Juliniere in one hand (Tyr 89, Thr 330 and Val 437) and NAT B11, Advira on the other hand (His, Ala, Ala respectively).

Discussion.

We have cloned and sequenced HC genes of four PVA isolates. An alignment of the derived amino acid sequence of four PVA isoaltes and PVA B11 revealed a high sequence homology (96.7%) between five studied virus isolates.Comparison between the HC gene of NAT and AT potyviruses or their strains was made by several authors (1, 16). Two HC protein regions (KITC-domain and PTK-domain) have been found to be required for transmission of potyviruses by aphid-vectors (3, 12). Those two domains are hihgly conserved among all aphid-transmissible potyviruses. The derived amino acid HC sequences of two AT PVA isoaltes and three NAT PVA isoalates were compared with that of ten different aphid-transmissible potyviruses (data not shown). An alignment of sixteen different HC proteins shows, that all amino acid changes between PVA-AT and PVA-NAT isoaltes distributed out of highly conserved regions of HC. Furthermore, mutant with the single mutation on infectious full-lenght clone of PVA NAT B11 made within CP N-terminus and resulted in substitution of Ser to Gly in DAS (Asp-Ala-Ser) tripeptide (DAS to DAG) was able to aphid-transmission (Puurand, unpublished data). These data suggest that the non-aphid transmissibility of PVA B11 is caused only by DAS sequence on CP N-terminus, but not by deffect in HC gene. However, an alignment of the HC sequences of five PVA isolates puts them into two subgroups (Fig. 2B) which is in accordance with the distribution to two subgroups of PVA isolates CP N-terminuses (Fig. 2A) (14). As it was shown by us previously PVA Juliniere seems to be an intermediate and is not aphid transmissible, but on the bases of the CP N-terminus and HC amino acid sequence alignment belongs to PVA AT subgroup (Fig.2). The comparison of five PVA HC-s revealed five amino acid changes between NAT and AT subgroups (Fig. 1, pos 88, 89, 153, 330, 437). In three out of five mutations (Fig. 1, pos. 89, 330, 437) PVA Juliniere HC represents amino acid sequence typical for AT isolates Rouge and USA. And two amino acids at pos. 88 and 153 (Fig. 1) are as those for NAT isolates B11 and Advira. It will be noted that differences at those four out of five positions (Fig.1 K 88 E; Y 89 H; S 153 G and T 330 A) led to a change in the properties of the amino acids (charge and/or hydrophobicity)
Dendrograms made on the multiple sequence alignment revealed similar clustering of CP N-terminal parts and HC proteins of PVA isolates.

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PVAB11HC YSTGDVFWRGFNRTFLENKPINLDHVCSSDFSVEECGSIAALICQSLLPCGKITCRACAA
PVAADVHC YSTGDVFWRGFNRTFLENKPINLDHVCSSDFSVEECGSIAALICQSLLPCGKITCRACAA
PVAJULHC YSTGDVFWRGFNRAFLENKPINLDHVCSSDFSVEECGSIAALICQSLLPCGKITCRACAA
PVAUSAHC YSTGDVFWRGFNRTFLENKPINLDHVCSSDFSVEECGSIAALICQSLLPCGKITCRACAA
PVAROUHC YSTGDVFWRGFNRTFLENKPINLDHVCSSDFSVEECGSVAALICQSLIPCGKITCRACAA
                         *                                *         *

PVAB11HC KNLNMDEDTFKEFQTQRAREISAVIISEHPNFACVSQFIDRYFSHQRVLNPNVNAYREIL
PVAADVHC KNLNMDEDTFKEFQTQRAREISAVIISEHPNFACVSQFIDRYFSHQRVLNPNVNAYREIL
PVAJULHC KNLNMDEDTFKEFQTQRAREISAVIISEYPNFACVSQFIDRYFSHQRVLNPNVNAYREIL
PVAUSAHC KNLNMDEDTFKEFQTQRAREISAVIISKYPNFACVSQFIDRYFSHQRVLNPNVNAYREIL
PVAROUHC KNLNMDEDTFKEFQTQRAREISAVITSKYPNFACVSQLIDRYFSHQRILNPNVNAYREIL
                                     * **         *           *

PVAB11HC KIVGGFTQSPYTHIQELNEILVLGGRATPEQLGSASAHLLEITRFVRNRTDNIKKGSLAL
PVAADVHC KIVGGFTQSPYTHIQELNEILVLGGRATPEQLGSASAHLLEITRFVRNRTDNIKKGSLAL
PVAJULHC KIVGGFTQSPYTHIQELNEILVLGGRATPEQLGSASAHLLEITRFVRNRTDNIKKGSLAL
PVAUSAHC KIVGGFTQSPYTHIQELNEILVLGGRATPEQLSSASAHLLEITRFVRNRTDNIKKGSLAL
PVAROUHC KIVGGFTQSPYTHIQELNEILVLGGRATPEQLSSASAHLLEITRFVRNRTDNIKKGSLAL
                                          *

PVAB11HC FRNKISAKAHVNTALMCDNQLDRNGNLIWGERGYHAKRFFSNYFDIITPGGGYKQYIERR
PVAADVHC FRNKISAKAHVNTALMCDNQLDKNGNLIWGERGYHAKRFFSNYFDIITPGGGYKQYIERR
PVAJULHC FRNKISAKAHVNTALMCDNQLDRNGNLIWGERGYHAKRFFSNYFDIITPGSGYKQYIERR
PVAUSAHC FRNKISAKAHVNTALMCDNQLDRNGNLIWGERGYHAKRFFSNYFDIITPGGGYKQYIERR
PVAROUHC FRNKISAKAHVNTALMCDNQLDRNGNLIWGERGYHAKRFFSNYFDIITPGGGYKQYIERR
                                     *                        *

PVAB11HC VPNGIRKLAIGNLIVTTNLEALREQLEGESIEKKAVTKACVSMSDNNYKYPCCCVTLDDG
PVAADVHC VPNGIRKLAIGNLIVTTNLEALREQLEGESIEKKAVTKACVSMSDNNYKYPCCCVTLDDG
PVAJULHC VPNGIRKLAIGNLIVTTNLEALREQLEGESIEKKAVTKACISMSDNNYKYPCCCVTLDDG
PVAUSAHC VPNGIRKLAIGNLIVTTNLEALREQLEGESIEKKAVTKACVSMSDNNYKYPCCCVTLDDG
PVAROUHC VPNGIRKLAIGNLIVTTNLEALREQLEGESIEKKAVTKACVSMSDNNYKYPCCCVTLDDG
                                               *

PVAB11HC TPLYSTFIMPTKNHLVIGNSGDPKFLDLPADISTQMYIAKSGYCYINIFLAMLVNVDESD
PVAADVHC TPLYSTFIMPTKNHLVIGNSGDPKFLDLPADISTQMYIAKSGYCYINIFLAMLVNVDESD
PVAJULHC TPLYSTFIMPTKNHLVIGNSGDPKFLDLPTDISTQMYIAKSGYCYINIFLAMLVNVDESD
PVAUSAHC TPLYSTFIMPTKNHLVIGNSGDPKFLDLPTDISTQMYIAKSGYCYINIFLAMLVNVDESD
PVAROUHC TPLYSTFIMPTKNHLVIGNSGDPKFLDLPTDISTQMYIAKSGYCYINIFLAMLVNVDESD
                                          *

PVAB11HC AKDFTKKVRDIIVPDLGEWPTLIDVATSCSLLSAFYPATSAAELPRILVDHDLKTMHVID
PVAADVHC AKDFTKKVRDIIVPDLGEWPTLIDVATSCSLLSAFYPATSAAELPRILVDHDLKTMHVID
PVAJULHC AKDFTKKVRDIIVPDLGEWPTLIDVATSCSLLSAFYPATSAAELPRILVDHDLKTMHVID
PVAUSAHC AKDFTKKVRDIIVPDLGEWPTLIDVATSCSLLSAFYPATSAAELPRILVDHDLKTMHVID
PVAROUHC AKDFTKKVRDIIVPDLGEWPTLIDVATSCSLLSAFYPATSAAELPRILVDHDLKTMHVID

PVAB11HC SYGSLNTGYHVLKANTIRQLIQFASNSLDSEMKHYRVG
PVAADVHC SYGSLNTGYHVLKANTIRQLIQFASNSLDSEMKHYRVG
PVAJULHC SYGSLNTGYHVLKANTVRQLIQFASNSLDSEMKHYRVG
PVAUSAHC SYGSLNTGYHVLKANTVRQLIQFASNSLDSGMKHYRVG
PVAROUHC SYGSLNTGYHVLKANTVRQLIQFASNSLDSEMKHYRVG
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Figure 1. Alignment of HC region amino acid sequences for five isolates of PVA. * indicates that a position in the alignment is not conserved.

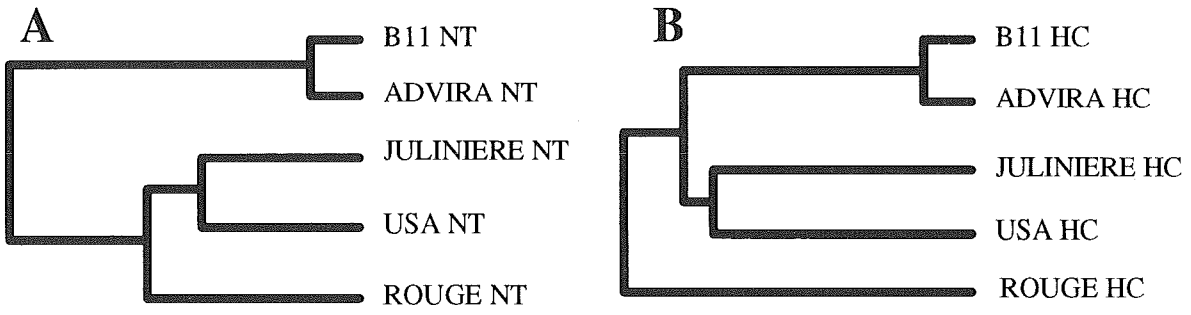

Figure 2. The dendrograms showing the relationships of the CP N-terminal regions of PVA isolates (A) and HC regions (B)

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TOWARDS GENERATING RESISTANCE TO BARLEY YELLOW MOSAIC VIRUS (BAYMV) IN BARLEY (*HORDEUM VULGARE* L.) AND CHARACTERIZATION OF THE RESISTANCE-MECHANISM

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Introduction

Yellow mosaic, caused by the Bymovirus barley yellow mosaic (BaYMV), is a disease of winter barley with increasing agronomic importance in Asia and Europe (1,2). Because of its transmission by the soil-borne plasmodiophorous fungus *Polymyxa graminis* Led. (3), efficient control measures are not available. So far, yield losses can only be prevented by growing resistant cultivars (1,2).

Besides traditional methods of plant breeding for virus resistance the tools of modern molecular biology permit to deliver defined virus genes into plant cells to provide protection. The first virus-resistance after transformation with a virus gene was reported in 1986 by Powell-Abel et al. (4): they transformed tobacco with the tobacco mosaic virus coat protein gene using Agrobacteria as vectors. Up to now, several successful investigations have been done - most of them with tobacco or other Solanaceae like potato and tomato which can be transformed easily using Agrobacteria (5). Since most monocotyledonous plants - including barley - have been recalcitrant to Agrobacterium transformation there are only two reports about cross protection in monocotyledonous plants (6,7). Experiments published in 1992 showed for the first time that in transgenic plants not only virus coat protein but also coat protein gene transcripts can interfere with virus replication (8,9,10).

After optimizing the biolistic method - the most widely used procedure for cereal transformation (11) - we started transformation experiments with the BaYMV coat protein gene by particle bombardment of highly embryogenic scutellar tissue of immature barley embryos. To characterize the resistance mechanism we have mutated the ATG start codon of the BaYMV coat protein gene rendering it untranslatable. A comparison to the translatable gene will show whether the coat protein or the transcript itself provides protection.

Material and Methods

Plant material and preparation for particle bombardment
Plants of the barley winter cultivar Igri were grown as described previously (12). Young seeds were harvested 10-14 days after pollination and surface sterilized for 1 min in 70% ethanol and for 15 min in 1% sodium hypochloride/0.2% Mucosol. After washing three times with sterile distilled water, scutella of immature embryos were dissected and placed scutellum-side up on solid medium.

Culture and selection conditions
Bombarded scutella were cultured 14-21 d in the dark at 26 °C on solid L2-medium (13) with 1 mg/l 2,4-dichlorophenoxyacetic acid. For selection, embryogenic calli were transferred to L2-medium without amino acids and with 15-20 µl/l Basta® (3-4 mg/l phosphinothricin) and were cultured at 24 °C under a 16 h light/8 h dark period. Selected regenerants, about 10 cm in size, were vernalized and grown to maturity in the greenhouse as described previously (12).

Osmotic treatment
To test the influence of osmotic treatments on the transformation efficiency, the osmolarity of the L2-medium (170 mOsm/kg) was increased up to 1020 mOsm/kg using higher amounts of maltose. The osmotic treatment consisted of a 5 h pre-treatment and a 48 h post-treatment.

Plasmid constructs
For the optimization of bombardment conditions we used the plasmid pAct1-D which contains the ß-glucuronidase (GUS)-gene (14). For stable transformation experiments we used the plasmid pDB1 with the ß-glucuronidase-gene and the phosphinothricin acetyltransferase (PAT)-gene, which has been described previously (15) [for the plasmid with the BaYMV coat protein gene see results].

DNA precipitation for microprojectile bombardment
The precipitation mixture contained 2 mg gold particles (1 µm, BioRad, München, FRG), 5-20 µg DNA, 16 mM spermidine-free base and 1 M $CaCl_2$. Sterile distilled water was added to a final volume of 125 µl. After vortexing for 3 min, the mixture was centrifuged for 10 sec at 10000 rpm. The DNA-coated particles were washed in 250 µl absolute ethanol and resuspended in 120 µl absolute ethanol. For each bombardment, 3.5 µl were spread onto the surface of the macrocarrier. In cotransformation experiments equal amounts (µg) of DNA from the 2 plasmids were used. The particle gun employed in our experiments was a PDS 1000/He gun (BioRad, München, FRG).

Histochemical GUS assay
GUS activity was determined histochemically by incubating scutella, calli or leaf tissue for 18 h at 37 °C in staining buffer (16). Chlorophyll of stained leaf tissue was extracted by incubation in absolute ethanol for 6-8 h at 65 °C.

Herbicide application
Two weeks after vernalization selected regenerants were sprayed with an aqueous solution of Basta® containing 150 mg/l phosphinothricin and 0.1 % Tween 20.

PCR-labelling of probe DNA
Probe DNA was labelled as described previously (15).

DNA isolation and Southern blot hybridization
Total genomic DNA was isolated from leaves harvested from selected plants using the protocol of Dellaporta et al. (17). Twenty micrograms of genomic DNA - uncut, digested with BamHI/SacI (to cut out the uidA-gene) and with HindIII (to determine the number of integration sites per genome) - were separated by electrophoresis in 0.8% agarose for 16 h at 25 V. DNA was blotted to Hybond N membranes according to a protocol described by Amersham, UK. The detection of introduced DNA was performed using a protocol of the digoxigenin chemiluminescent method (18) with modifications as described by Becker et al. (15). Hybridization signals were visualized on Hyperfilm™-MP (Amersham, UK).

Results

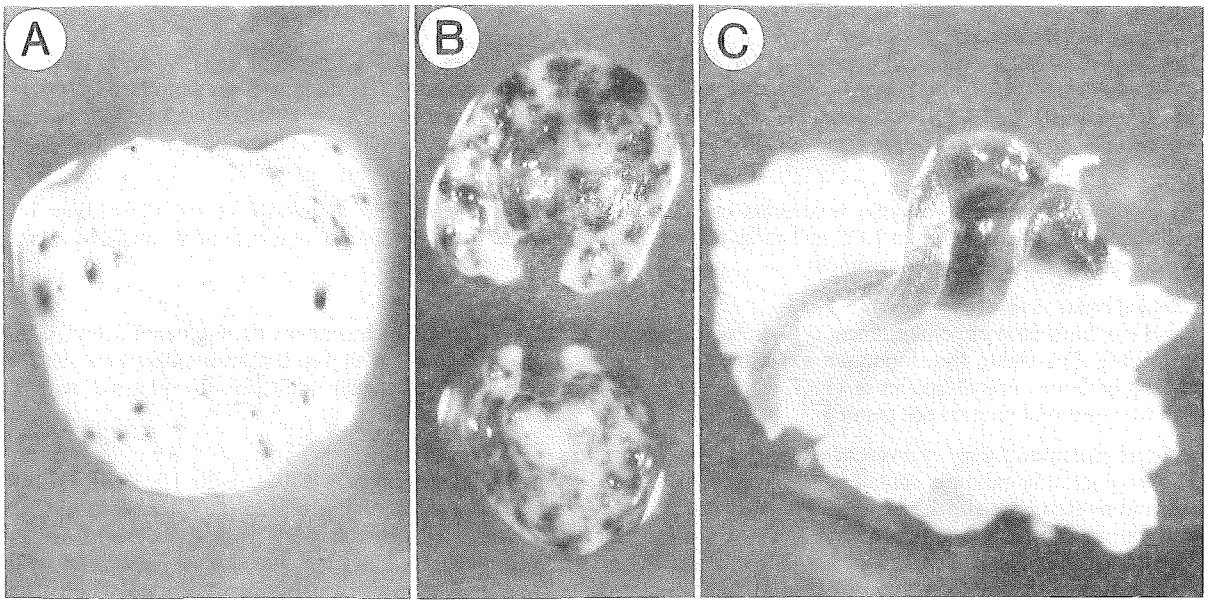
I. Parameters for particle bombardment

Bombardment conditions for scutellar tissue of immature barley embryos of the winter cultivar Igri were optimized by transient gene expression using the plasmid pAct1-D which contains the GUS-marker gene. Bombarded scutella were stained 2 d after bombardment. The optimized parameters are shown in Fig. 1.

Fig. 1: Optimized parameters for particle bombardment of scutellar tissue of immature barley embryos.

Distance between	
-rupture disk and macrocarrier	2.5 cm
-macrocarrier and stopping screen	0.8 cm
-stopping screen and target cells	5.5-8.7 cm
Gas pressure	900-1300 psi = 63-91 bar
Partial vacuum	28 inch Hg = 65 mbar
Size of gold particles	1 µm
Amount of particles per shot	60 µg
Amount of DNA per shot	0.15-0.6 µg
Osmotic pre-treatment	5 h 680-850 mOsm/kg

Fig. 2 A: Transient GUS-expression on a scutellum which was bombarded after a 5h-pre-treatment on L2-medium with 170 mOsm/kg. **B:** Transient GUS-expression on 2 scutella which were bombarded after a 5h-pre-treatment on L2-medium with 850 mOsm/kg. **C:** GUS-expressing shoot on a callus derived from a scutellum which was bombarded after a 5h-pre-treatment on L2-medium with 850 mOsm/kg and stained histochemically 1 month after bombardment.

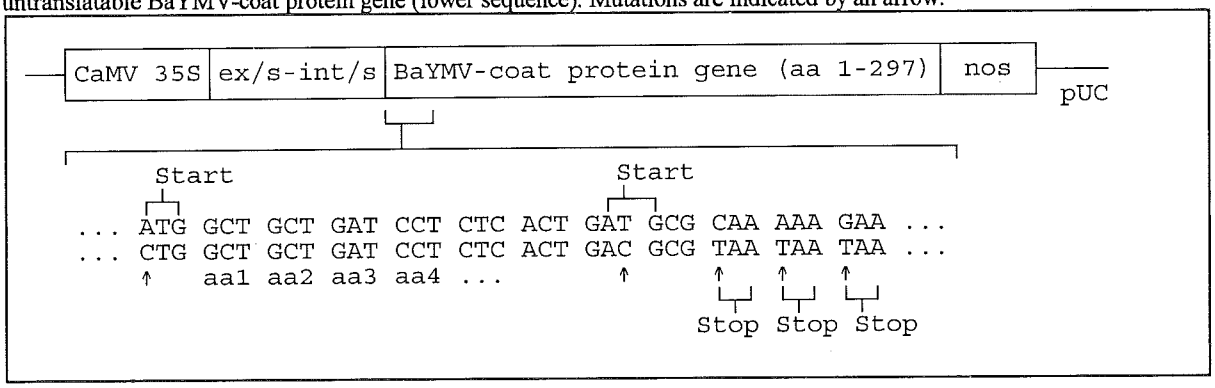


The most important parameter was an osmotic treatment before bombardment [Fig. 2 A, B]. The highest increase of transient transformation events was achieved by a 5h-pre-treatment on L2-medium with 850 mOsm/kg. An average of 2134±292 (mean±SE; n=6) blue GUS-signals per plate with 10 Scutella could be determined. To confirm that an osmotic pre-treatment increases also the number of stable transformation events we repeated these optimization-experiments, but this time we made the histochemically GUS-assay 1 month after bombardment. 21 % of calli derived from scutella which were bombarded after a 5h-pre-treatment on L2-medium with 850 mOsm/kg showed a strong blue staining area or a GUS-expressing shoot/root [Fig. 2 C].

II. Mutagenesis of the BaYMV coat protein gene

The BaYMV-coat protein gene is under control of the CaMV 35S promotor and the exon 1 and intron 1 of the maize *Shrunken1*-gene, which increase transient gene expression of barley (19). To characterize the resistance-mechanism we generated by site-directed mutagenesis (Chameleon™ double-stranded, site-directed mutagenesis kit; Stratagene; Heidelberg; FRG) 5 point mutations to render the BaYMV-coat protein gene untranslatable [Fig. 3]: the first mutation changed the translational start ATG to CTG - the second changed a translational start in a different reading frame from ATG to ACG and the other three created three stop codons (TAA). The nucleotide changes have been confirmed by sequencing.

Fig. 3: Schematical representation of the transformation vectors with the translatable (upper sequence) and the untranslatable BaYMV-coat protein gene (lower sequence). Mutations are indicated by an arrow.



III. Selection of transformants and analysis of T_0-plants

Scutella of immature embryos of the barley winter cultivar Igri were bombarded after an osmotic treatment with the optimized parameters [Fig. 1]. For cotransformation experiments the plasmid pDB1 - which contains the marker-genes GUS and PAT - and the plasmids which contain either the translatable or the untranslatable BaYMV coat protein gene were used. After 14-21 d embryogenic calli were transferred to amino acid-free selection medium containing 15-20 µl/l Basta® (3-4 mg/l Phosphinothricin). Selected regenerants were vernalized, transferred to the green house and screened histochemically for GUS activity. Furthermore, putative transformants were sprayed with an aqueous solution of Basta® containing 150 mg/l Phosphinothricin. Up to now, 2 plants showed no necrosis after Basta application and 1 regenerant showed blue staining in the histochemical GUS assay.

Discussion

The aim of our project is to generate resistance to BaYMV and a characterization of the resistance-mechanism. Therefore we optimized the biolistic transformation method for highly embryogenic scutellar tissue of immature embryos of the barley winter cultivar Igri. The most important parameter was an osmotic treatment of scutella 5 h prior to and 48 h after bombardment. Both transient and stable transformation frequencies were dramatically increased as reported also for embryogenic suspension culture cells of maize (20). This enhancement might result from plasmolysis of the cells [Fig. 2 B] leading to reduced cell damage after penetration of particles. With these optimized parameters cotransformation experiments with the marker-genes coding for GUS and PAT and with the translatable/untranslatable BaYMV coat protein gene were carried out. Despite of the high number of transient transformation events we got up to now only 2 plants which showed no

necrosis after Basta application and 1 regenerant which showed blue staining in the histochemical GUS assay. Compared to Wan and Lemaux (21) the number of independently transformed barley plants is rather low. However, if the BaYMV coat protein genes are cointegrated, virus inoculation experiments of the T_1-plants will be done to show whether the coat protein or the transcript itself provides protection.

Acknowledgements

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BARLEY YELLOW DWARF AT ILLINOIS: PROGRESS AND PROSPECTS

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Introduction

Barley yellow dwarf (BYD) has been studied at the University of Illinois for over four decades by scientists employed by the state of Illinois and by the United States Department of Agriculture. The longest term focus of these research programs has been development of spring oat and winter wheat lines with tolerance to BYD. Twenty BYD tolerant oat germplasm lines, 9 oat cultivars, and 3 wheat cultivars have been released. Another focus of research has been production of antibodies useful for detection and diagnosis of barley yellow dwarf viruses (BYDVs). Monoclonal antibodies developed at the University of Illinois against PAV, MAV, and RPV strains of BYDVs have been used in virus surveys in the U.S., Europe, and Australia.

Currently there are three scientists at the University of Illinois whose research programs focus on BYD. Dr. Frederic L. Kolb is a plant breeder working on management of BYD. The goals of his program are development of BYD tolerant spring oat and winter wheat lines and determination of the usefulness of imidacloprid insecticide for management of BYD. Dr. Leslie L. Domier is a virologist studying the molecular biology of BYDVs. His current projects include identification of host genes associated with tolerance to BYD and estimation of the genetic variability of natural BYD populations. Dr. Cleora J. D'Arcy's program is focused on the luteovirus group, with emphasis on BYDVs. Recent and current research projects of her laboratory group are described herein.

Illinois BYD Survey

Little is known about the strains of BYDVs present in many areas of the world, and about the stability or variability of the strains over time. From 1989 to 1993 a survey to determine the incidences of BYDVs in spring oats and winter wheat in Illinois was conducted. Leaf samples were collected from 50 randomly selected plants in each field surveyed. For the first two years of the survey all samples were tested for PAV, MAV, and RPV strains of BYDVs in both double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with polyclonal antibodies and triple antibody sandwich ELISA (TAS-ELISA) with monoclonal antibodies for detection. Results indicated that the two detection systems were in agreement >99% of the time (D'Arcy *et al.*, 1992), so only TAS-ELISA was used during the last three years of the survey.

Results of the survey were relatively consistent across the two crops and across the five years of the study (Tables 1 and 2).

Table 1. BYDVs in spring oats in Illinois, 1989-1993.

	1989	1990	1991	1992	1993	%
PAV	74	93	47	105	87	15.3
RPV	14	16	13	66	26	5.1
MAV	0	0	8	16	-	1.1
Total	550	350	550	650	550	

Table 2. BYDVs in winter wheat in Illinois, 1989-1993.

	1989[1]	1990	1991	1992	1993	%
PAV	1	81	125	60	206	13.3
RPV	1	10	12	16	36	2.1
MAV	0	0	13	7	-	0.9
Total	350	700	850	700	1300	

[1] BYDVs poorly detected in samples from mature fields; numbers not included in totals.

PAV strains were most common in oats and wheat, followed by RPV and MAV. In both crops usually about 10-15% of the plants were infected with PAV, except in oats in 1990, when 26.6% of the plants sampled were infected. RPV and MAV infections were much less common. RPV was detected in both crops at low levels in all years of the survey. No MAV was detected in either oats or wheat in 1989 or 1990.

This survey has shown that three strains of BYDVs are found in oats and wheat in Illinois, but that PAV strains predominate. In addition, the results demonstrate that monoclonal antibodies can be used successfully in field surveys for BYDVs. In the future we plan to reexamine periodically the distribution and incidences of these BYD strains in Illinois crops and, in addition, to determine the incidence of RMV strains.

BYD in Maize

For the past several years, symptoms typical of BYD infection have been noted in sweet corn (*Zea mays* L.) in Illinois and elsewhere in the midwestern U.S. These symptoms include either yellow or purple-red marginal discoloration of leaves and sometimes stunting of plants. Discoloration typically begins at the tip of the leaf, but may extend more than half of its length. RMV serotypes have been the most common BYDVs detected in midwestern sweet corn. Since the impact of BYDVs on production of maize (both sweet and dent) in the midwestern U.S. is poorly understood, we have begun a research program on BYD in maize. The goals of the program are to: (1) determine the economic impact of BYD in maize (both sweet and dent), (2) identify sources of tolerance or resistance to BYD in maize, and (3) determine the pattern of inheritance of tolerance or resistance in selected lines. Research on BYD in sweet corn led by Ronald L. Itnyre is described herein.

Five Pillsbury/Green Giant sweet corn hybrids were inoculated with BYDV-RMV in 1992 and 1993 at Urbana, IL with a modified bug bazooka (Wiseman *et al.*, 1980). In 1992 samples were collected from the tips of the uppermost leaves of inoculated plants; in 1993 samples were collected from the tips of a top, middle and bottom leaf of each plant. All samples were stored at -80C until they were tested for RMV by DAS-ELISA. The uppermost ear from ten plants were collected and weighed.

Results from three of the five sweet corn hybrids are shown in Table 3. In 1992 the incidence of virus infection detected in the top leaf samples was very low, probably due to the highly uneven distribution of RMV in the corn plants (Hammond *et al.*, 1983). The use of a three leaf composite sample in 1993 helped to reduce this problem, but did not eliminate it. There also was significant spread of virus infection into control plots, despite the use of border rows. However, since these plants became infected later in the season the detrimental effects of BYD were reduced. Yields of two hybrids were reduced approximately 25% when inoculated with RMV in 1992. In 1993 no individual hybrid had a significant decrease in ear weight, but there

Table 3. Yield loss and BYDV-RMV incidence in three sweet corn hybrids.

	1992 Yield (kg/10 ears)	1992 Incidence (%)	1993 Yield (kg/10 ears)	1993 Incidence (%)
H5 inoculated	1.2[1]	27	1.9	71[1]
H5 control	1.6	13	2.2	22
H10 inoculated	1.3	22	1.9	48
H10 control	1.3	14	2.1	14
H99 inoculated	1.1[1]	12	2.1	34
H99 control	1.5	20	2.2	11

[1]Inoculated plots significantly different from control plots.

was a significant reduction in ear weight averaged over the five hybrids used in the experiment (data not shown).

BYDV-RMV infection can cause significant yield reductions in some sweet corn hybrids. This yield loss, like symptom development, is dependent on several factors, including hybrid, time of planting, and environmental conditions.

In situ Immunogold Localization of BYDV Proteins

Little is currently known about the timing and location of production of BYDV proteins in infected host cells. The BYDV-PAV genome contains 6 open reading frames (ORFs); the function of only one gene product, the 22kDa coat protein produced from ORF3, has been determined. The goals of this project, led by Petra H. Nass, are to identify the sequence and location of BYDV protein accumulation *in situ* by immunogold localization. The first proteins which have been studied are the 22kDa coat protein (ORF3) and the 50kDa readthrough protein of ORF5, which is immediately downstream of ORF3.

Labeling densities of the 22kDa coat protein in oat cells between 2 and 6 days post inoculation (dpi) indicate that this protein is most likely produced in the cytoplasm (Table 4).

Table 4. Labeling densities of BYDV-PAV 22kDa protein in infected and control oat cells.

	Cyto-plasm	Nucleus	Cell Wall	Vacuole	Mito-chondria
Control	0.0	0.0	0.8	0.6	0.2
PAV - 4 dpi	5.1	1.4	0.2	0.6	0.0
PAV - 6 dpi	17.2	-	-	-	-

Labeling was first detected in the cytoplasm (2 dpi), and later in the nucleus (4-5 dpi). Coat protein found in the nucleus probably diffuses into that organelle after disruption of the nuclear membrane (Nass *et al.*, 1995).

The 50kDa readthrough protein has recently been detected in extracts from BYDV-infected leaf tissue (Cheng *et al.*, 1994) and is thought to be a structural protein on the external surface of BYD virions (see next section). The increase in 50kDa protein in infected tissue, as measured by either ELISA or immunogold labeling, parallels that of 22kDa protein. Labeling of 50kDa protein by polyclonal antibodies occurs mainly in the cytoplasm, at about 1/10 the

frequency of 22kDa protein labeling (data not shown). These results provide evidence for co-production of the 22kDa and 50kDa proteins of BYDV in the cytoplasm of infected plant cells.

Further studies are being made of *in situ* production and localization of BYDV + and - sense RNAs and the 17kDa protein. These studies will help to elucidate the replication mechanisms of BYDV and may provide information useful in development of novel management strategies for the disease.

High Resolution Electron Microscopy of BYD Virions

The fine structure of several plant viruses has been studied by electron microscopy, particularly when virus crystals were available. Low quantities of purified BYD virions have limited such studies for these viruses. Dr. Shu-Ling Cheng, in collaboration with Dr. Ya Chen at the University of Wisconsin-Madison and Dr. Mark Yeager at the Scripps Research Institute, has recently used cryo-scanning electron microscopy (cryo-SEM) and cryo-transmission electron microscopy (cryo-TEM) to study the surface structure of purified BYD virions.

For cryo-SEM highly purified BYDV-PAV was fixed with 1% glutaraldehyde and plunge frozen in liquid ethane. The preparations were freeze-dried and transferred to a cryo-SEM, where they were sputtered with chromium prior to examination. For cryo-TEM virus from the same preparations was plunge frozen in liquid propane, transferred to a cryo-TEM and images were captured by a slow scan camera.

Both methods revealed BYD virions with an average diameter of 28 nm with hexagonal two-dimensional profiles. In cryo-SEM knob-shaped structures were present on the surface of virions; in cryo-TEM spikes averaging 7 nm in length were seen protruding from virion surfaces. The number of surface knobs or spikes varied considerably, from zero on about half of all particles observed to 3 or 4 per particle. About 90% of the knob structures were cleaved when virions were incubated with trypsin. It is likely that the original number of these structures was reduced during virus purification and/or electron microscopy preparation procedures.

Our working hypothesis is that the surface spikes visualized on BYD virions are the 50kDa readthrough protein. The structure of BYDV may be similar to that of ϕX174, in which the readthrough protein forms knob-like spikes at the vertices of the 27 nm icosahedral virion.

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BREEDING OF POTATO BASIC MATERIAL WITH HYPERSENSITIVITY TO POTATO VIRUS S (PVS)

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Summary

Results in potato breeding for hypersensitivity to PVS are summerized 30 years after the beginning. The sources of resistance from *Solanum tuberosum* ssp. *andigena* are listed. PVS attack of 15 hypersensitive clones was serologically tested from natural attack in the field (9 years), by a contact test of plants growing in a cold frame (3 years), and by grafting test (2 years). For serological tests leaves were used grown from excised-bud assay of 20 tubers per clone.
Hypersensitivity largely protected potato in the field against PVS. The test of seed potatoes from the field and the contact test made it possible to discard susceptible clones before grafting with PVS carring shoots. Hypersensitive reaction differentiated hypersensitive and highly relatively resistant clones. Grafting seldom did not led to PVS infection of hypersensitive clones. Detailed information are given for 13 additional traits. This basic material is improved in important traits to a level, that it can be used in variety breeding.

Introduction

Potato virus S (PVS) belonging to the group of carla-viruses is mostly spread by leaf contact and is very common in potato production. It causes no, or very slight, symptoms and its yield reduction reaches up to 15 % (11). In contrast, if potato plants suffering from PVS infection are superinfected by other pathogens, considerable increase of damage might occure (12). Consequently there is a certain interest to control spread of PVS in potato . This can be achieved by using tested virus-free seed potatoes, obtained after heat and chemical therapy (13) or by using resistance breeding.
Breeding strategies for PVS resistance have used two types of resistance against PVS . Minor genes conditioning infection resistance is reducing the PVS attack in the field as in varieties 'Adretta', 'Narew', 'Uran' (9 , 7) . Localized hypersensitivity is assumed to be controlled by one dominant gene Ns (11 ,14, 15) and is expected to protect against mechanical transmission (14). At Groß Lüsewitz breeding for PVS hypersensitive resistance was initiated as early as 1964 by Rothacker and continued by Oertel until 1991. Combination of both types of PVS resistance was intended in parts of the material . In this paper the results are summerised .

Material and methods

Several accessions of *Solanum tuberosum* ssp. *andigena* L. (=*adg*) were used as sources of hypersensitivity to PVS (table 1) .

Table 1 : Sources of hypersensitivity to PVS from *adg* according to OERTEL et al. (1990)

Number in gene bank Groß Lüsewitz	country of origin	received from / as
58.3/129/3	Argentina	gene bank Saint Petersburg
59.3/551/1 III	Argentina	gene bank Saint Petersbrug
59.3/566/1 II	Argentina	gene bank Saint Petersburg
59.3/593/1 I	Argentina	gene bank Saint Petersburg
65.3/234/6	North of Argentina	Prof. Brücher
65.3/253/1	North of Argentina	Prof. Brücher
65.3/253/2	North of Argentina	Prof. Brücher
65.3/1685	Bolivia	PI 258 907

In addition the Hungarian variety 'Szignal' was used as parent too, the source of Ns in it could be *adg* from BAERECKE (1967). Relative resistance originated from 'Adretta' and several breeding clones .

Common methods were applied in assessing of PVS attack in clones. Excised-bud assay was performed to obtain leaves for serological tests. Prior to 1990 RIDT (Radial Immuno-Diffusion Test) was used for PVS detection , later on DAS-ELISA . The following plant material was assayed :

1. natural infection of breeding stock seed in the field (20 tubers tested annually),
2. attack of young breeding material by a contact test . In a cold frame 10 plants each of a test clone were planted side by side with a PVS infected clone.Two tubers per plant were harvested for excised-bud assay. The contact test was repeated next two years. Clones with >15% attacked plants were discarded.
3. attack of those clones by a grafting test , which remained free from or rarely attacked by PVS during test 1 and 2 . PVS infected shoots were grafted onto 20 plants of a clone to test. Plants on which the youngest axillar shoots or axillar leaves became necrotic or on which several kinds of leaf necroses occured a few weeks after grafting were considered to react hypersensitively. Clones without symptomes pass for relatively resistant. This test was repeated a second year.

Tuber characteristics were mostly assessed according to usual breeding practice. The results were expressed in notes 1 to 9 if not otherwise indicated. Note 9 expresses the desirable, very best expression, note 1 means the worst level. Tuber impression in general considers all the visible aspects of shape, surface, size, and disease symptoms. External tuber defects involve pear-like form, squared shape, formation of the heel-end, deformation by *Rhizoctonia solani* Kühn. Culinary quality was determined according to GRASSERT et al. (1985) and expressed as an index (Speisewertzahl), considering appearance and discoloration after cooking and the flavour. The index was converted to the scale 1-9. Rust spots within the tuber are assumed to have physiological reasons.The susceptibility to damage was assessed by a pendulum device (4 , 5).The pendulum index between 0 and 100 was converted to the scale 1-9.

Results and discussion

How hypersensitivity protects potato in the field and in special tests is shown in table 2. Results of 15 selected and in crossing program used clones from our basic material are listed and compared with a highly relatively resistant variety and a susceptible standard.

Table 2: PVS attack of 15 PVS-hypersensitive potato clones, a highly relatively resistant variety and the susceptible clone 77.9152/23 in per cent of plants tested. Natural attack in stock seed from the field at Groß Lüsewitz, attack by contact test and grafting test.

clone	natural attack in breeding field										contact test	grafting test
	1986	1987	1988	1989	1990	1991	1992	1993	1994	x	3 years	2 years
76. 9101/14	0	10	10	0	5	0	0	0	0	3,3	0	22
76. 9156/ 6	0	0	0	0	5	-	12	0	0	1,9	2	60
77. 9157/ 3	0	0	0	-	25	0	0	0	0	2,8	3	45
79. 9191/41	5	13	0	0	0	0	0	0	0	2,0	7	22
82. 9277/11	0	0	0	5	0	0	0	0	0	0,6	0	66
82. 9277/17	0	0	0	0	0	0	0	0	0	0,0	0	84
82. 9292/ 7	0	5	0	0	0	0	0	-	-	0,7	5	33
82. 9494/43	0	0	0	0	0	0	0	0	10	1,1	6	30
83. 9345/17	0	0	0	0	0	0	0	0	0	0,0	4	86
83. 9351/ 2	0	0	0	5	0	0	0	0	0	0,6	2	84
83. 9352/34	0	0	0	0	0	0	7	0	0	0,8	9	68
83. 9355/ 8	0	0	0	0	0	0	0	0	0	0,0	0	78
83. 9355/12	0	0	0	0	0	0	0	0	0	0,0	0	63
84. 9378/26	-	0	0	0	0	0	0	0	-	0,0	0	100
84. 9378/29	-	0	0	0	0	0	0	0	0	0,0	5	95
x	0,4	1,9	0,7	0,7	2,3	0	1,3	0	0,7	0,9	2,9	62
variety A	5	10	10	20	50	10	10	40	5	17,8	30	100
77. 9152/23	100	100	70	100	100	100	100	100	90	95,6	100	100

40 % of PVS-hypersensitive clones remained in field and/or contact test without PVS. In case of attack in one year generally none of the plants carried PVS to next year, although there was only one (the same) source of seed potatoes and no therapy was applied. The variety A showing highly relative resistance was attacked by PVS between 5 and 50 %, while on the highly susceptible clone 77.9152/23 almost 100 % PVS was detected.

The test of seed potatoes from field and the contact test made it possible to discard susceptible clones before grafting, by which highly relatively PVS-resistant and hypersensitive clones are differentiated.
However, inoculation by grafting resulted in 22 to 100 % attack of daughter tubers of hypersensitive clones (table 2). Seldom we obtained 0 % PVS after grafting over the years as shown in table 3 .

Table 3: Results of grafting test in several years. Percentage of hypersensitively reacting clones and of thoses remaining free from PVS after grafting.

year	number of clones tested		%	clones without PVS after grafting	
	grafted	hypersensitive	hypersensitive	number	%
1987	103	53	52	0	0
1988	57	39	68	7	12
1989	42	33	79	1	2
1992	21	16	76	2	12
1993	18	13	72	1	6
1994	16	12	75	1	6

Attack by grafting test in table 2 correlated r = -0,75 with field attack and r = - 0,30 with part of infections in contact test. Because of very small clonal differences as well in field attack as in contact test these correlations should not be overestimated. It could be possible that hypersensitivity and relative resistance combined lead to lower attack in grafting (as 76.9101/14, 79.9191/41, 82.9292/7, 82.9494/43), but did not affect or only partly affect PVS transmission by aphids in the field and cold frame. This problem has to be clarified. For practical purposes is sure that hypersensitivity keeps the potato largely PVS free in the field, nevertheless there is no guarantee for each single plant or tuber to remain PVS free in the field, but the resistance mechanism seems to lead to loss of PVS again. Further investigation has to be performed in mechanism and inheritance of PVS resistance.

Good PVS resistance has to be combined with sufficient expression of other traits of the potato to be used by variety breeding. Our crossing program served to improve the formerly poorly expressed characteristics such as susceptibility to PVY, PLRV or PVM, and low tuber size, which were described as problems in breeding to PVS resistance at Groß Lüsewitz in the past (10). Table 4 shows data in average of several years for a few important traits considering agronomical value of the PVS-resistant clones.

Table 4: Tuber characteristics and resistance to virus diseases of 15 PVS hypersensitive clones and three varieties

clone	external tuber traits						internal tuber traits				resistance to		
	1	2	3	4	5	6	7	8	9	10	PLRV	PVY	PVM
76. 9109 /14	6,3	5,5	5,0	6,7	5,3	936	14,4	6,3	0	3,9	7	5	4
76. 9156 / 6 *	5,2	4,5	3,5	5,8	5,5	828	16,1	4,9	0	5,3	7	9	7
77. 9157 / 3 *	5,7	5,0	3,7	5,8	4,4	1283	14,4	5,4	5	5,9	8	9	4
79. 9191 /41	6,1	5,9	3,9	6,1	4,5	1073	16,0	5,0	0	4,9	8	7	5
82. 9277 /11	5,8	5,5	4,5	6,0	5,5	967	13,3	-	5	4,8	5	6	6
82. 9277 /17	5,2	6,3	3,4	5,8	4,2	1484	13,0	6,2	5	4,5	7	9	7
82. 9292 / 7	6,0	6,3	5,0	5,7	6,0	928	14,3	-	0	7,0	7	3	5
82. 9294 /43	6,0	5,3	5,0	6,0	6,3	945	17,0	4,5	10	6,8	4	5	4
83. 9345 /17	5,6	5,6	4,2	6,3	6,3	1170	16,4	5,9	20	5,7	6	5	3
83. 9351 / 2	6,6	5,9	5,0	6,7	6,4	1050	16,2	5,6	25	4,9	4	4	4
83. 9352 /34	5,9	5,8	5,1	6,5	6,0	868	17,2	-	0	3,2	6	3	5
83. 9355 / 8 *	5,7	5,9	4,3	6,1	5,6	1225	16,0	5,1	30	3,7	8	9	3
83. 9355 /12	6,0	5,9	5,1	6,9	5,9	850	17,7	5,2	15	4,2	7	6	6
84. 9378 /26	6,1	5,5	6,0	6,7	6,3	940	17,1	6,2	0	1,3	3	4	4
84. 9378 /29	6,8	5,8	7,0	7,5	7,2	1009	18,7	4,6	30	2,4	7	3	5
variety A	6,5	5,9	5,9	6,9	6,6	1067	17,6	5,8	0	4,9	7	5	-
variety G	6,3	5,6	5,5	6,8	6,9	1167	13,9	4,7	0	4,7	6	7	-
variety Q	8,0	7,1	7,4	7,9	7,3	949	15,7	6,2	0	2,7	5	3	-

Explanation: 1= in general impression, 2 = size , 3 = external defects , 4 = beauty of shape , 5 = eye depth , 6 = yield (g per plant) , 7 = starch content (%) , 8 = culinary quality (considering appearance and discoloration after cooking and flavour), 9 = rust spots (%), 10 = susceptibility to damage (assessed by a pendulum device), * = clones handed over to variety breeders.

Results in table 4 show that these clones largely could correspond with expectations of variety breeders. In general tuber impression was middle to good. The yield, tuber size, beauty of shape and eye depth seemed not to be a problem now, but external defects occured more frequently and more intensively than in case of these varieties.
Among internal tuber traits out of rust spots there was not a markable difference between variety level and basic material, but the single clones differed from each other. However, in a few cases too many internal rust spots occurred.
Besides resistance to PVS the particular advantage of this basic material consists in complex resistance to most important virus diseases. This complex virus resistance could considerably reduce the efforts to produce seed potatoes. While four of these 15 clones combine hypersensitivity to PVS with extreme resistance to PVX and PVY just as highly relative resistance to PLRV, a few others are a bit too susceptible to PVY . These clones mature middle early to middle late , while variety A matures between early and middle early .
These results are presented 30 years after the beginning of the potato breeding with hypersensitivity to PVS. While it's inheritance is simple, the hypersensitive reaction is not so easy to recognize in each case. Disorders of plant growth out of daughter tubers can make it impossible to decide the type of PVS resistance.The earlier hope of rapid use of hypersensitivity to PVS in variety breeding (2 , 1) was too optimistic because of problems in other traits (3 , 16) and different reaction to several PVS isolates (8). Yet now there is basic material in our institute which should be suitable to be used as cross parents in variety breeding. The second, third and twelfth clone of table 2 and 4 were handed over to German breeders in 1994.

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MONOCLONAL IMMUNOENZYME TEST SYSTEM FOR ARABIS MOSAIC VIRUS DETECTION

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Introduction

Arabis mosaic virus (ArMV), the type member of the nepovirus group, is an isometric, RNA-containing, nematode-transmitted virus.

The particles contain two RNA species of m.w. $2.8 \cdot 10^6$ (RNA-1) and $1.4 \cdot 10^6$ (RNA-2). ArMV has a polyhedral capsid composed of a single type of the coat protein molecules of m.w. 54 kD (1). This virus infects strawberry, raspberry, other berries, some vegetables and flowers.

In this paper we report work on production and characterization of monoclonal antibodies (Mab's) to ArMV. Application of Mab's for the development of high-sensitive test system suitable for routine detection of ArMV is discussed.

Material and Methods.

The ArMV type strain, purified by the method of Davies and Clark (2), and rabbit polyclonal antibodies (Pab's) to ArMV were kindly provided by O.V. Borisova (Department of Virology, Moscow State University).

Intrasplenic immunization of mice was performed as described by Spitz et al. (3). Intraperitoneal immunization and production of hybridomas were carried out as described by Plechko et al. (4). Mab's isotypes were identified using kit of the subclass-specific antibodies (Calbiochem). Ascitic fluid was produced by intraperitoneal injection of 10^7 hybrid cells into syngenic mice that had been primed intraperitoneally with 0.5 ml pristane (Sigma) 3 days before.

Mab's were purified from ascitic or cultural fluids using ammonium sulfate precipitation followed by affinity chromatography on Protein-A Sepharose (Pharmacia). Purity of Mab's was checked by gradient SDS gel electrophoresis. The binding constant of anti-ArMV Mab's were determined by noncompetitive ELISA (5).

Plant material was homogenized in PBS (5 ml of PBS per 1 g of plant material) and clarified by centrifugation at 5000g for 15 min. The extracts were stored in 50 % glycerol at -20°C.

Horseradish peroxidase (HRP, Boehringer-Mannheim) was conjugated with Mab's according to method of Tijssen et al. (6). ELISA procedures were modified from Clark and Adams (7).

Cultural fluids were tested by triple antibody sandwich ELISA (TAS-ELISA). The best Mab's combination for ArMV detection was determined by double antibody sandwich ELISA (DAS-ELISA) and TAS-ELISA with rabbit Pab's against ArMV as capture.

Latex-test for ArMV detection was carried out as described in (4). Polyacroleine latex granules were kindly provided by Yu.V.Lukin, Institute of Bioorganic Chemistry, Moscow).

Results

Four fusion experiments using P3.X63.Ag8.653 myeloma cells yielded a total of nine hybrid cell lines.

Four stable hybrid cell lines were established after two cloning steps. Two hybrid lines designed using intrasplenic immunization produced IgM isotype Mab's, whereas two lines designed using intraperitoneal immunization produced IgG isotype Mab's.

Some characteristics of these lines are presented in Table 1. Mab's 3B4 and 1A10 were conjugated with HRP. To select an optimal immunoassay format we compared DAS-ELISA, TAS-ELISA and latex test. These methods were used to determine minimal detection level of ArMV in the purified preparation and in plant extracts. The experimental results are summarized in Table 2.

Table 1. Properties of Mab's studied.

Monoclonal antibody	Isotype	K_a, M^{-1}	Mab's titre in	
			ascitic fluid	cultural fluid
3B4	IgG2b	$4.1 \cdot 10^{11}$	-	$4.0 \cdot 10^3$
4A2	IgG2b	$2.3 \cdot 10^{11}$	-	$2.0 \cdot 10^3$
1A10	IgM	$2.1 \cdot 10^{10}$	10^5	$1.5 \cdot 10^3$
3G3	IgM	$2.3 \cdot 10^{10}$	10^5	$1.2 \cdot 10^3$

Table 2. The reactivity of the Mab's directed against ArMV in different ELISA formats and in latex test.

Mab's	Min. detectable level in purified ArMv preparation, ng per ml			Max dilution of plant extracts		
	DAS[1]	TAS[2]	latex[3] test	DAS	TAS	latex test
3B4	2-3[a]	2-3	6-8	1:1280[a]	1:1280	1:160
4A2	4-6[a]	6-8	10-12	1:640[a]	1:640	1:80
1A10	30-60[b]	-	1000	1:12000[b]	-	1:80
3G3	60[b]	-	1000	1:12000[b]	-	1:80

1) - ELISA format: Mab - antigen - 3B4-HRP[a] or 1A10-HRP[b] - o-phenylenediamine (OPD)

2) - ELISA format: anti-ArMV Pab's - antigen - Mab's - goat anti-mouse-HRP conjugate - OPD.

3) - latex immunoassay: antigen - Mab's immobilized on latex polymeric granules.

Epitope specificity of Mab's was demonstrated by competitive binding assay (8). HRP-marked Mab's were challenged by unlabeled Mab's, which were titrated in serial dilutions. IgM isotype Mab's 1A10 and 3G3 didn't compete with IgG isotype Mab's 3B4 and 4A2. 4A2 Mab from 40 to 60 % decreased the binding of 3B4-HRP (Fig. 1 A; curve 1); 3G3 Mab decreased the binding of 1A10-HRP at high concentration only (Fig. 1 B; curve 1). The last can be explained that 3G3 and 1A10 Mab's are IgM isotype, what caused limits at theirs binding. Thus we supposed that all four Mab's reacted with different epitopes on the virus coat protein.

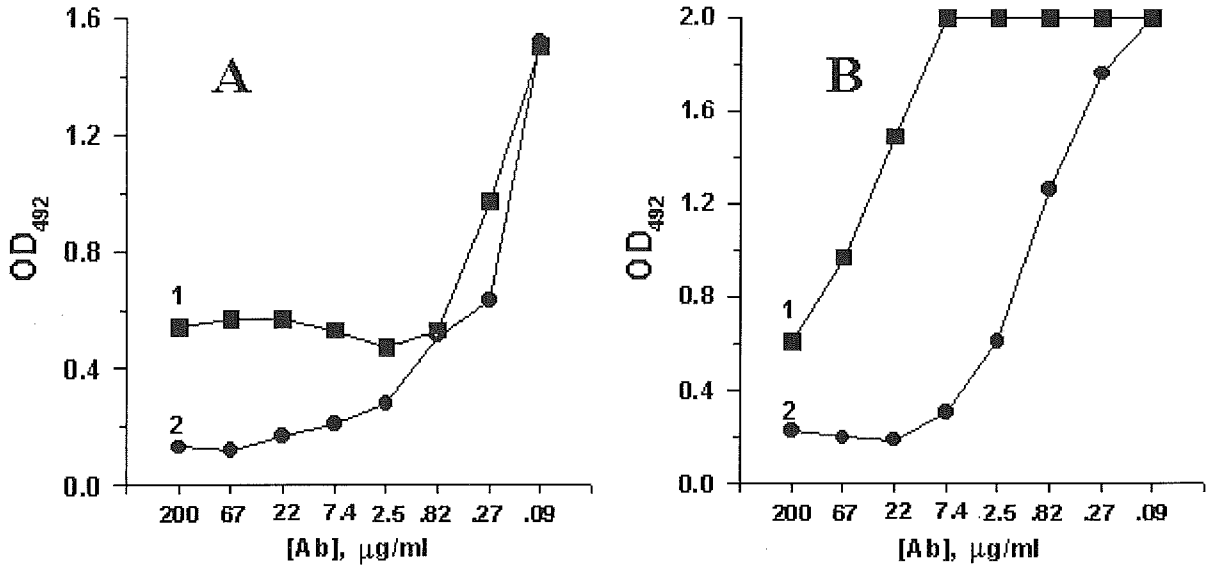

Fig. 1. Competitive binding assay. A: Mab's 4A2 (1) and 3B4 (2) are compete with 3B4-HRP conjugate. B: Mab's 3G3 (1) and 1A10 (2) are compete with 1A10-HRP conjugate.

Discussion

Four stable hybridoma cell lines secreating Mab's specific for ArMV antigen were obtained in four fusions experiments. We wanted Mab's that would be suitable for routine ArMV detection in plant extracts. As was shown in the preliminary experiments the level of nonspecific reactions with IgM isotype Mab's was rather high. Moreover, sensitivity of test system based on IgG isotype Mab's 3B4 and 4A2 was essentially above. Monoclonal test-system based on Mab 3B4 as capture and 3B4-HRP conjugate allowed to detect ArMV to 2-3 ng per ml as well as combined test system with rabbit Pab's as capture and 3B4-HRP conjugate.

It was shown that latex test and DAS-ELISA with Mabs 3B4 and 4A2 have closely sensitivity. Using Pab's for nepovirus detection by latex test don't permit sufficiently sensitivity for reliable detection of nepovirus (1). Therefore, latex test with Mab's can have widely application in phytovirological practice.

The results of competitive binding assay can, with certain limits , supply information on the topological relationships of the epitopes, especially when the antibodies do not compete with each other. The epitope delineated with 3B4 Mab differed from the one identified by 1A10, 3G3 and, probably, 4A2 Mab's. We suppose that the epitopes reacting with 3B4 and 4A2 Mab's to overlap whereas the epitopes detected by 1A10 and 3G3 are distinct.

The monoclonal test system was used in Moscow State Botanical Garden Russian Academy of Sciences for the ArMV detection for 700 plant samples of Cucumis sativus, Fragaria vesca, Fragaria ananassa, Rubus idaeus, Ribes, Artiplex, Nicotiana, Lonicera.

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PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST BARLEY YELLOW DWARF VIRUS. DETECTION OF BaYDV IN THE FIELD SAMPLES FROM CENTRAL REGION OF RUSSIA.

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Introduction

Barley yellow dwarf virus (BaYDV) is the type member of the luteovirus group. The virus is found in low concentration in infected plants and is limited to the phloem (1). BaYDV is small (approximately 25 nm) isometric particle that is transmitted in a persistent manner by aphid vectors. Five isolates of BaYDV originally were differentiated by the species of aphids which transfer them (2). The MAV, PAV and SGV isolates are serologically related. The RPV and RMV isolates are distantly related to either MAV and PAV (1).

The purpose of this study was to develop a monoclonal test system for the BaYDV diagnostics in field samples.

Material and Methods.

BaYDV isolates were purified by the method of Hammond et al. (3) from naturally infected oat and barley plants of Central region of Russia. Intraperitoneal immunization and production of hybridomas were carried out as had described by Erokhina (4).

Mab's isotypes were identified using the subclass-specific antibodies kit (Calbiochem).

Ascitic fluid was produced by intraperitoneal injection of 10^7 hybrid cells into syngenic mice that had been primed intraperitoneally with 0.5 ml of pristane (Sigma) 3 days before.

Mab's were purified from ascitic or cultural fluids using ammonium sulfate precipitation followed by affinity chromatography on Protein A Sepharose (Pharmacia). Purity of Mab's was checked by gradient SDS gel electrophoresis.

The binding constants of anti-BaYDV Mab's were determined by non-competitive ELISA (5). Horseradish peroxidase (HRP, Boehringer-Mannheim) was conjugated with Mab's according to method of Tijssen et al. (6). Plant extracts were prepared as directed in (4).

ELISA procedures were performed as described in (4). Cultural fluids were tested by triple antibody sandwich ELISA (TAS-ELISA). Antigen-coated plates (ACP) and double antibody sandwich (DAS) ELISA formats were used to define Mab's sensitivity and specificity.

Results

About 200-300 mkg of the virus were purified from 1 kg of the plant material. A few isolates of BaYDV (U-92, N-93, T-93) of unknown composition were used for the test system development.

Immunization procedure was carried out using U-92 isolate. Five stable hybrid cell lines were established after two fusions of mouse spleen cells with Pai and P3.X63.Ag8.653 myeloma cells. Some characteristics of Mab's produced by these lines are presented in Table 1.

Table 1. Some characteristics of anti-BaYDV Mab's.

Monoclonal antibody	Derived from myeloma line	Isotype	K_a, M^{-1}	Titre in cultural fluid
4B5	P3.X63.Ag8.653	IgG2b	$1.5 \cdot 10^{11}$	$4.0 \cdot 10^4$
3C3	P3.X63.Ag8.653	IgG2a	$8.8 \cdot 10^8$	$4.0 \cdot 10^4$
1D2	Pai	IgG3	$1.1 \cdot 10^{12}$	$2.0 \cdot 10^4$
1C5	Pai	IgG2a	$1.6 \cdot 10^{11}$	$2.5 \cdot 10^3$
3C2	Pai	IgG2a	n.d.	$2.0 \cdot 10^4$

n.d. - non determined.

In order that to determine composition of BaYDV isolates, the polyclonal immunoenzymatic kit to five isolates of BaYDV (Agdia, USA) was used. Five Pab's specific to different BaYDV isolates were used as capture in DAS-ELISA. Three isolates of BaYDV: U-92, N-93 and T-93 were examined for their composition. We compared 4B5 Mab HRP labeled and anti-BaYDV Pab's alkaline phosphatase (AP) labeled.

It was determined that T-93 isolate contains PAV, MAV and SGV isolates; N-93 contains largely PAV isolate, and to a lesser degree MAV and SGV isolates. U-92 contains PAV, MAV and to a lesser degree RPV and SGV isolates. The results were similar for 4B5 Mab and anti-BaYDV Pab's (Diagram 1; a, b).

We tested each antibody in the coating step by DAS-ELISA to define its specificity. 4B5 and 3C3 Mab's reacted strongly with all isolates in DAS-ELISA as "lower" (immobilized on the plate) and "upper" (peroxidase conjugated) antibodies. 1D2 Mab bound with N-93, T-93 and U-92 isolates only as "upper" antibody. Reaction of other two Mab's varied for different BaYDV isolates.

Mab's showed clearly defined specificity when they had applied to BaYDV detection in plant extracts. We were fortunate to discover field samples that contained PAV isolate either RPV one. In the experiments with this samples, 4B5 Mab reacted with both extracts, whereas 3C2, 1C5 and 1D2 Mab's were RPV-specific (Diagram 2).

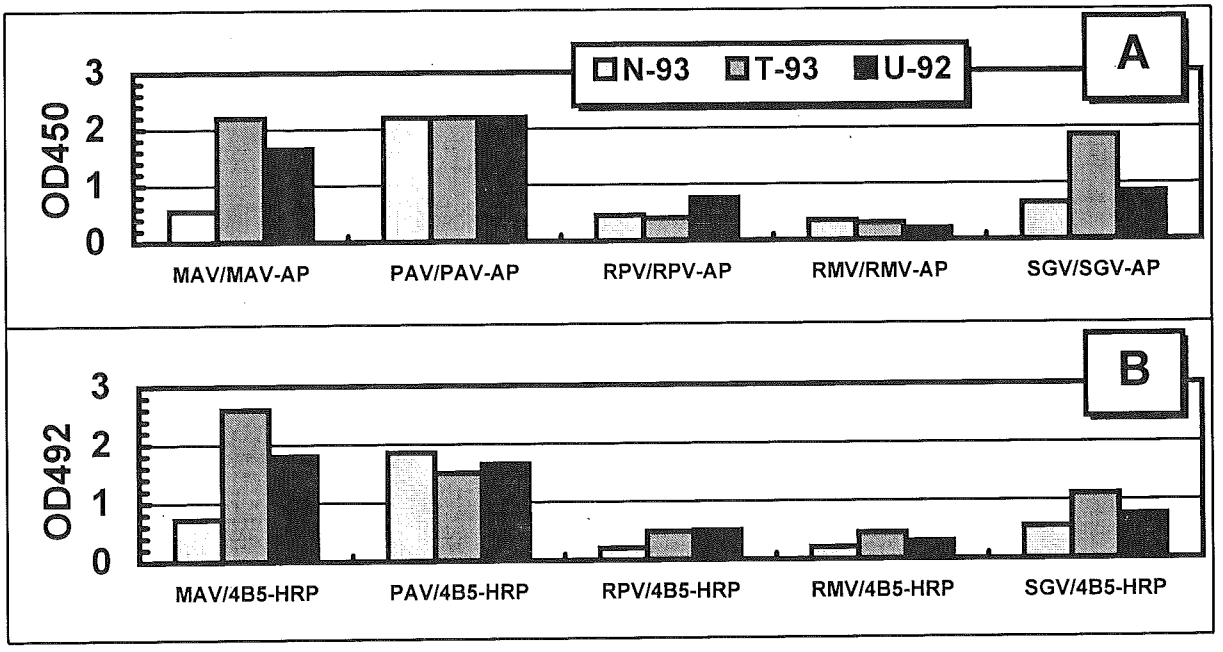

Diagram 1. Composition of BaYDV isolates N-93, T-93 and U-92 from field samples of the central region of Russia.

A: DAS-ELISA with polyclonal immunoezymatic kit against BaYDV isolates;
B: DAS-ELISA with anti-BaYDV Pab's in capture and 4B5 Mab HRP-labeled.

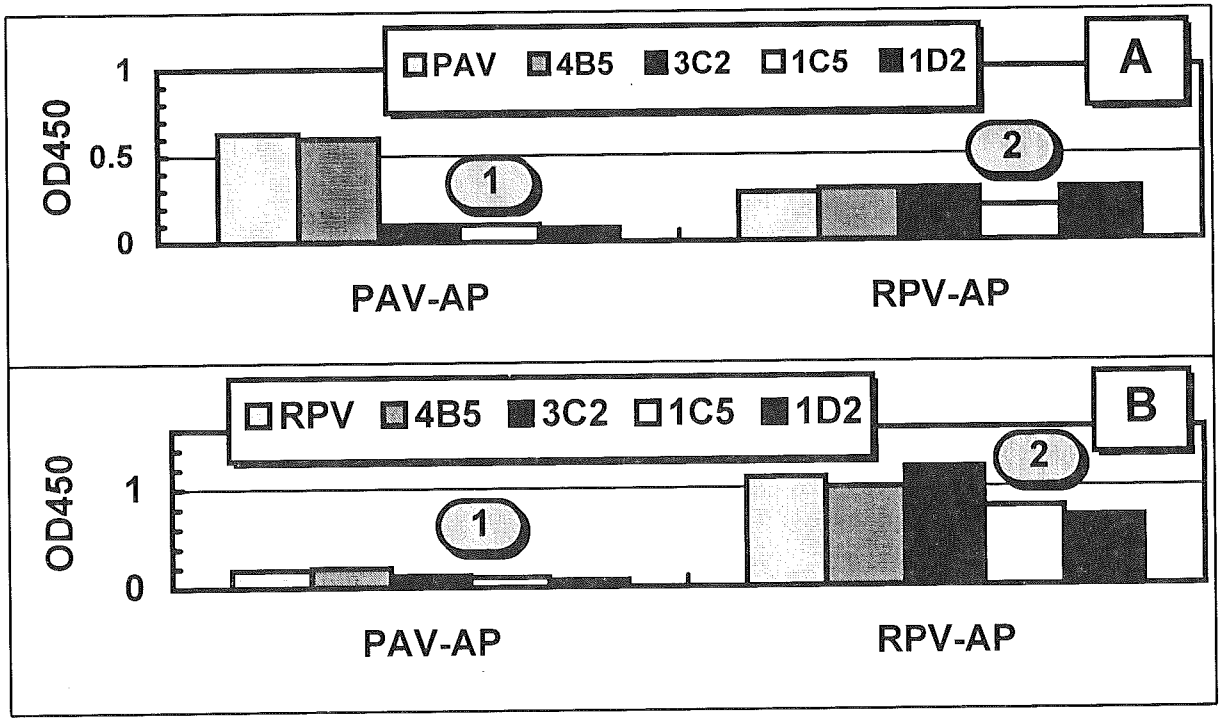

Diagram 2. Mab's specificity with PAV - (A) and RPV (B) containing plant extracts.

1: anti-PAV AP-labeled conjugate;
2: anti-RPV AP-labeled conjugate.

The sensitivity of 4B5 and 3C3 Mab's were in the range between 7 to 8 ng of BaYDV per ml, when they had used in the coating step in DAS-ELISA. For 1C5, 3C2 and 1D2 Mab's the lowest determinable concentration was from 15 to 60 ng per ml. In plant extracts BaYDV was determined at a dilution from 1:16 to 1:128.

All Mab's did not react with four different isolates of potato leafroll virus which also belong to luteoviruses.

Discussion

The five BaYDV-specific Mab's can be used for the virus detection in plant samples. The RPV-specific 3C2 Mab permits to differentiate RPV from PAV. These isolates were found in 80-90 % of plant samples from central region of Russia. 4B5 Mab reacted with all purified virus preparations (used in this study) as well as with PAV and RPV containing plant extracts. We supposed that this antibody bind with epitope common for PAV and RPV isolates. 3C2, 1C5 and 1D2 Mab's reacted with RPV containing extracts only. Unfortunately MAV and SGV containing extracts were inaccessible for as. Any samples studied did not contain a considerable amount of RMV isolate.

Mab's had been used for BaYDV detection in large number of field samples of wheat, oats and barley obtained from central region of Russia and the results compared favourably with tests using polyclonal antibodies.

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RESISTANCE TO BEET NECROTIC YELLOW VEIN VIRUS (BNYVV) INFECTION IN *NICOTIANA BENTHAMIANA* EXPRESSING BNYVV-SPECIFIC SINGLE CHAIN ANTIBODY FRAGMENTS (SCFV)

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Introduction

A novel approach to establish virus resistance in plants is based on the expression of virus-specific antibodies or single chain fragments (scFv) (1, 2). The scFv are monovalent antigen binding molecules consisting of the variable regions of the heavy (VH) and the light chains (VL) of antibodies which are connected by a linker peptide. It has been shown that *Nicotiana benthamiana* expressing scFv specific for the coat protein of artichoke mottled crinkle virus has a reduced susceptibility for this virus and the development of symptoms is delayed (1).

Antibody or scFv-mediated resistance seems to be an attractive alternative to pathogen-derived resistance as it circumvents biosafety problems arising from phenomena such as transcapsidation (3, 4) or recombination (5) of the expressed viral genes with genes of other viruses which have been observed occasionally (3, 4, 5). We are attempting to establish scFv-dependent resistance against beet necrotic yellow vein virus (BNYVV) in plants. BNYVV is a fungus-transmitted positive strand RNA virus causing the economically important rhizomania disease of sugarbeet (6) which in susceptible varieties causes a severe inhibition of the growth of tap roots and a remarkable reduction of the sugar content.

Material and Methods

Cloning of VH and VL encoding sequences in pCOCK for expression as scFv

Oligo (dT)-primed cDNA was obtained using poly(A)$^+$ RNA from hybridoma cell line 14CG6 as template in a reverse transcription reaction. In the first PCR the sequences encoding the variable regions and the neighbouring constant regions of the antibody heavy and light chains were amplified by Taq DNA polymerase (Gibco BRL) using appropriate primers (7) and the cDNA as initial template. DNA fragments with sizes of 650-700 bp encoding VHCH1 and VLCL were obtained and these DNA fragments were used as template for the amplification of the VH and VL encoding DNA. The primers used for these reactions also contained the sequences recognized by the restriction endonucleases which were used for the cloning of the amplification products in the expression vector pCOCK (8). The DNA fragments encoding VH were integrated first into pCOCK. Recombinant plasmids with the VH encoding DNA were isolated from *Escherichia coli* HB2151 transformants and the DNA fragments for VL were ligated into these plasmids. The HB2151 transformant with pCOCK-scFv14CLb1 encoding a functional scFv was selected from transformed colonies grown on 2xYT agar plates supplemented with 100µg/ml ampicillin and 1% glucose. Expression of scFv was studied after addition of IPTG to transformed HB2151 cells grown up to an OD_{600} of 0.8 in induction medium (2xYT, 0.05% glucose, 100 µg/ml ampicillin).

Constructs for plant transformation

Different scFv-encoding DNA fragments with and without signal peptide-encoding sequences were obtained by PCR using appropriate primers. These primers also included the cleavage sites of BspHI or XbaI for the integration of the amplified DNA into the NcoI and XbaI sites of pRT103 (9) or pRT213. One of the primers contained the whole signal peptide-encoding sequence of the phytohemagglutinine gene of *Phaseolus vulgaris* (10). The plant scFv expression cassettes from pRT103 and pRT213 were recloned in pLX222 (11) and the resulting plasmids were introduced into *Agrobacterium tumefaciens* for transformation of *Nicotiana benthamiana* by the leaf disk method (12).

Dot blot immunoassay for the detection of functional scFv

A preparation of *E.coli*-expressed BNYVV coat protein was dotted onto a Quiabrane nylon membrane. After blocking, the membrane was incubated with scFv-containing bacterial culture supernatants or with plant extracts. ScFv bound to the coat protein were detected by a colour reaction after further washings and incubations with MAb 9E10 specific for the myc-tag (13) of the scFv and anti-mouse IgG alkaline phosphatase-conjugated antibodies.

Results and Discussion

Expression of scFv in bacteria

The scFv which we have expressed in plants are derived from MAb 14CG6. This MAb binds to an epitope at the carboxy terminal end of the coat protein. This epitope is accessible along the entire length of the viral particle (14). The DNA sequences encoding VH and VL of MAb14CG6 were cloned sequentially in the expression vector pCOCK. In these cloning steps the VH and VL encoding sequences become connected by a DNA sequence encoding a linker peptide. In addition the lac promoter and coding sequences for the bacterial signal peptide of PelB (15) and the myc-tag marker peptide (13) are added to the ends of the scFv open reading frame. Among several HB2151 transformants one clone containing pCOCK-scFv14CLb1 (Fig. 1a) was selected which expressed scFv after induction of the lac promoter. In Western blotting analyses these scFv specifically bound to immobilized BNYVV coat protein. Comparative studies with different fractions of the bacterial cultures revealed that only relatively low amounts of scFv were present in the culture medium. Most of the scFv were found in the insoluble cell fraction.

In order to compare the efficiencies of antigen detection by MAb 14CG6 and the scFv derived from it, the antigen titers were determined in a dot blot immunoassay. Serial dilutions of a BNYVV coat protein preparation were dotted onto a nylon membrane and this membrane was incubated with MAb 14CG6 or a concentrated sample of a scFv-containing culture supernatant. As little as 0.1 ng of antigen were detected by the scFv as well as the MAb. The scFv did not bind to petunia asteroid mosaic virus (PeAMV) which was used as a control.

Expression of scFv in *Nicotiana benthamiana*

Agrobacterium tumefaciens was used to transform *N. benthamiana* with several plant scFv expression cassettes. This plant was chosen since transformation of sugarbeet still is very difficult to achieve and a few BNYVV isolates are available which infect *N. benthamiana* (R. Koenig, unpublished results). The scFv plant expression cassettes initially were constructed in pRT103 (9) and pRT213. Analogous scFv constructs with coding sequences for the PelB signal peptide, the plant derived signal peptide of the phytohemagglutinine (PHA) gene of *Phaseolus vulgaris* (10) or without any signal peptide-encoding sequences were either cloned behind the 35S CaMV promoter sequence in pRT103 or behind the enhanced 35S promoter sequence and the TMV Ω-leader region of pRT213 (Fig. 1, b-d).

Plants transformed with the respective expression cassette were selected on the basis of their tolerance towards the antibiotic kanamycin. Extracts of transgenic plants grown in the greenhouse were analysed for production of functional scFv in the immunodot blot assay. In addition, the amount of neomycine phosphotransferase (NPTII) was determined by ELISA. There was no direct correlation between the amount of NPTII and scFv expression in comparison of several transgenic lines. In extracts of plants transformed with constructs for cytoplasmic expression no or only very low expression of functional scFv could be found (Table I). Higher but nevertheless varying amounts of scFv could be detected in plants transformed with constructs for extracellular scFv expression.

Attempts to detect virus resistance

The possible establishment of virus resistance was checked with F1 plants from the transgenic line 23 which expresses relatively low amounts of scFv. Non-transformed *N. benthamiana* plants were used as controls. Seeds of the more efficiently expressing transgenic line 116 (Table 1) were not yet available. In two experiments we observed a delay of virus infection which was more pronounced with a more dilute virus inoculum (plant sap diluted 1:16) than with a more concentrated one (plant sap diluted 1:4). In a third experiment this effect was less clear, possibly because due to seasonal variation in this experiment infections became established earlier than usually and the testing was started correspondingly too late. In all three experiments the transgenic plants showed fewer symptoms than the nontransgenic ones. Further experiments with plants of the strongly expressing transgenic line 116 will be started soon.

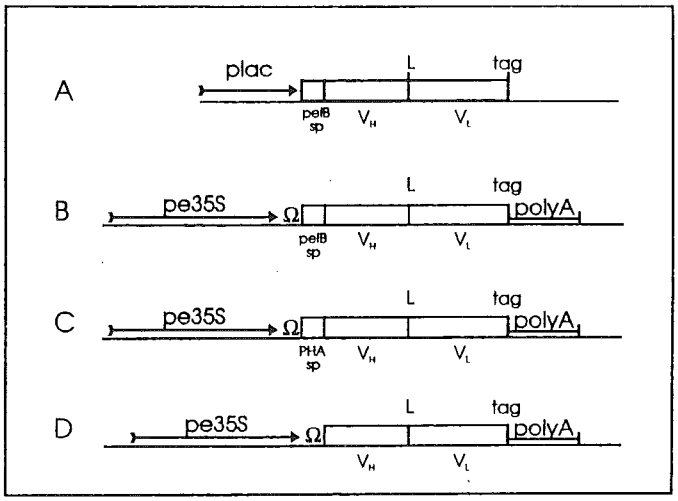

Figure 1. Constructs for the expression of scFv in bacteria (A) and in plants (B-D) in pCOCK (A) and in pRT213 (B-D). Abbreviations: plac: promoter of *E.coli* lac operon; pelB sp, PHA sp, V_H, L, V_L, tag: DNA fragments encoding signal peptide of pectate lyase, signal peptide of phytohemagglutinine, VH, linker peptide, VL and myc-tag peptide, respectively; pe35S: enhanced 35S promoter of CaMV; Ω: translational leader region of TMV; polyA: polyadenylation signal of CaMV.

Table 1. Detection of neomycin phosphotransferase (NPT II) levels by means of ELISA and of scFv by a modified dot blot immunoassay (see text) in extracts of kanamycin tolerant regenerated *N. benthamiana* plants. The plants had been grown in the greenhouse for seed production.

species/line	signal peptide PHA/PelB	amount of NPTII in ELISA assay (ng/mg)	relative scFv-expression level (dotblot assay)*
N.b./control	-	**0**	-
N.b./115	**PelB**	**18**	-**
N.b./33	**PelB**	**2**	-**
N.b./22	**PHA**	**22**	+
N.b./23	**PHA**	**30**	++
N.b./77	**PHA**	**<0.5**	+++
N.b./116	**PHA**	**<0.5**	++++++
N.b./27	-	**23**	-
N.b./28	-	**0.5**	- (?)
N.b./89-1	-	**7**	-
N.b./118	-	**6**	-
N.b./51	-	**5**	- (?)

Appendix:

* - means: no expression of functionally active scFv; - (?): possibly very low expression of scFv-further analysis is necessary; +, ++, +++: lower or higher amounts of scFv expressed in comparison to a scFv standard.

**: plants kept in sterile culture originally produced low amounts of scFv.

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EVALUATION OF MAIZE MATERIAL OF THE GENE BANK GATERSLEBEN FOR RESISTANCE TO THE VIRUSES SCMV AND MDMV

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Introduction

During the last years an increasing level of virus diseases in maize crops in Germany was found. This is mainly due to the sugarcane mosaic virus (SCMV). At present the maize dwarf mosaic virus (MDMV) occurs relatively seldom. According to the taxonomy of SHUKLA et al. (1) both viruses belong to the sugarcane mosaic virus subgroup of potyviruses. A distinction between the two viruses on the basis of symptoms is not possible. An early infection (from the end of May to the middle of June) can reduce the plant height by 16.9 %, the total plant weight by 37.1 % and the total corn-cob weight by 27.8 % (2). Since both viruses are transmitted in a non-persistent manner by aphids a control of the vectors seems not recommendable. Although early sowing and the establishment of fast-covering crops can help to minimise the virus infection rate the only promising way to control the virus is the breeding for and the cultivation of resistant varieties. The selection of resistant inbred lines and their utilisation in practical plant breeding is a necessary precondition for this.

According to American investigations (3) the inbred lines B 68, Oh 1 EP, Pa 11 and Pa 405 exhibit immunity or a high degree of resistance to the two viruses both in the greenhouse and under field conditions. Also the Canadian inbred lines CO 125 and CM 105 proved to be resistant (4, 5). The genetic basis for the resistance to MDMV and SCMV was examined in American studies (6, 7) and by means of diallelic crosses as well as back crosses with the susceptible and the resistant parent, respectively (8). Depending on the used genetic material 2 to 5 genes seem to be involved in resistance to the two viruses (6, 7). Meanwhile the major gene Mdm 1 responsible for MDMV resistance could be located by means of the restriction fragment length polymorphism method (RFLP) on the chromosome 6 near the centromer between the markers UMC 45 and BNL 6.29 (9). The biological basis for the resistance is still unknown. The existing findings lead to the presumption that all resistances found so far possess the same genetic background. Therefore, it seemed promising to test material with a different geographical origin to find additional resistance genes. The Gene Bank Gatersleben with its more than 1200 accessions from all over the world provides good preconditions for such a survey.

Materials and methods

Between the 01.01.1993 and the 30.05.1995 we tested 654 accessions for resistance to SCMV and MDMV. Forty seeds per accession were sown in pots with a diameter of 9 cm (5 seeds per pot). About 8 to 10 days after sowing 20 plants were inoculated mechanically in the three-leaf stage with SCMV and MDMV, respectively, using the isolates SCMV-Seehausen and MDMV-Bulgaria. For evaluation of resistance we determined the disease index and the symptom index (8). The disease index (10) is calculated by means of the following formula:

$$\text{disease index} = 4W+3X+2Y+Z$$

W, X, Y, Z represent the percentage of plants showing symptoms 7, 14, 21, and 28 days after the initial inoculation, respectively. The obtained values are related to resistance and susceptibility as follows :

resistance class	behaviour	disease index
I	resistant	0 ... 350
II	intermediate	351 ... 650
III	susceptible	651 ... 1000

For the determination of symptom index values we noted the symptom severity at 4 weeks p.i. Every plant was examined for symptoms using the following scoring scale:

score 1 no symptoms
score 2 single or a few narrow stripes with mosaic pattern on the youngest leaves
score 3 some narrow or wider stripes with a mosaic pattern on several leaves
score 4 typical mosaic symptoms on all leaves

For the calculation of the symptom index we used the following formula:

$$\text{symptom index} = \frac{a + 2b + 3c + 4d}{n}$$

The letters a, b, c, and d equal the number of plants with the scoring 1, 2, 3, and 4, respectively. The calculated values allow a classification as follows:

resistance class	**behaviour**	**symptom index**
I	resistant	1.00 ... 1.99
II	intermediate	2.00 ... 2.99
III	susceptible	3.00 ... 4.00

The three accessions found to be resistant under greenhouse conditions in 1993 were tested in 1994 under field conditions (provocation trial). For this, 40 seeds per accession were sown on the 22.04.1994 at the trial station 'Kühnfeld' of the Faculty of Agriculture. Sources of infection were provided by means of border rows ('Bermasil') inoculated mechanically with SCMV and MDMV, respectively. Infection of the trial itself took place by aphids occurring naturally in the field. Visual scorings were carried out on the 11.06., 21.06., 02.07., 30.07., and 27.08.1994. All plants were checked serologically in an ELISA on the 28.08. 1994.

Results

The results of the evaluation of 654 maize accessions from the Gene Bank Gatersleben for resistance are presented in Table 1. In most cases the accessions showed the same behaviour considering the two viruses. So we found 481 susceptible accessions, 28 intermediate and 5 resistant one's.

Table 1: Results of testing 654 accessions from the Gene Bank Gatersleben for resistance to SCMV and MDMV in the greenhouse

		SCMV		
	resistance class	susceptible	intermediate	resistant
MDMV	susceptible	481	13	0
	intermediate	117	28	0
	resistant	5	5	5

A number of 140 types showed different response to SCMV and MDMV. So we found 117 types that were intermediate and 5 types that were resistant to MDMV but susceptible to SCMV. Five accessions with resistance to MDMV reacted intermediate referring to SCMV. On the other hand, 13 accessions were intermediate to SCMV and susceptible to MDMV. At present time an interpretation of the results is not possible. Further investigations have to be carried out but it should be mentioned that obviously the MDMV seems to have a lower virulence to maize what is seen in the resistance test as well. Investigations by FUCHS et al. (11) in the central and southern regions of Germany proved a weaker incidence of MDMV under field conditions compared with SCMV.

Further information on the accessions found to be resistant in the greenhouse are shown in Table 2. Obviously, immunity does not occur because single plants could be infected. An exception was 'ICAR 54' that showed immunity to MDMV.

Table 2: Evaluation of the resistant accessions

Accession	greenhouse				field		
	disease index		symptom index		number of plants tested	serologically positive (ELISA)	
	SCMV	MDMV	SCMV	MDMV		SCMV	MDMV
Zea mays L. conv. *mays* var. *caesia* Alef. (ZEA 365/1978)	75	10	1,2	1,1	35	5	1
Zea mays L. conv. *dentiformis* Körn. var. *flavorubra* Körn., 'Szegedi Sarga' (ZEA 111/1982)	118	90	1,3	1,4	27	26	0
Zea mays L. conv. *dentiformis* Körn. var. *flavorubra* Körn. 'ICAR 54' (ZEA 195/1981)	96	0	1,6	1,0	30	0	0
Zea mays L. conv. *dentiformis* Körn. var. *flavorubra* Körn. from Korea (ZEA 1063/1983)	15	6	1,2	1,2	n.t.	n.t.	n.t.
Zea mays L. from the Georgian Republic (ZEA G285/1985)	25	100	1,2	1,3	n.t.	n.t.	n.t.

n.t. = not tested

The three accessions found to be resistant in the greenhouse in 1993 were exposed to a natural infection by aphids in 1994. At all scoring dates an evaluation of symptoms was impossible since all plants were dwarfed and had light and dark green spots and mottling on their leaves. A record of scoring notes is therefore missing. After the serological test on the 28.08.1994 only 'ICAR 54' proved to be resistant while 'Szegedi Sarga' and the var. *caesia* Alef. appeared to be highly susceptible and slightly susceptible, respectively. A further characterisation of the resistance of 'ICAR 54' has yet to come.
In the year 1995 we will test 17 accessions for resistance under field conditions including 'ICAR 54' and the resistant accessions from Korea (ZEA 1063) and from the Georgian Republic (ZEA G 285) which were resistant in the greenhouse in 1994. The other 14 types showed a different response to the two viruses in the greenhouse.

Discussion

In the greenhouse test of 654 maize accessions from the Gene Bank Gatersleben we could determine 5 types with resistance to SCMV and MDMV. Two of the three accessions already tested in the field failed to be field resistant. These results let assume that the investigation on resistance is very difficult, expensive and time-consuming but a screening in the greenhouse should be always followed by a verification under field conditions. For the remaining three resistant types as well as 14 accessions with different response to SCMV and MDMV under greenhouse conditions this will be carried out in 1995.
Furthermore, it has to be considered that the used material did not react like inbred lines but like populations. Therefore phenotypic expression is not always uniform. In addition, it has to be pointed out that several accessions are not adapted to the climatic conditions of Central Europe what makes investigations in the field difficult.
A characterisation of the found resistances has yet to come. A comparison with already known resistant inbred lines seems imperatively to answer the question whether new resistance genes are involved or not. Appropriate crossing tests will be carried out in 1995. So far we could not find forms resistant to SCMV and susceptible to MDMV. Reverse cases could be observed. However, it has to be considered that the SCMV is more virulent than the MDMV. This was also seen in the field trial. Although we provided similar sources for infection for both viruses by means of mechanically infected border rows we could detect only single plants infected with MDMV at the end of the growing season. Therefore it is impossible to deduce from the results of the field trial a statement regarding resistance to MDMV.
The intermediate forms obviously express a quantitative resistance characterised by an elongated incubation period, a weaker symptom expression and a reduced virus concentration. This type of resistance is difficult to characterise genetically because of its polygenic mechanism of inheritance.

Summary
The evaluation of maize material from the Gene Bank Gatersleben is not yet finished. Up to now 654 accessions were tested in the greenhouse finding 5 resistant forms. A genetic characterisation has still to be done. The results of the field trial showed that it is imperative for interesting accessions to repeat the greenhouse test under field conditions. A decision whether new resistance mechanisms are involved is not possible until a comparison with already described resistance mechanisms is carried out; appropriate investigations are in progress.

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CHARACTERISTIC OF PHYTOVIRUS CAPSID PROTEINS FROM DIFFERENT TAXONOMIC GROUPS

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Introduction

One of the criteria uniting the plant viruses into a certain taxonomic group is the presence of identical epitopes in some members of this group. Due to development of methods in molecular biology it was possible recently to investigate the structural characteristics of virus-specific proteins. In addition to the standard biological tests, quantitative immunochemistry procedures, peptide mapping, translation of the virus RNA in vitro and sequencing this allows to plot more precise classification scheme within taxonomic groups. All those methods have been used by us in studies of the viruses identified in the south of the Russia. Far East as well as the viruses not described previously in literature to characterize them and classify as a certain taxonomic group.

Material and methods

Potexviruses: potato virus X, white clover mosaic virus, potato aucuba mosaic virus, Hydrangea ringspot virus, Plantago asiatica mosaic virus; potyviruses: potato virus Y (YBKo, YBKn, YBKc), potato virus A, soybean mosaic virus, bean yellow mosaic virus, onion yellow dwarf virus, turnip mosaic virus and new viruses - Tradescantia albiflora virus, Hippeastrum mosaic virus and Trifolium montanum mosaic virus; carlaviruses: potato M and S-viruses; bromoviruses: brome mosaic virus and three strains of Vicia unijuga mosaic virus were studied. Elaborated and modified methods of rocket immunoelectrofhoresis, double antibody "sandwish"-test and blocking variant ELISA, immune electron microscopy allowed to reveal strain-, virus- and

groupspecific epitopes of capcid proteins and to investigate their antigenic specificity.

Results and discussion

Comparative study on physico-chemical and antigenic properties of nine potyviruses were carried out to demonstrate belonging of new Tradescantia albiflora virus (TAV) and Trifolium montanum mosaic virus (TMMV) to the group of potyviruses. Structural proteins of TMMV and TAV were compared with proteins of Turnip mosaic virus (TMV) – a typical representative of potyviruses. The hard polypeptides with m.m. 38.0, 34.0 and 36.0 kD were established by gel papain limited proteolysis (for TMV, TAV and TMMV, respectively). The main protein of these viruses, was determined to cleave into more 9–12 polypeptides. In this case, 6 –7 of them coincide in stability in all three viruses thus pointing to homology of structural components and testifying to the affinity among viruses. Most probably the peptides bearing analogues are the intermediate products of hydrolysis.

When comparing the RNA of five potyviruses TMMV, TAV, TMV, onion yellow dwarf virus (OYDV) and bean yellow mosaic virus (BYMV) it was shown electrophoretically that there is one zone with m.m. 3.2–3.5 mD within the limits of m.m. RNA potyviruses. The RNA of BYMV, TAV and TMV were transtated in the system of lyzates of rabbit reticulocytes. The BYMV and TAV RNA synthesized a heterogenous set of peptides with m.m. from 200 to 14 kD. The hard polypeptides were most likely to be proteins-precursors from which the virus-specific, proteins were synthesized (1, 2). Peptide with m.m. 75.0 kD was consistent with a protein of cytoplasmic inclusions that is rather typical of potyviruses. Peptide with m.m. 30.0 kD was bound among the products of BYMV RNA translation thus corresponding to the cover protein. The TAV RNA in contrast to BYMV RNA did not code a protein similar to m.m. of cover protein. The TAV RNA coded two low molecular peptides with m.m. 15.0 and 14.0 kD which were not observed among the products of

BYMV RNA translation. These two peptides may be products of TAV protein degradation.

The data obtained by us about capsid proteins and the BYMV, TMV, TMMV and TAV RNA testified to belonging of TMMV and TAV to the same taxonomic group.

Comparison of the primary structure of related proteins of the potyviruses under study was continued in revealing the identical epitopes of capsid proteins by means of immunochemical methods. DDR was successful to show affinity of three potyviruses BYMV, soybean mosaic virus (SMV) and PVY. Results of rocket immunoelectrophoresis demonstrated an extent of affinity in the percentage ratio for all the potyviruses studied. To improve accuracy of immunoanalysis two variants have been used: ELISA blocking and double antibody "sandwich" - method (DASM). The DASM possibilities were estimated using the mice and chicken immunoglobulins and $F(ab')_2$ - rabbit antibody fragments as primary antibodies. The native rabbit antibodies were used at the 3d stage as the detecting ones. They were recognized by peroxidase conjugate with protein A and donkey antibodies. In developing the method and fitting the optimal conditions for DASM it was obtained that the optimal concentration of IgG for the sorption at the first stage amounted to 2-5 mkg/ml for mice, 30-50 mkg/ml for chicken and 15-20 mkg/ml for $F(ab')_2$-fragments. The rabbit antiserum dilution was 1:3000 for PVY, BYMV and OYDV; 1:15000 for TMV and 1:500 for SMV.

It was showed experimentally that better results were obtained with $F(ab')_2$-fragments of rabbit antibodies. In the developed systems of DASM the viruses under study were revealed in concentration 30 ng/ml used as the primary antibodies of IgG mice, about 60 ng/ml - IgG chicken and up to 5-10 ng/ml rabbit $F(ab')_2$-fragments.

Thus, the taxonomic position of new potyviruses identified by us on Trifolium montanum mosaic virus and Tradescantia albiflora virus was established.

There is the evidence obtained which permits to classify the potato aucuba mosaic virus and Plantago asiatica mosaic virus

as typical members of Potexvirus from possible ones.
It was shown there were 50% of identifical group-specific epitopes in carlaviruses - potato virus M and potato virus S and this accounts for their frequent cross reactions in detecting the immunochemical methods.
The Vicia unijuga mosaic virus (spot, mosaic and necrotic strains) was proved to be a new member of the group.
Finally, the physico-chemical and antigenic properties of capsid proteins in poty-, potex-, carla-, rhabdo- and bromoviruses were analyzed. Antigenic specificity was examined in three strains of PVY (PVYo, PVYn, PVYc).
The group-specific epitope of PVYn corresponding to position of 198-208 polypeptide chain was localized. New viruses; Vicia unijuga mosaic virus, Trifolium montanum mosaic virus, Tradescantia albiflora virus, Plantago asiatica mosaic virus and the Far East strain of cereal north mosaic virus, were placed in the taxonomic groups in the course of study on functional properties of protein structural components and by antigenic specificity.

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EVIDENCE OF RESISTANCE TO BEET WESTERN YELLOWS VIRUS IN OILSEED RAPE

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Introduction

In recent time very high levels of infection of winter oil seed rape by beet western yellows luteovirus (BWYV) were detected in Germany, Great Britain, France, the Czech Republic, and the USA (1-7). In yield trials plots of cv. 'Jet Neuf' with high levels of natural infection resulted in 10% reduction in seed yield compared to yield from plots in which infection level was reduced by spraying with aphicides to control the vector (1). Yields from single plants of one tested breeding line and cv. 'Liporta', inoculated with BWYV at the four to six leaf stage were reduced by 40% and 50% respectively (4). In a two-year experiment at Aschersleben, Germany, virus-free experimental plots of the oilseed rape cv. 'Falcon' and 'Zeus' yielded 12% to 34% more seed than plots with 90% to 100% BWYV infection (Graichen, unpublished). The symptoms of BWYV infection on oilseed rape were visible in our investigations during the late autumn until early spring as conspicuous anthocyanous discoloration and growth reduction.
Yield losses in oilseed crops caused by BWYV infection can be prevented by resistance breeding to BWYV. Differences in the incidence of BWYV infection in 14 *Brassica* cultivars under natural infection conditions were reported from the USA (8). The cultivars ranged from highly resistant to highly susceptible to infection by BWYV.
An effective method based on glasshouse and field experiments was developed for screening oilseed rape genotypes and related species to evaluate their reaction to BWYV and revealed some highly resistant *Brassica* genotypes.

Materials and methods

In the glasshouse experiments a total of 652 genotypes of summer and winter oilseed rape cultivars, actual breeding lines and resynthesized rape forms were screened for resistance to a highly virulent BWYV isolate (BN 5 ASL) from oilseed rape. The seedlings were inoculated with BWYV using five to 10 viruliferous *Myzus persicae* Sulz. propagated on virus infected oilseed rape. After the inoculation period of 72 to 96 hr plants were sprayed with pirimicarp and triforin. At two weeks interval the plants were sprayed with pirimicarp and triforin for control of aphid and fungi infestation.
The percentage of infected plants and the relative virus concentration (absorbance value at *405* nm) were assessed approximatly eigth weeks after virus inoculation by means of DAS-ELISA using a BWYV polyclonal antiserum. The cv. 'Sollux' which showed very high degree of susceptibility to BWYV (sc) was used as positive controls.
In field experiments 86 selected cultivars, breeding lines, resynthesized rape and single plant progenies were drilled each in two rows. At the end of September/beginning of October one row of each genotype containing 30 plants was inoculated with BWYV by colonisation with viruliferous *M. persicae*. At the same time the control rows were sprayed with pirimicarp. In the

beginning of May the virus symptoms were visually assessed as : grade 1. no symptoms; grade 3. mild chlorosis and weak growth reduction; grade 5. distinct chlorosis and growth reduction; grade 7. distinct chlorosis and distinct growth reduction.
BWYV-infected plants of few genotypes were indexed for virus when necessary by ELISA. The results of visual assessment and ELISA were compared with the results of the highly susceptible cv. `Sollux'.

Results and conclusions

All of the 652 rape genotypes were susceptible to BWYV in glasshouse experiments. The average absorbance value was in the range of 1.18 to 2.13 indicating high virus concentration in infected plants. Lower absorbance values were recorded only in samples of plants of the cvs. `Fertödi' and `Gorscanski'.
A further exception was the resynthesized rapeseed produced in 1988 in the Institute of Agronomy and Plant Breeding, Göttingen (9), from which plants were selective immune to BWYV (Table 1). After selfing of four plants the progenies showed different degrees of resistance to BWYV. From the progeny R 54-15 all 17 plants were immune to BWYV until four month after inoculation. But the repeated indexing by ELISA eight months after virus inoculation and further indexing of more plants resulted in a higher ratio of susceptible plants.
Two other progenies R 54-3 and R 54-5 yielded resistant as well as susceptible plants, whereas the ratio of resistant to susceptible plants was 4.9 : 1 and 8.7 : 1. In the fourth progeny R 54-7 the ratio was 1 : 2.3.

Table 1. Resistance to infection with BWYV of the resythesized rapeseed R 54

P	r : s*	S1	r : s
R 54	**16 : 48**	54 - 3	**73 : 15**
	(1 : 3)		*(4.9 : 1)*
		54 - 5	**70 : 8**
			(8.7 : 1)
		54 - 7	**7 : 16**
			(1 : 2.3)
		54 - 15	**17 : 0**
			15 : 2**
			24 : 14
			(2.4 : 1)

* - resistant : susceptible
** - repeated ELISA 8 month p.i.

Crosses between the susceptible cvs. 'Falcon', 'Mansholts' and 'Samourai' and resistant S1 plants of R 54 resulted in F1 plants which showed uniform susceptibility (Table 2).

Table 2. Uniform susceptibility of the crosses of oilseed rape cultivars with the resynthsized rapeseed R 54

crosses	F1 ELISA results n*	$\bar{x}$ E405
Samourai 11.4	0 : 14	1,672
Samourai 1 x R 54	1 : 20	0,417
2 x R 54	4 : 16	0,445
3 x R 54	3 : 18	0,598
4 x R 54	0 : 21	0,665
Mansholts 5.1	0 : 14	1,659
Mansholt's 1 x R 54	0 : 21	0,786
2 x R 54	0 : 21	0,650
3 x R 54	0 : 21	0,488
Sollux (sc)	0 : 28	1,294

* - number virus-free : virus-infected plants

In field experiments the plants of most genotypes showed chlorosis and growth reduction. Only the cultivars 'Fertödi' and 'Gorscanski' showed no chlorosis but a little growth reduction. In contrast to the other genotypes which were higly susceptible both under glasshous and field experiments all plants out of the four progenies of the resynthesized rapeseed R 54 were totally symptomless in the field after virus inoculation at the end of September and repeated inoculation in the beginning of March. BWYV was not detectable by ELISA from a total of 120 inoculated plants from the four single plant progenies. But the indexing of some other genotypes resulted in high degree of infection and high absorbance value recorded for the positive samples.
In further investigations 38 *B. oleracea* and 45 *B. rapa* genotypes were screened for resistance to BWYV, among them the parents of the resynthesized rapeseed R 54 the cabbage 'Stone head' and the *B. rapa* ssp. *pekinensis* no. 67. Whereas the cabbage 'Stone head' was highly susceptible to BWYV like all *B. oleracea* genotypes all the 62 inoculated *B. rapa* ssp. *pekinensis* no. 67 plants were immune to BWYV after repeated virus inoculation. But on the other hand, all six turnip oilseed (*B. rapa* ssp. *oleifera*) cultivars the other original form of the current oilseed rape cultivars were highly susceptible.
Experiments with dihaploid lines from crosses with resynthesized rapeseed R 54 are underway to verify the genetic architecture of BWYV resistance.

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COMPARISON OF THE HOST RANGES OF LUTEOVIRUS ISOLATES FROM OILSEED RAPE AND SUGAR BEET FOR THE ASSESSMENT TO THEIR OCCURRENCE IN BOTH CROPS

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Introduction

In connection with the very high infection by BWYV and the considerable increase of the winter oilseed rape (*Brassica napus* L. ssp. *napus*) production there was the question in which manner on the one hand winter oilseed rape is a danger for sugar beet (*Beta vulgaris* L. var. *altissima*) crops in regard to the infestation by BWYV and on the other hand the beet mild yellowing virus (BMYV) of sugar beet is a danger of oilseed rape crops.
BWYV and BMYV have many properties in common, although the American isolates of BWYV have a wider host range, including lettuce and *Brassica* crops (1). Both viruses belong to the luteovirus group and have isometric particles with approximately 26 nm in diameter.
By host range study using 25 virusisolates it was investigat the transmissibility of BWYV isolates from oilseed rape to sugar beet and of BMYV isolates from sugar beet to oilseed rape. Futhermore, the host range was compared of one BWYV and one BMYV isolates.

Materials and Methods

Virus isolates

For experiments to study the ability of isolates to infect oilseed rape (cv. Sollux), sugar beet (cv. Ponemo) and *Capsella. bursa-pastoris* L. were used 15 isolates of BWYV and 10 isolates of BMYV, which were obtained from different regions of Germany, from Great Britain, France, New Zealand and the USA. One isolate of BWYV and one isolate of BMYV from Germany were used for comparison of the host range. The isolates were separately maintained in oilseed rape and sugar beet plants.

Virus transmission experiments

Experiments to transmit luteovirus isolates on oilseed rape, sugar beets and *Capsella bursa-pastoris*

For aphid transmission tests virus-free *Myzus persicae* Sulz. maintained on Chinese kale (*B. rapa* L. ssp. *pekinensis*) or pepper (*Capsicum annuum* L.) were allowed a 24-hr access to leaf samples in sealed petri dishes. After acquisition, using about 10 viruliferous aphids every seedling of oilseed rape, sugar beet and *C. bursa-pastoris* plants at the stage of two true leaves was inoculated with either BWYV or BMYV for an inoculation access period of 72 to 96 hr. After the inoculation period plants were sprayed with pirimicarp. In two weeks the plants were sprayed with pirimicarp and triforin for control of aphid and fungi infestation. Non-viruliferous aphids were transferred directly from Chinese kale or pepper to 8 seedlings of oilseed rape, sugar beet and *C. bursa-pastoris* to ensure that the aphids were free of BWYV and BMYV.

Experiments to transmit BWYV and BMYV on host range

For host range studies plants from 116 species belonging to 24 families were inoculated at of two to four true leaves stage with either BN 5 ASL or BM 2 GA isolates using five to ten viruliferous *M. persicae* reared on infected oilseed rape and sugar beet plants respectively. The inoculation period was between 72 to 96 hours before killing aphids by spraying with pirimicarp. The treatments were repeated every two weeks. The inoculated plants were grown separately in regard of the both isolates in two insect-proof glasshouses. Four plants were maintained of each species as uninoculated control in glasshouses. Many plants were examined by aphid back transmission tests on oilseed rape and sugar beet plants.

Serological detection method

The presence of viruses was assessed in inoculated plants by means of double antibody sandwich (DAS)-ELISA using polyclonal antisera showing cross-reactivity with BMYV, BWYV and potato leaf roll virus (PLRV) (Richter et al. 1983, Kühne et al. 1985). For positive-negative threshold the mean absorbance value for four healthy control wells was used. ELISA results were verified in many cases by back transmission test with *M. persicae* from the inoculated host plant to oilseed rape and sugar beet.

Results

Transmission of BWYV/BMYV isolates to oilseed rape, sugar beet and *C. bursa-pastoris*

BWYV

Serological assays by DAS-ELISA showed that all the 15 BWYV isolates from oilseed rape, cabbage and spinach infected oilseed rape and *C. bursa-pastoris* plants to a high degree. The mean absorbance value for positive samples of oilseed rape ranged from 13 isolates over 1.00 and of *C. bursa-pastoris* from 7 isolates over 2.50. None of oilseed rape plants showed symptoms in the greenhouse. The isolates BN MA 43, BO SIP and BO GEM 8 produced strong growth reduction and caused the death of some plants of *C. bursa-pastoris*. But no infections were detectable in 447 sugar beet plants inoculated with the 15 BWYV isolates.

BMYV

Transmission of the 10 BMYV isolates to sugar beet and *C. bursa-pastoris* was successful. In comparison with the BWYV isolates the number of BMYV infected plants and absorbance value were distinctly increased. Virus symptoms were not visible on sugar beet plants under greenhouse conditions. The BMYV isolates BM 8 ITB and BM 10 MA only produced weak reductions of growth and yellowing of older leaves on *C. bursa-patoris*. In none of the total 280 oilseed rape plants inoculated with BMYV isolates virus infections were detectable.

Comparison of host range

BWYV

The BWYV isolate BN 5 ASL was able to infect 60 out of a total of 116 species, subspecies or varieties in 14 families, among them 35 members in the family of Brassicaceae. Futhermore, 4 species were infected from Compositeae, 7 from Fabaceae, 4 from Solanaceae and further 9 species of 9 families. Nine of these species, *Camelina sativa, Fumaria officinalis, Lepidium campestre, L. ruderale, Lupinus luteus, Ornithopus sativus, Phacelia tanacetifolia, Trifolium resupinatum*, and *Zinnia peruviana* have not been previously reported to be a host of BWYV. In

many samples the absorbance value measured over 2.00. Only *S. oleracea* was infected by BWYV among the Chenopodiaceous plants inoculated.
Infections was not detected in *Avenae byzantina* and *Solanum tuberosum*, which ranged as hosts for BWYV, as well as all species in the families Cucubitaceae, Euphobiaceae, Gramineae, Plantaginaceae, Polygonaceae, Resedaceae, Rubiaceae, Umbelliferae and Urticaceae that were tested.

BMYV
In comparison to BWYV the BMYV isolate BM 2 GA infected a smaller number of host plants. A total of 21 out of 100 species and varieties in 10 families were only infected. It was transmissible to all 7 subspecies and varieties of *B. vulgaris* and further 14 species. Three of these, *A. retoflexus*, *F. officinalis*, and *F. convolvulus* were detected for the first time to be hosts of the virus. Out of the 35 species of Brassicaceae only *C. sativa*, *C. bursa-pastoris* and *Sinapis alba* were infected by BMYV.
All species, subspecies and varieties in the genus of Brassica (10), and in the families of Fabaceae(10), Gramineae(3), Plantaginaceae(2), Solanaceae(12), Umbelliferae(3) and Urticaceae(2) used in the study were not susceptible to the virus.

Discussion

In our study significant differences were detected in the host range of BWYV and BMYV isolates. None of the 15 luteovirus isolates of BWYV from different countries used in our experiments was able to infect sugar beet plants. Transmissions to rape and *C. bursa-pastoris* were successful to high degree.
On the other hand, it was not possible to transmit 10 BMYV isolates from sugar beet to oilseed rape, but they were readly transmitted to sugar beet and *C. bursa-pastoris*. The mean of absorbance value of BWYV infected *C. bursa-pastoris* was very high with the value of 2.306 and indicating a very high virus concentration in infected plants. In contrast the mean absorbance for BMYV infected *C. bursa-pastoris* was lower ($\bar{x}$ A*405* =0.507).
The direct comparison of the host range of the BWYV isolate BN 5 ASL and BMYV isolate BM 2 GA resulted in great differences with respect to the number of host plants and the membership of families and genuses. The isolate BN 5 ASL was transmissible to 60 species, subspecies and varieties in 13 families, but not to the genus *Beta*.
In contrast BMYV isolate BM 2 GA was only transmissible to 21 species, subspecies and varieties in 10 families, including all 7 subspecies and varieties of the genus *Beta*. Transmission was not successful to all species of genus *Brassica* and all species in *Fabaceae* and *Solanaceae* of which were susceptible to BWYV.
Eleven species were infected by both BWYV and BMYV isolates used in our experiments. But the absorbance value was distinctly higher in all samples from common hosts infected by BWYV
The authors conclude from their own results and the analysis of the comprehensive bibliographical references that the BWYV of *Brassica*-species it concerns the turnip yellows virus (TuYV) distinctly differing from the BMYV of the sugar beet in its host range, in the virus concentration in common hosts, and in the aphid transmissibility.Vanderwalle (2) and Roland (3) were the first to report on an aphid- but not on sap-transmissible virus on turnip, causing yellowing and red edge. Burkhardt (4), (5) reported on the attack by TuYV on cruzifera in Westfalia and the whole northern part of Germany for 1957 and the following years. Heinze (6) detected a number of Brassicaceae as host plants for persistent aphid-transmissible TuYV.

The difference between luteoviruses established in this study is specially of practical importance, since the natural host range include economically important crops. It can be assumed that

increased cultivation of winter oilseed rape and the high incidence of BWYV established, no endangering of sugar beets crops regarding the attack by luteovirus isolates from oilseed rape is to be expected.

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PRUNUS NECROTIC RINGSPOT ILARVIRUS: NUCLEOTIDE SEQUENCE OF RNA3 AND THE RELATIONSHIP TO OTHER ILARVIRUSES BASED ON COAT PROTEIN COMPARISON

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Introduction

Prunus necrotic ringspot virus (PNRSV) belongs to the genus *Ilarvirus* of the family *Bromoviridae*, which also includes the genera *Alfamovirus*, *Bromovirus* and *Cucumovirus* (1). Members of this family have a positive-sense single-stranded RNA genome, which is divided into three species, designated RNA1, 2, and 3 in order of decreasing size. Whereas RNA1 and RNA2 are monocistronic and encode nonstructural proteins involved in viral RNA synthesis RNA3 is bicistronic and codes for the putative movement protein, P3a, (2) and the coat protein (CP). P3a is translated directly from RNA3, whereas the CP is translated from a subgenomic mRNA, RNA4, which is also encapsidated. The complete nucleotide sequences of the RNA3 of tobacco streak virus (TSV; 3) and prune dwarf virus (PDV; 4) are available, and the sequence data suggest that the RNA3 of ilarviruses share a genome organisation similar to other members of *Bromoviridae.*
Two properties are common for ilarviruses as well as alfalfa mosaic virus (AlMV): first the formation of nonisometric nucleoprotein particles proportional in size to the encapsidated RNAs, and second the requirement of CP to initiate viral genome replication in host plants. This differentiates them significantly from other genera of the *Bromoviridae*. Furthermore, the coat proteins of several ilarviruses and AlMV are interchangeable in the process of genome activation, although they have no apparent sequence or serological homology (5).
In order to obtain more information about the genomic organisation of ilarviruses and the molecular mechanism of genome activation by homologous and heterologous coat proteins, we have determined the complete nucleotide sequence of the PNRSV RNA3. In addition, the comparison of sequences from different isolates as well as related viruses would be helpful to elucidate the evolutionary relationships among ilarviruses and their relationship to other genera of the *Bromoviridae*.

Material and Methods

The PNRSV isolate (Cat. No.: PV-0096, DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen) originated from *Prunus mahaleb.* The ApMV-G isolate was obtained from A. Eppler, BRD. Both viruses were propagated on cucumber (*Cucumis sativus*) cv. Riesenschäl. The viruses were purified according to (6); RNA was extracted from virus particles as described by (8). Viral RNA (5 µg) was polyadenylated with *Escherichia coli* poly (A) polymerase (GIBCO/BRL) and subsequently used for first-strand cDNA synthesis with oligo-(dT) and hexanucleotide random primers according to (9). Double-stranded cDNA was made blunt-end with T4 DNA polymerase and inserted into the *Hin*cII site of pT7T3-19U (Pharmacia). Sixteen PNRSV cDNA were selected for nucleotide sequencing. Sequence data were collected and analysed using either the GCG sequence analysis software package (9), or the PC-program package DNASIS (Pharmacia).

Results

The complete nucleotide sequence of PNRSV RNA3 deduced from cDNA including the ultimate 5'-end consisted of 1943 residues. It is the smallest RNA among the RNAs 3 of the members of *Bromoviridae* sequenced so far. Computer-assisted analysis revealed two large ORFs located on PNRSV RNA3. The 5'-proximal ORF (ORF3a) starts at nucleotide (nt) 174 and terminates with a UGA triplet at nt 1023-1025, while the 3'-proximal ORF (ORF3b) starts at nt 1100 and terminates with a UAG triplet at nts 1772-1774. No other ORFs longer than 200 nts were found on either plus or minus strands.
The 5'-proximal ORF3a, 852 nts, encodes a polypeptide of 283 amino acids with a predicted molecular weight of 31.4 kDa. The 3'-proximal ORF 3b, 675 nts, codes for a protein with 224 amino acids with a predicted molecular weigtht of 24.9 kDa. ORF 3b was expressed with different pQE vectors (Quiagen) in *E. coli.* The purified fusion protein reacted strongly with an antiserum against PNRSV on Westernblots, indicating that ORF 3b representis the CP.
The 5'-noncoding sequence of PNRSV RNA3 consists of 173 nts. Similar to AIMV, TSV and PDV, it is markedly enriched in U (40%), when compared with the total nucleotide composition. Three repeats of 7-10 nucleotides were found in the 5'-NCR of PNRSV. The function of these repeats is not clear. An intercistronic region of 74 nts separates the two ORFs. The 3'-NCR of PNRSV RNA3 comprises of 169 nts.

Discussion

The overall organisation of PNRSV RNA3 is very similar to that of the RNAs 3 of PDV (4), TSV (3), AIMV (10), brome mosaic virus (BMV; 11) and cucumber mosaic virus (CMV; 12). Based upon biochemical and mutation analysis as well as amino acid sequence comparison, it has been proposed that 3a proteins of the *Bromoviridae* function as movement proteins (2,13). The deduced amino acid sequence of PNRSV 3a protein was aligned with analogous proteins of other members of the *Bromoviridae*. The alignment analysis indicated that there is a sequence similarity in the core region of the 3a proteins of tripartite viruses. A domain of 17 amino acids starting at amino acid 101 of PNRSV protein 3a is highly conserved with 59% overall similarity among PDV, TSV, AIMV, CMV and BMV. When comparing the 3'-NCRs of PNRSV, AIMV, TSV, PDV and ApMV, it was found, that the terminal sequence of 18-23 nucleotides was conserved and had the potential to form a stem-loop structure flanked by AUGC-boxes. In case of AIMV and TSV it has been shown that these stem-loop structures, flanked by AUGC-boxes represent the binding sites with a high affinity for CP (14). In addition it has been pointed out that the AUGC-box 3, starting from the 3'-end of AIMV, is essential both for binding to CP and infectivity. This common structural feature in the 3'-NCR of ilarvirus and AIMV RNAs may be the prerequisite for specific interaction with a homologous or heterologous coat protein leading to genome activation. The consensus in the 3'-NCR of AIMV and ilarviruses also suggests a close evolutionary relationship among these viruses.
For ilarviruses and AIMV, the coat protein not only encapsidates the virions, but is also involved in genome replication. Examination of deduced PNRSV and ApMV coat protein sequences revealed that their N-termini were rich in basic amino acids and have the potential to form an amphipathic helix with the majority of the charged residues aligned on one side as suggested for secondary protein structures interacting with RNA (15). In addition and similar to TSV the N-termini of PNRSV and ApMV CPs have the sequences CRICNHTHAGGCRSCKKCH and CKYCGHTHPGACVNCKWCH, respectively. These represent zinc-finger motifs CZ2-4CZ2-15XZ2-4X (where X can be either His or Cys ,and Z any amino acid) that have been found in many nucleic acid binding proteins and have been proposed for TSV to be responsible for nucleic acid CP interaction. In analogy, the N-termini of PNRSV and ApMV coat proteins may play a role in binding of genomic RNA during encapsidation and activation of genome replication, as proposed for TSV and AIMV CP N-termini. However, the absence of the zinc-

finger motif in the coat protein of PDV suggests that this motif is not a common structural feature of all ilarviruses and, therefore, not a general requirement for coat protein activation of replication.

PNRSV and ApMV belong to the same subgroup of ilarviruses and are serologically distantly related (16). Due to an unexpected homology of our PNRSV CP sequence with a recently published ApMV sequence (17) the sequence of the CP-ORF of an ApMV isolate from Germany (ApMV-G) was determined. This sequence was compared with that of other ilarviruses. ApMV-G CP had 88.1% identity with that of ApMV-I. A four-residue deletion was found in ApMV-G CP when compared with that of ApMV-I. At nucleic acid level, the CP genes of the two isolates shared 87.3% identity and three one-base insertions in ApMV-G CP gene led to a frameshift of 12 amino acids, in comparison with that of ApMV-I. The amino acid sequence of ApMV-PV32 published recently (17) had only 50.9% and 49.3% identity with that of ApMV-G and ApMV-I, respectively, but 91.6% identity with that of PNRSV. In analogy to the guidelines for the taxonomy of potyviruses (18) with respect to sequence divergence between different strains and viruses, it is proposed that ApMV-PV32 should be regarded as a strain of PNRSV.

PNRSV and ApMV CPs were also compared with those of other members of the *Bromoviridae*. No significant homology between CP sequences from members of the genera *bromovirus* and *cucumovirus* was found by multiple alignments, however, a similarity became evident in the C terminal part of the ilarviruses and AlMV CP sequences. The results of the multiple alignment were used to generate a dendrogram, illustrating the tentative phylogenetic relationships in the family *Bromoviridae* (Fig. 1). It emphasizes the high similarities found for members of the same subgroup of ilarviruses, whereas a larger divergence occurs between members of different subgroups. Also the close relationship between ilarviruses and AlMV becomes evident, whereas the remaining genera of the family are clearly separated.

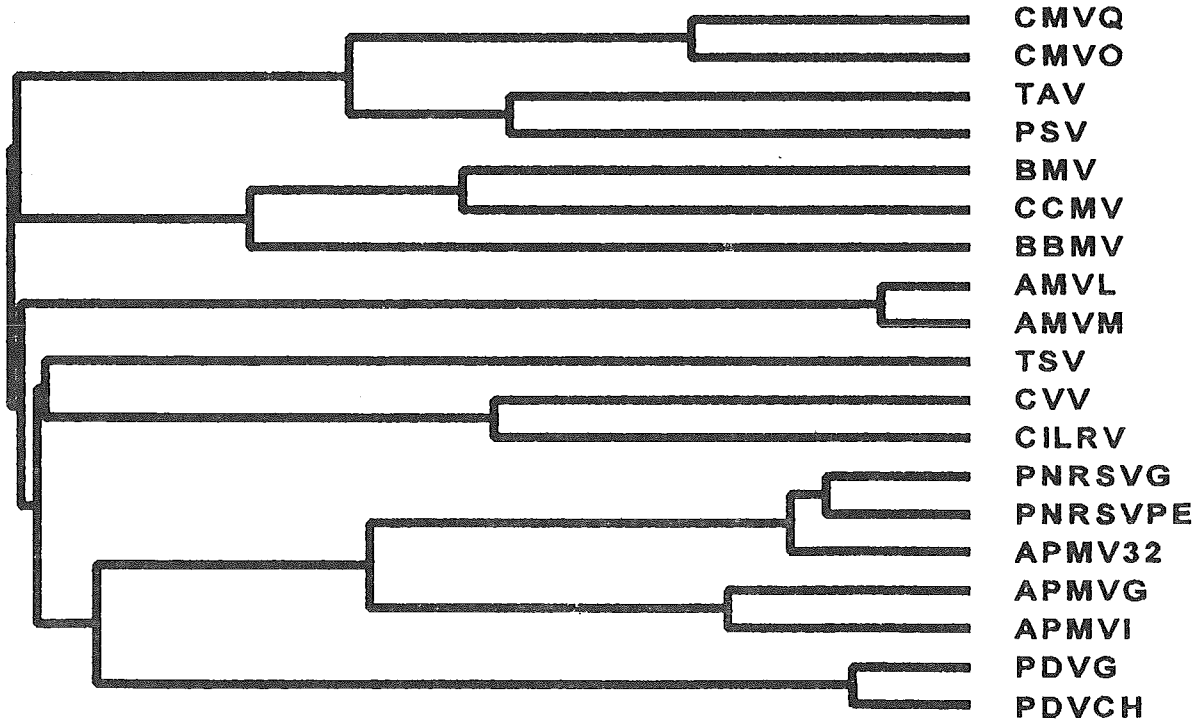

Fig. 1 Dendogram of selected members of the *Bromoviridae* according to CP amino acid sequences. (CMV cucumber mosaic virus strain-Q and O; TAV tomato aspermy virus; PSV peanut stunt virus; BMV brome mosaic virus; BBMV broad bean mottle virus; CCMV cowpea chlorotic mottle virus; AMV alfalfa mosaic virus strain L and M; TSV tobacco streak virus; CVV citrus variegation virus; CiLRV citrus leaf rugose virus; PNRSV prunus necrotic ringspot virus strain PE and G, ApMV apple mosaic virus strain 32, G and I; PDV prune dwarf virus strain G and CH).

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RESULTS OF TEN-YEAR SELECTION OF WINTER BARLEY FOR TOLERANCE TO BARLEY YELLOW DWARF VIRUS

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Introduction

The barley yellow dwarf luteoviruses (BYDV) cause a worldwide important disease of *Poaceae* (1). Cultivation of resistant or tolerant varieties is the most effective and environmental-tolerated method to control the disease.
Already in the 50th researchers started screening work to select barleys with tolerance to BYDV. The first detected, recessive tolerance gene, yd_1, found in 'Rojo' (2), was not included in practical breeding because of its low level of tolerance. In several Ethiopian barleys field tolerance was found (3), (4). A semidominant gene, located on chromosome 3 (5) and named Yd_2 , was proved to be responsible for this tolerance reaction (6). The gene was used to develop BYDV tolerant spring barley varieties. It was also transmitted in the winter barley variety 'Vixen' (7). Some other varieties of winter barley with unknown BYDV tolerance source were bred, e.g. 'Post' (8), 'Perry' (9), 'Surry' (10) and 'Maury' (11).
In 1984 we have started our investigations on BYDV tolerance in winter barley, including the following tasks:

1. development of useful methods for testing barley under natural and artificial infection,
2. isolation and characterization of the actual virus strains existing in the field and
3. characterization of the BYDV resistance reaction of winter barleys, especially of the Gatersleben World Collection, but also of cross progenies and doubled haploid (DH) lines.

The present paper describes some of the main results.

Material

Up to now more than 1500 entries of the Gatersleben Winter Barley Collection, 183 progenies (F_3/1988 to F_8/1995) and 410 doubled haploid lines (produced by Dr. Peterka, Quedlinburg, using *Bulbosum*-technique) of crosses of 'Post' and 'Vixen' (tolerant) with 'Rubina', 'Borwina' and 'Viresa' (cultivars of high yielding type: long, loose ears, but BYDV susceptible) have been tested under natural infection conditions. Besides the cross progenies were also evaluated at morphological characters, e.g. density of spike, number of kernel rows and stem length. From 1988 to 1995, 132 entries with the highest level of tolerance in the field were examined in the greenhouse under artificial inoculation. 'Erfa', 'Rubina' (susceptible) and 'Perry', 'Post' and 'Vixen' (tolerant) served as standards for BYDV reaction.

Methods

1. Transmission tests

The specifity in BYDV transmission by different aphid species (12) was used to characterize the actual virus situation in the region of Aschersleben. Since 1992 every year in spring and autumn barley plants with BYDV symptoms were collected in the field. Pieces of leaves of each source plant were placed in petri dishes. A single dish was used for each aphid species, e.g. *Rhopalosiphum padi* (L.), *Sitobion avenae* (F.) and *Rhopalosiphum maidis* (Fitch), respectively, which was allowed a 48-h acquisition feeding at about 20 °C. About 10 aphids

from each dish were transferred to 10 barley seedlings of the susceptible variety 'Erfa' for an inoculation feeding period of 48 h, usually at about 20 °C. Then the aphids were killed by insecticide. The test plants were placed in a growth chamber, where the symptom expression was observed for 7 weeks. 49 days past inoculation (dp.i.) the plants were harvested for DAS-ELISA (double antibody sandwich-ELISA, using polyclonal antisera: PAV-Aschersleben, MAV and RPV-Sanofi, France; absorbance values measured at wave length 405 nm).

2. Resistance tests

Several preinvestigations have been done to find out the optimal conditions for field and greenhouse BYDV resistance tests. In the field the effect of following features on virus attack were tested: density of infection stripes (2, 4 or 6 rows on 1 m), sowing date of entries (1st/3rd week of September) and number of kernels per plot (24/40). In the greenhouse duration of acquisition (24/48 h), number of viruliferous aphids per plant (1,3,5...15), duration of inoculation (24/48 h) and the age of plant at the inoculation time (seedling, three-leaf-stage and adult plant) were investigated.

Field trials

In the field the so-called preselection test has been carried out under natural infection. To increase the natural infection pressure in the third week of August, so called infection stripes of 'Erfa' (4 rows) were sown, spontaneously attacked and infected by viruliferous aphids at favourable weather. The entries (one plot with two rows, 1 m long, 24 kernels) were sown very early, usually in the 1st week of September, between the infection stripes (five plots between two stripes) in 3 repetitions.

Estimation of BYDV reaction: In autumn the number of plants per plot was counted (BBCH 12...15). In the following spring the rate of overwintering was determined. At or near by heading (BBCH 37...55) BYDV reaction of each single plant was visually recorded based on a scale from 1 = plant without symptoms to 9 = dead of plant. On the basis of these ratings the degree of resistance (DR) was calculated according to SCHMIDT et al. (13):

$$DR = 100 - \frac{\Sigma\, n_b \times (b - 1) \times 100}{N \times (B - 1)}$$

n_b = number of plants per class of resistance b
b = class of resistance according to calculation table
N = total number of all estimated plants
B = the lowest class of resistance (note 9) according to calculation table

Greenhouse tests

Inoculation: 20 seedlings per entry and test were inoculated with PAV- or MAV-like strains of BYDV using greenhouse-reared aphids of the species *Rhopalosiphum padi* (L.) or *Sitobion avenae* (F.) with a 48 h acquisition period and at an approximate rate of 5 to 10 per seedling. 2 d p.i. the aphids were killed by insecticide. Further cultivation of plants was carried out in the greenhouse or in a growth chamber (20 °C, 70-90 % relative humidity, light for 16-h-day).

Estimation of BYDV reaction: The visual estimation of foliar symptoms on a scale from 1 to 9 was made for each plant 49 d p.i. The degree of resistance was calculated as described above. Moreover, also 49 d p.i. virus concentration of plants was established by ELISA technique.

Results and Discussion

In dependence on the weather, the infection conditions and the actual virus situation the attack of the infection stripes and consequently the number of field-selected virus tolerant entries differed from year to year. PAV- and MAV-like isolates predominated in 1992 and 1993. These results confirm early investigations in Aschersleben (14), (15). In 1993 and 1994 also RPV-like isolates were proved. Except 1986/87 every year a preselection was possible. For example in 1992/93, appr. 17 % of the several years tested entries reacted tolerant and 23 % moderate tolerant. The remaining barleys were susceptible (Fig. 1a). In comparison to these results only 3 % of the first time tested 121 barleys were tolerant, and 3 % moderate tolerant (Fig. 1b).

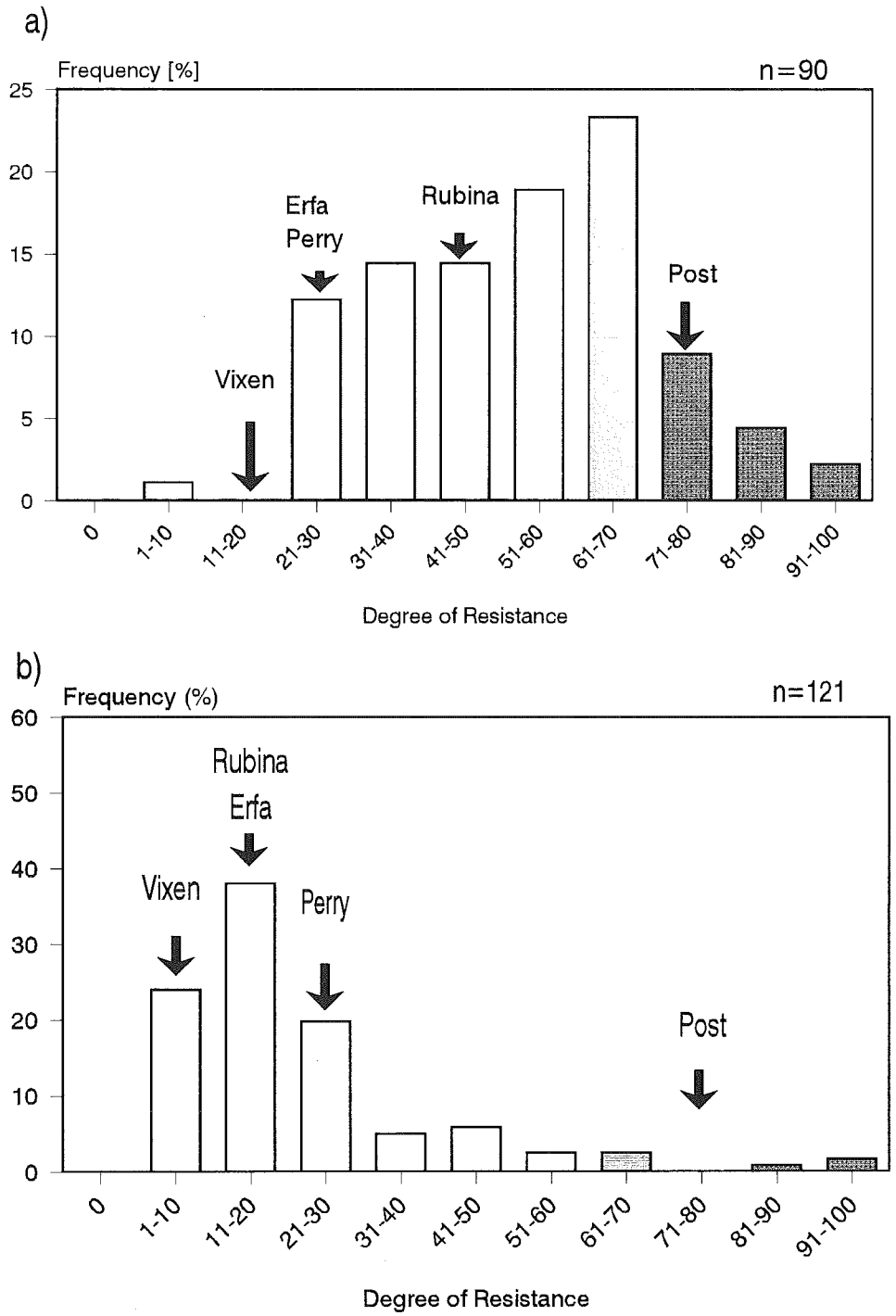

Fig.1: BYDV-field test in 1992/93; a) several years tested material, b) material in the first test year

A relatively stable level of tolerance, comparable to 'Post', the tolerant standard, was expressed in 12 barleys (Tab. 1). A geographical concentration of tolerant barleys was found in Korea (4) and Russia (3). The others come from Bulgaria, Sweden, USA and Japan.
In 1992/93 a very high virus attack was observed in the field. ELISA of infected plants have shown that they were often infected not only with BYDV but also with wheat dwarf virus (WDV). In the following years this virus was also found. The incidence of WDV complicates our field tests for BYDV tolerance and in future we will concentrate our work on selection under artificial infection.

Tab. 1: Expression of BYDV tolerance in Gatersleber winter barleys in the field and with artificial inoculation (PAV/*Rhopalosiphum padi*; MAV/*Sitobion avenae*) in comparison to standard varieties

Variety/ Entry	Degree of Resistance					
	Field			Greenhouse		$\bar{x}_{ext}$
	1992	1993	1994	PAV	MAV	PAV
Post	86,6	73,0	84,1	97,8	97,0	1,84
Perry	61.5	27,7	60,2	92,2	88,3	1,54
Vixen	46,8	18,9	49,2	99,3	87,0	1.43
Rubina	59,1	32,4	53,8	44,4	64,8	1,49
Erfa	62,7	21,7	50,9	29,5	52,9	1,52
HHOR 3158	94,6	83,6	87,8	99,6	99,7	1,43
HHOR 3263	91,7	69,5	74,6	76,8	97,8	1,73
HHOR 3318	91,3	77,6	43,8	95,8	100,0	1,51
HHOR 3486	87,5	65,7	67,3	78,5	93,1	1,62
HHOR 3488	92,5	60,2	71,6	86,4	80,9	1,77
HHOR 4389	85,8	60,0	92,3	50,2	96,4	1,37
HHOR 4559	94,3	73,7	76,1	47,9	77,5	1,65
HHOR 4436	87,2	68,5	76,6	85,5	98,8	1,48
HHOR 4932	90,4	79,6	67,5	86,8	98,8	1,72
HHOR 10589	94,8	98,9	90,4	89,4	85,2	1,42
HHOR 10859	n.t.	96,0	90,3	100,0	100,0	1,38
HHOR 10860	n.t.	90,9	84,9	100,0	100,0	1,48

Attack of infection stripes:
1991/92 40 %
1992/93 80 %
1993/94 60 %

n.t. = not tested

In 1992 we isolated a MAV-like strain from natural infected plants as a prerequisite for artificial inoculation tests. The MAV-like isolate causes weaker symptoms than the PAV-like one, seen in the attack of susceptible standards 'Rubina' and 'Erfa' (Tab. 1). But 'Perry' and 'Vixen' seem to be more susceptible to MAV- than to PAV-isolates. According to their reaction to the two different BYDV-isolates the entries could be grouped as follows:

1) high tolerant to PAV- and MAV-strains: HHOR 3158, HHOR 3318, HHOR 10859 and HHOR 10860,
2) medium level of tolerance to PAV- and MAV-strains: HHOR 3488 and HHOR 10589,
3) higher tolerance level to MAV- than to PAV-strains: HHOR 3263, HHOR HHOR 3486, HHOR 4436, HHOR 4932,
4) MAV-tolerant but PAV-susceptible: HHOR 4389 and
5) PAV- and MAV-susceptible: HHOR 4559.

In the tolerant barleys plants without or with weak leaf symptoms are predominant in comparison to the susceptible ones, in which most of the plants are yellowed and dwarfed. However, differences in virus concentration between tolerant and susceptible barleys not existed in DAS-ELISA (Tab. 1). The same was observed in the 3 F_7- and 10 F_8-lines with 'Post'-tolerance. Only these 13 lines combine BYDV-tolerance with high yielding plant type.

In the last year a cross programme including doubled haploid technique was started to study inheritance of the BYDV-tolerance of the selected Gatersleben winter barleys. Up to now, 'Post' tolerance was investigated more in detail using DH lines of crosses 'Post' x 'Rubina'

and 'Viresa' x 'Post'. BYDV reaction of 58 lines of 'Viresa x Post' shows, that this tolerance is probably quantitatively inherited (Fig. 2).

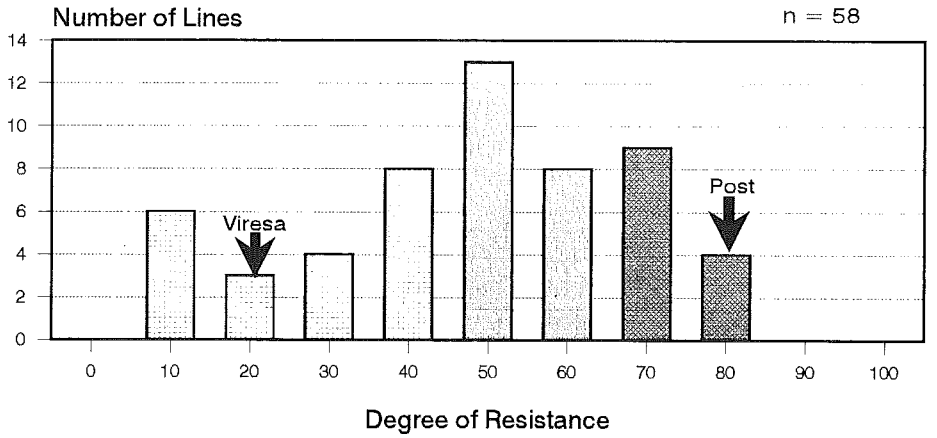

Fig.2: Reaction of doubled haploid lines of the cross 'Post' x 'Viresa' to BYDV-PAV infection

Some of the selected BYDV-tolerant entries also posses resistance to BaMMV, BaYMV-1 and -2. Now their resistance reaction to fungi diseases, e.g. *Puccinia hordei* Otth and *Drechslera teres* (Sacc.) Shoem., is tested.

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Tolerance to Barley Yellow Dwarf Virus in Oats

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Introduction

Barley yellow dwarf virus (BYDV) is recognised as a serious pathogen of small grain cereals. Some sources for tolerance have been found (1, 3, 4, 5, 6) in different *Avena* species and heritabilities are high enough in order to incorporate these tolerances into high yielding cultivars (2, 5). In 1992 a program was initiated to develop oat germplasm with tolerance to BYDV. Therefore the wild oat collection of the IPK Gatersleben (164 accessions) and some accessions of the Canadian Avena collection (CAV collection) have been screened for tolerance to BYDV. Results of comparisons between BYDV-tolerant standard genotypes, mostly from the European Oat Disease Nursery, and some selected wild oat accessions are shown.

Materials and Methods

The plants were inoculated in the two-leaf stage (1992 and 1994) and 4-leaf stage (1993) with a PAV-isolate[1] of BYDV using *Rhopalosiphum padi* as vector. In 1992, 15 plants per accession have been inoculated and compared with control plants without replication or randomisation. The most tolerant accessions (plant height and discolouration) have been selected for further examinations in 1993 and 1994. In 1993 and 1994 seeds were dehulled and pregerminated in petri dishes. Seeding was done in pots. Inoculation was done in the greenhouse using *R. padi* as vector. The aphids were reared on BYDV-infected oat plants and the inoculation was done in the greenhouse transferring small leaf piece's with 5-10 aphids to every plant. After a two-day inoculation feeding period the plants were sprayed with an insecticide (Filitox) and planted outdoors in complete randomised block design with three replications. The inoculated and control variants were planted side by side. The following data were recorded:

- a visual score of the leaf discolouration on a 1 to 9 scale (1= no discolouration ... 9= severe reddening of the leaves),
- the plant height of the main culm in cm,
- the number of ears per panicle,
- infections were confirmed by ELISA (9) with a kit supplied by Loewe Biochemica.

Results

Severe reddening and stunting of the most accessions occurred in all years but more drastic in 1992 and 1994 than in 1993, due to the later inoculation in 1993. Among all accessions the perennial variety *Avena macrostachya* was the most tolerant line (Tab. 1, Fig.1 and 2) and the only one with reduced virus content according to the absorbance values after ELISA (data not shown). In *A. macrostachya* there was only in some plants a very poor stem development with no remarkable panicle formation.

Some hexaploid wild oats are found reaching the tolerance level of some of the most tolerant standards Saia (*A. strigosa*) and IL 86- 4189 (*A. sativa*) (Tab. 1). The most tolerant accessions of *A. sterilis* (AVE 942/83; CAV 3301; CAV 2608) and *A. occidentalis* (CAV3887; CAV 3890) show a lower reddening of the leaves but especially in 1994 a strong stunting and reduction in number of ears per panicle (Tab. 1). In the latter feature *A. fatua* CAV 4578 has shown the greatest tolerance (Tab. 1, Fig. 3).

For the comparisons between different *Avena* varieties the relative differences between inoculated and control plants, referred to the inoculated variant, have been calculated for the plant height and ears per panicle. There are mostly significant correlation coefficients between latter variables (Tab. 2) with the exception toward the plant height in 1993.

Tab. 1 Leaf discolouration, plant height and ears per panicle of oat genotypes after inoculation with PAV-strain of BYDV in 1993 and 1994

Variety	Cultivar/ line	discol.-score** 1993 Inoc.*	1994 Inoc.	plant height (cm) 1993 Inoc.	Contr.	rel. Diff. ***	plant height (cm) 1994 Inoc.*	Contr.	rel. Diff. ***	ears per panicle 1993 Inoc.	Contr.	rel. Diff. ***	ears per panicle 1994 Inoc.	Contr.	rel. Diff. ***
A. macrostachya	CAV5264	1,0	1,6	25	33	24,2	36	34	-7,9						
A.sterilis	CAV2608	1,3	2,3	69	84	18,0	79	125	36,5	10	12	21,6	8	40	79,4
A.occidentalis	CAV3887	1,0	2,3	82	90	8,0	56	90	37,5	11	10	-5,4	7	14	50,2
A.sterilis	AVE 942/83	1,0	2,5	128	135	5,5	88	126	30,2	11	8	-46,7	11	23	53,2
A.occidentalis	CAV3890	1,0	2,6	101	104	3,3	85	112	23,8	15	15	1,0	13	26	50,2
A.sterilis	CAV3301	1,7	2,9	107	112	4,7	70	122	42,9	11	12	9,5	7	12	43,4
A.fatua	CAV4578	1,3	3,7	118	129	8,4	84	110	23,6	13	14	9,6	7	9	19,5
A.strigosa***	**Saia**	2,3	3,8	128	139	8,2	105	133	21,5	33	37	12,0	19	44	55,7
A.fatua	AVE 2094/84	3,7	3,9	137	154	10,8	84	162	48,1	34	45	23,2	25	61	59,2
A.sativa***	**IL 86-6404**	3,0	4,1	107	119	10,3	65	120	46,3	19	24	19,6	11	47	75,7
A.fatua	AVE 2095/90	3,0	4,2	143	157	9,1	91	159	42,6	30	36	16,3	25	85	70,5
A.sativa***	**IL 86-5698**	3,3	4,2	103	113	8,4	63	111	42,8	14	15	9,5	7	24	68,9
A.fatua	AVE 1441/89	3,0	4,4	131	149	12,0	100	152	34,1	30	43	28,9	20	46	55,6
A.hybrida	CAV5802	2,0	4,4	129	139	7,0	85	155	45,3	24	29	15,9	10	46	77,4
A.fatua	AVE 2109/86	3,7	4,5	134	161	16,6	71	162	56,0	35	43	19,4	19	82	76,6
A.fatua	AVE 527/82	3,7	4,5	137	149	8,1	87	157	44,6	26	39	33,6	11	58	81,9
A.fatua	AVE 1764/85	3,7	4,6	136	153	11,5	69	163	57,4	30	35	15,1	29	78	63,4
A.sativa	Alfred	5,0	4,8	113	114	0,5	50	121	58,3	25	29	16,3	7	66	89,7
A.sativa***	**IL 86-4189**	1,3	4,8	102	107	4,7	85	113	24,5	20	23	13,9	19	44	56,0
A.fatua	AVE 1516/83	3,7	4,8	124	142	12,7	87	145	40,1	23	29	22,2	26	48	45,5
A. occidentalis	CAV3897	2,0	5,0	116	130	10,7	72	117	38,8	24	25	4,6	8	22	63,8
A.sativa	Gramena	5,0	5,3	103	112	7,5	45	123	63,6	22	34	35,4	6	69	91,9

* Inoc. ... Inoculated plants; mean of 3 replications with 8 or 10 plants respectively

** discolouration-score: 1= no symptoms; 9= severe reddening of leaves

*** tolerant oat lines according to Endo (1957) and Kolb et al. (1991)

**** relative difference between inoculated and control variant referred to the inoculated variant

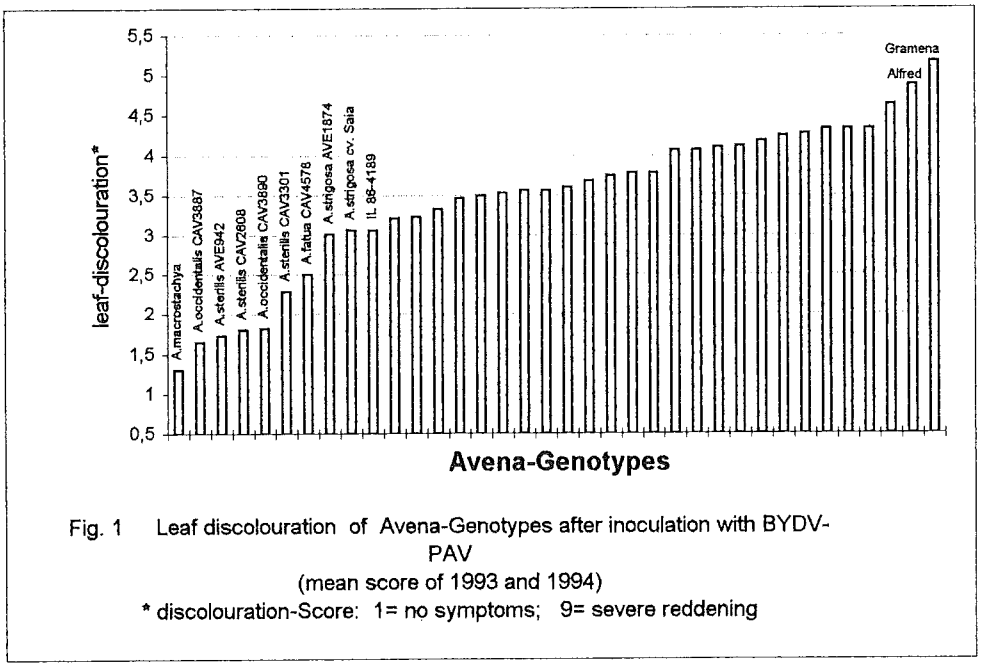

Fig. 1 Leaf discolouration of Avena-Genotypes after inoculation with BYDV-PAV
(mean score of 1993 and 1994)
* discolouration-Score: 1= no symptoms; 9= severe reddening

Tab. 2 Coefficients of correlations between relative differences** of variables of BYDV-tolerance experiments in 1993 and 1994

		discolor.score*		relative differences** between inoculated and control variant in plant height		ears per panicle	
	year	1993	1994	1993	1994	1993	1994
discolor.score*	1993	1,00					
	1994	0,70	1,00				
plant height	1993	-0,03	-0,23	1,00			
	1994	0,73	0,65	-0,29	1,00		
ears per panicle	1993	0,63	0,55	0,33	0,42	1,00	
	1994	0,63	0,45	0,05	0,73	0,44	1,00

* discolouration score: 1= no symptoms; 9= severe reddening of leaves
** relative difference between inoculated and control variant referred to the inoculated variant

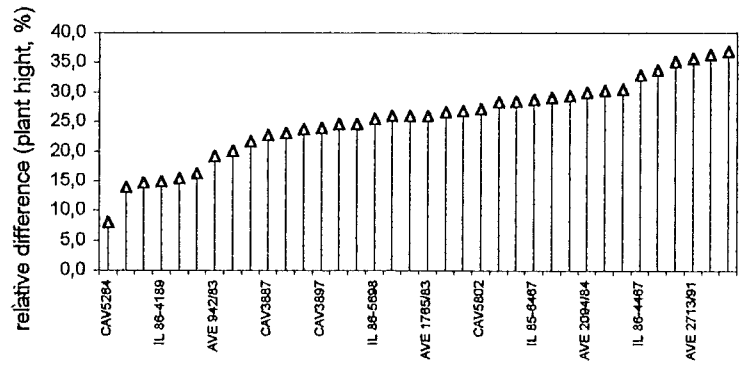

Fig. 2 Relative Difference in the plant hight between inoculated and control variant (refered to the inoculated variant) of oat genotypes in the BYDV-tolerance experiments (mean of 1993 and 1994)

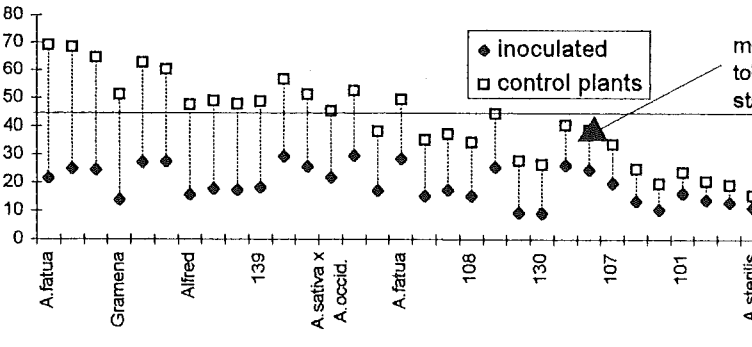

Fig. 3 Ears per panicle of oat genotypes (mean of 1993 and 1994 BYDV-experiments)

According to Fig. 3 most tolerant accessions have developed only low ear numbers per panicle in the control plants. On the other side some of the *A. fatua* accessions outyielding both susceptible cultivars Gramena and Alfred with and without inoculation (Fig. 3).

Discussion

If susceptible oat plants are infected with BYDV they show severe stunting, lower tillering and seedsetting. But there are large morphological differences with and without infection between *Avena* species. Therefore it is necessary to use the relative differences between inoculated and control plants for comparisons between different *Avena* accessions, which is also discussed previously (7, 8).

In general the most tolerant accessions show lower differences between inoculated and control variants in all assessed features and lower discolouration (Tab. 1). This is supported by the significant correlation coefficients between the relative differences of the different features within the years (Tab. 2). The exception of missing significant correlation coefficients toward relative differences in plant heights in 1993 may at first be due to the later inoculation in 1993, which resulted in smaller differences between inoculated and uninoculated plants (Tab. 1). Secondly the control plants were also infected by BYDV recognized by ELISA. Therefore the differences between inoculated and control plants have to be considered as unreliable in 1993. But on the other hand there is a good confirmation of the tolerances in IL 86-4189, IL 86-6404, IL 86-5698 and Saia, described in experiments by other authors (5, 6), so that results of 1993 can be exploited.

The high tolerance of *A. macrostachya* confirm the results from (3) . Additionally it may assumed, that *A. macrostachya* reduced more than the other genotypes the virus replication within the plant tissue.

Those genotypes, which outyielding both susceptible cultivars Gramena and Alfred in the number of ears per panicle, are obviously the highest plants and perhaps may be used to enrich the variability for yield components, especially for the number of kernels per panicle.

To incorporate the tolerances into high yielding cultivars the backcross method will be used, which was successfully applied by (1).

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STUDIES OF POTATO VIRUSES: ANTIGENIC ANALYSIS, STRUCTURE, TRANSMISSION AND DETECTION

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Potato virus X (PVX) was the first potato virus whose particles and coat protein (CP) structure we studied in detail. Localization of epitopes with monoclonal antibodies (MAbs), combined with tritium planigraphy studies, carried out in collaboration with Dr. Baratova's group (Moscow State University), showed that the N-terminus of the CP forms PVX-specific antigenic determinant(s) located at the virion surface. The C-terminal region is buried, and will open up ot the surface only after the cleavage of N-terminus (1), (2). This finding indicated that the structure of the potexvirus PVX CP differs from the structure of the CPs of well studied tobamovirus - tobacco mosaic virus (TMV) and potyvirus - potato virus Y (PVY).

At present time our interest is focused on potato A potyvirus (PVA). It is transmitted by mechanical inoculations or by aphids in a non-persistent manner. PVA isolate B11 is not transmitted by aphids, and its CP N-terminal octapeptide contains the sequence DAS, which is associated with non-aphid-transmissibility in other potyviruses. MAb A5B6, which binds to this region, reacted strongly in ELISA with three out of four other non-aphid-transmissible (NAT) PVA isolates, but not with three aphid-transmissible (AT) ones (3). We supposed that differences in the N-terminal region of the PVA may underlie most of the differences in aphid transmissibility. The cloning, sequence analysis and comparisons of PVA AT and NAT isolates RNA genome 3' regions involved CP genes was undertaken and sequences were compared to those of the 3' regions of several published PVA isolates (4), (5), (6).

Multiple sequence alignments and phylogenetical relationships based on these alignments revealed that different PVA isolates originated from distinct geographical regions confirm to generally accepted rules for the classification of potyvirus isolates. Both share a highly conserved CP amino acid sequence (94 to 99%) and highly conserved 3' nontranslated region (3'-NTR) (92 to 99%). The 3'-terminal region of the RNA of PVA isolates seems to consist of three independent domains, which have different evolutionary rates. The CP N-terminal variable domain clustered PVA isolates into AT and NAT subgroups (Fig. 1). Grouping the NAT isolate Juliniere into the AT subgroup was confirmed also by our serological data (3). Sequence data of a virus described as tamarillo mosaic virus (TaMV) were also included. TaMV is an AT virus, originally reported by Chamberlain as PVY (7) and later believed to be an indipendent member of the potyvirus group (8). TaMV causes yield loss and fruit blemishing in tamarillo in New Zealand. The amino acid sequence of the TaMV CP (9) showed 93% sequence similarity to PVA-B11. A more detailed examination of the amino acid sequences of the N-terminal part of the CP of PVA AT and NAT isolates revealed amino acid differences at ten positions (Fig 1). Amino acids at five of these positions (pos. 2, 7, 9, 10 and 23) are conserved among the PVA isolates within subgroups, but differ between them. We would like to stress here that differences at these five positions (G 2 E; G 7 S; T 9 A; P 10 L and G 23 S) led to a change in the properties of the amino acids (charge and/or hydrophobicity). TaMV has two (pos. 2 and 7) of the five conserved amino acids within AT subgroup.

AT PVA isolates CPs contain a DAG triplet at position 5 to 7 in the N-terminus, whereas the CP of the NAT isolate Juliniere from the AT subgroup contains DTG at the same positions (Fig. 1). It seems likely, that the conversion from A (Ala) to T (Thr) at the second position of the DAG triplet might

contribute the loss of aphid transmissibility of PVA isolate Juliniere. Our recent findings support this conclusion. The helper component protein (HC-pro) of Juliniere apparently does not contain the changes in amino acids believed to be crucial for aphid transmission (Andrejeva, unpublished data). Other PVA NAT isolates cluster in the NAT subgroup and contain a DAS triplet at position 5 to 7 (Fig. 1). Our preliminary data indicate that the conversion from G (Gly) to S (Ser) in the DAG tripeptide might be the factor responsible for the loss of aphid transmissibility of PVA isolates PVA-B11 (Puurand, unpublished data), Ali, Datura and Advira. Here we have to mention, that just after DAG triplet or its analogs (at position X) in both subgroups we found an acidic E (Glu) residue (Fig. 1), which, according to Atreya *et al.* (10) means a reduction in aphid transmissibility. On the other hand, naturally occuring PVY AT isolate D has also E in this position (11). Our data allows to suggest, that position X immediately after DAG triplet isn't crucial for aphid transmissibility of PVA. We also propose that the prefectly conserved amino acids (pos. 2. 9, 10, 23) (Fig. 1) in both AT and NAT subgroups have an important function in the CP of PVA and *via* that in viral life cycle, but they can have other function other involvement in the aphid-transmission of virus. Analysis of amino acid sequences of N-terminal parts of CPs of NAT PVA isolate Juliniere and TaMV from PVA isolates AT subgroup seems to confirm this suggestion. The central domain (core part and C-terminus of CP), highly conserved among all PVA isolates (94 to 99%), contains motifs typical for potyviruses and believed to be involved in virus assembly (12). 3' -NTR, the third domain, also separates PVA isolates into two subgroups on the basis of its length and homology. These groups are not identical with those based on the CP N-terminus. Biological functions of 3' -NTR are not yet clear.

		1 2 567 910 15 20 23 25 30
B11	NAT	A*E*TL**DAS**E*AL*AQKSEGRKKERE*S*NSSKAVAVKD
Ali	NAT	A*E*TL**DAS**E*AL*AQKSEGRKKEgE*S*NSSKAVAVKD
Advira	NAT	A*E*TL**DAS**E*AL*AQKpEGRKKEgE*S*NSSKAVAVKD
Datura	NAT	A*E*TL**DAS**E*AL*AQKSEGRKKEgE*S*NSdKAVAVKD
Juliniere	NAT	A*G*TL**DTG**E*TP*AQKSEGRKqEgE*G*NSSKAVAVKD
USA	AT	A*G*TL**DAG**E*TP*AQKSEGRKKEgE*G*NSSrAVAVKD
CANADA	AT	A*G*TL**DAG**E*TP*AQKSEGkKKEgE*G*NSgKAVAVKD
MAI	AT	A*G*TL**DAG**E*TP*AQKcEGkKKEgE*G*NSgKAVAVKD
L. Industry	AT	A*G*TL**DAG**E*TP*AQKtEGRKrEgE*G*NSgKAVAVKD
Rouge	AT	A*G*TL**DAG**E*TP*AQKtEGRKrEgE*G*NSgKAVAVKD
TaMV	AT	A*G*TL**DAG**E*a t*AQKaEGkKKEgE*V*sSgKAVvVKD

Fig. 1 Alignment of the CPs N-terminal parts of ten PVA isolates and TaMV. The sources for the sequences are as follows: NAT PVA isolate B11 (4); NAT PVA isolates Ali, Advira, Datura, Juliniere and AT PVA isolates Lighte Industry and Rouge (present paper); AT isolates USA and MAI (6) ; AT isolate PVA-Canada (5); TaMV (9). Sequence analysis was done with the aid of the PCGENE program (CLUSTAL).

One of the goals of our study was to obtain MAbs that would be suitable for the serological detection of economically important potato viruses. We have prepared MAbs to PVX, PVY, potato virus M (PVM), potato virus S (PVS), and potato leafroll virus (PLRV) and developed different immunoassays for the quantitative determination of those viruses. The analysis of the incidence of the potato viruses in Estonia was carried out, and as a result, a tentative picture of the spread and distribution of potato viruses in Estonia was obtained. Out of 5 987 potato tubers analysed 827 or 13.8% were infected. PVX is the most widely spread virus in Estonia (6.7%) and PLRV is the less spread one (3.5%). The results of the regional spread of potato viruses are presented in Fig 2. Our results show that Harju, Kohtla-

Järve and Haapsalu regions have higher levels of potato virus infection that other regions. In these regions a very high infection rate with PLRV was noticed. At the same time island Hiiumaa is almost virus infection free and therefore could be recommended for growing seed potatoes. It was also found that the most common combination of viruses in potato tubers infected with more than one virus were PVX and PVS, or PVX and PVM. Though, all the other combinations of infection could also found.

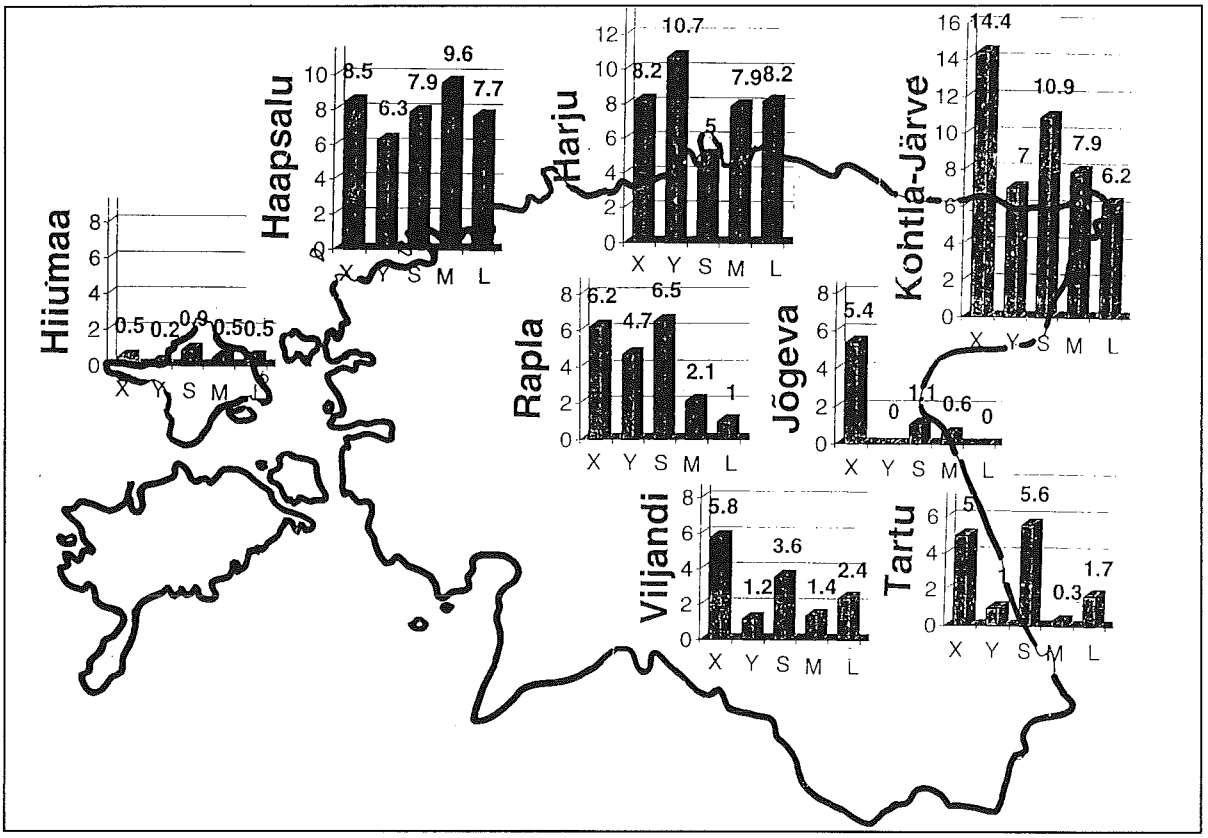

Fig. 2. Regional spread of potato viruses in Estonia
Viruses infected potato (%) in regions

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GENETIC ANALYSIS OF STRAINS OF BARLEY MILD MOSAIC AND BARLEY YELLOW MOSAIC VIRUSES.

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Introduction

Two fungus-borne viruses, barley mild mosaic (BaMMV) and barley yellow mosaic (BaYMV) viruses, are involved in yellow mosaic disease of barley. The use of resistant barley cultivars is the only practical measures to control the disease. However, all the resistant barley cultivars currently used in Japan are susceptible to particular virus strains. Two strains of BaMMV and six strains of BaYMV have been identified based on the pathogenicity toward barley cultivars and serology (1, 2).

Both BaMMV and BaYMV have bipartite ssRNA genomes. Partial nucleotide sequences have been determined for the genomes of two BaMMV strains Ka1 and Na1 (3), and complete nucleotide sequence for the genome of BaYMV strain II-1 (4, 5). In this study, to examine relationships between the RNA components and the pathogenicity, mechanical inoculation with a mixture of two virus strains was made onto different barley cultivars, and RNA combinations in infected plants were analyzed by RT-PCR. Plants with different RNA combinations were used for further inoculation experiments. In addition, the capsid protein genes of BaYMV strains were sequenced.

Material and Methods

BaMMV and BaYMV strains were maintained in barley plants (cv. Ishukushirazu for BaMMV and cv. New Golden for BaYMV) by mechanical inoculation. Five barley cultivars used in this study are listed in Table 1. Barley seedling at the one and half leaf stage were inoculated with leaf homogenate in 0.1 M phosphate buffer pH 7 and then incubated in growth cabinets with the constant temperature (13-15°C) and the sun light. Co-inoculation was made using a mixture of equal amounts of sap from plants infected with each virus. Mosaic symptoms were first observed on the third leaves usually three to four weeks after inoculation, and these leaves with symptoms were used for RT-PCR.

Six pairs of 'forward' and 'reverse' primers were made for specific detection by RT-PCR of RNA1 and RNA2 of BaMMV-Ka1, BaMMV-Na1 and BaYMV-II-1. Primer sequences were based on the nucleotide sequences reported for BaMMV-Ka1 RNA1 (3), BaMMV-Na1 RNA1 (3), and BaYMV-II-1 RNA1 and RNA2 (4, 5), and on the sequences of the 3' non-coding regions in RNA2 of BaMMV-Ka1 and BaMMV-Na1 obtained using selected clones from the oligo dT-primed cDNA libraries (3).

Nucleic acid (a mixture of RNA and DNA) was extracted from approx. 5 mg of leaf tissue using the Sepa Gene kit (Sanko Junyaku Co. Ltd.), and dissolved in 50 µl distilled water. First strand cDNA was synthesized from 1 µl of the nucleic acid in a 5 ul reaction mixture containing a reverse primer (0.25 µg) and SuperScript reverse transcriptase (BRL) by incubation at 42°C for 15 min, followed by incubation at 99°C for 5 min to inactivate the enzyme. Whole of the cDNA mixture was used to make a 25 µl PCR mixture containing a

forward primer (0.25 μg) and AmpliTaq DNA polymerase (Perkin-Elmer). The PCR program was 30 cycles each consisting of 94°C for 1 min, 65°C for 2 min and 72°C for 3 min, followed by 72°C for 7 min. Aliquot of PCR products (5 μl) was analyzed by agarose gel electrophoresis.

For sequence analysis of the capsid protein (CP) genes of BaYMV strains, nucleic acid was extracted form leaf tissue infected with each virus. cDNA containing whole of the CP gene was amplified by RT-PCR, and cloned using pBluescript II.

Table 1. Barley cultivars used in this study and their reactions to BaMMV strains Ka1 and Na1, and BaYMV strain II-1 (1, 2)

Barley cultivar (resistance gene)	BaMMV-Ka1	BaMMV-Na1	BaYMV-II-1
New Golden	S	S	S
Ishukushirazu (*ym3*)	S	S	R
Misato Golden (*Ym*)	R	S	R
Shiromugi 6	S	R	S
Tosan Kawa 73	S	R	S

S: Susceptible, R: Resistant.

Table 2. Combinations of viral RNA components in infected barley plants after co-inoculation with BaMMV-Ka1 and BaMMV-Na1

Barley cultivar	Number of infected plants	RNA combination*								
		$K_1N_1K_2N_2$	$K_1N_1K_2$	$K_1K_2N_2$	$N_1K_2N_2$	$K_1N_1N_2$	K_1N_2	N_1K_2	K_1K_2	N_1N_2
Ishukushirazu	25	3	2	3		1	3	2	8	3
New Golden	18			1	1	1	2		10	3
Misato Golden	7				1			3		3
Shiromugi 6	9	1				1	4		3	
Tosan Kawa 73	5			1					4	

Number of plants with each RNA combination is shown.
K_1:Ka1-RNA1, N_1:Na1-RNA1, K_2:Ka1-RNA2, N_2:Na1-RNA2.

Results

Barley seedlings of five cultivars were inoculated with a mixture of BaMMV-Ka1 and BaMMV-Na1. Percentage of infected plants varied with the cultivar (11 to 62 %). Analysis of the infected plants by RT-PCR showed different combinations of RNA components (Table 2). In Ishukushirazu and New Golden, in addition to single (K_1K_2 and N_1N_2) and mixed ($K_1N_1K_2N_2$) infections, combination of RNA 1 and RNA 2 from one strain and RNA 1 or RNA 2 from the other strain ($K_1N_1K_2$, $K_1K_2N_2$, $N_1K_2N_2$ and $K_1N_1N_2$), and combination of RNA 1 and RNA 2 from different origins (pseudorecombinants; K_1N_2 and N_1K_2) were observed (Table 2). Fewer combinations were observed in Misato Golden, Shiromugi 6 and

Tosan Kawa 73. The RNA combinations observed in Misato golden, which is susceptible to BaMMV-Na1 but resistant to BaMMV-Ka1, all contain N_1. In contrast, the RNA combinations in Shiromugi 6 and Tosan Kawa 73, both of which are susceptible to BaMMV-Ka1 but resistant to BaMMV-Na1, all contain K_1 (together with N_1 in two plants of Shiromugi 6). However, either K_2 or N_2, or both, were found in infected plants of these three cultivars.

Plants with RNA combinations $K_1N_1K_2N_2$ (mixed infection), $K_1N_1K_2$ or $K_1K_2N_2$ were selected for further inoculation experiments. After inoculation with $K_1N_1K_2N_2$ or $K_1N_1K_2$, all the infected plants of Misato Golden had N_1, whereas those of Shiromugi 6 and Tosan Kawa 73 had K_1. After inoculation with $K_1K_2N_2$, Misato Golden was not infected, but Shiromugi 6 and Tosan Kawa 73 were infected and had K_1.

Plants with pseudorecombinants K_1N_2 or N_1K_2 were also used for inoculation to the five barley cultivars. Ishukushirazu and New Golden were infected with either K_1N_2 or N_1K_2. Misato Golden was infected with N_1K_2, but not with K_1N_2. Shiromugi 6 and Tosan Kawa 73 were infected with K_1N_2, but not with N_1K_2. There were significant differences in the symptoms on New Golden induced by K_1N_2 and N_1K_2. K_1N_2 induced severe mosaic, necrosis and yellowing, and these symptoms were similar to those induced by BaMMV-Ka1. However, N_1K_2, like BaMMV-Na1, induced only mild mosaic. Moreover, on Ishukushirazu, K_1N_2 and BaMMV-Ka1 induced severer mosaic symptoms than N_1K_2 and BaMMV-Na1 did.

After co-inoculation of New Golden with a mixture of BaYMV-II-1 and BaMMV-Ka1 or BaMMV-Na1, single and mixed infections were observed, but no exchange of their RNA components observed. Plants having mixed infections with BaYMV-II-1 and BaMMV-Ka1 or BaMMV-Na1 were also used for inoculation to the five barley cultivars, but only single and mixed infections were observed.

The CP genes of the six known BaYMV strains (I-1, I-2, I-3, II-1, II-2 and III) were cloned using RT-PCR and sequenced. The CP genes of these strains, all of which start with an alanine codon (GCU) and end with an UAA termination codon, encode 297 amino acids. The sequence of the BaYMV-II-1 CP gene is identical to that reported previously (4). Comparisons of the CP genes of the six strains reveal one to eleven nucleotide differences, which lead to zero to three amino acid variations. A new BaYMV strain IV was isolated from a yellow mosaic-diseased plant of Masakadomugi, a cultivar which has the resistance gene *ym3* and is resistant to six other BaYMV strains. The CP gene of this strain also encodes 297 amino acids, but its deduced CP has nine to eleven amino acid differences from those of six other strains.

Discussion

Co-inoculation with BaMMV-Ka1 and BaMMV-Na1 gave different combinations of RNA components. All the RNA combinations observed in infected plants of the five cultivars contain at least one RNA1 component and one RNA2 component, indicating that both RNA species are needed for infection. The RNA combinations in infected plants of Misato Golden, Shiromugi 6 and Tosan Kawa 73 indicate the involvement of BaMMV RNA1, but not RNA2, in pathogenicity toward these cultivars. Inoculation experiments with pseudorecombinants also demonstrated that exchange of RNA2 did not alter the pathogenicity. Moreover, the experiments with pseudorecombinants suggest that RNA1 is related to the symptom severity.

Exchange of RNA components was not observed after co-inoculation with BaYMV-II-1

and BaMMV–Ka1 or BaMMV–Na1. Although both BaYMV and BaMMV belong to the genus *Bymovirus*, they have no serological relationship (2) and very low sequence homology (3). Thus, to confirm that BaYMV RNA1 is involved in pathogenicity, co–inoculation experiments using different BaYMV strains are required. Sequence data of the capsid protein genes of seven BaYMV strains would be useful for specific detection of BaYMV strains by RT–PCR.

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THE DESIGN AND ANALYSIS OF ANTI-HLVd RIBOZYMES

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Hop latent viroid (HLVd) belongs to small autonomously replicating (e.g.1), coat protein-free, circular, pathogenic RNAs fully adapted to the host metabolism. Viroids form a thermostable rod-like secondary structures, which are characterized by a serial arrangements of double-helical sections and internal loops. Viroid structure is also highly stabilized against degradation with major plant nucleases (2) and therefore, it can be easily spreaded by agrotechnique. It is probably why HLVd is worldwide distributed with nearly 100% incidence. HLVd is a deleterious to production in certain hop cultivars, as it causes changes in the composition of lupulin (3). Therefore, it is of interest to develop effective methods for HLVd eradication. A ribozyme appproach could contribute to viroid elimination.

The artificial hammerhead ribozyme (HR) as engineered by Haseloff and Gerlach (4) forms a sequence-specific endonuclease, which can be used as a tool for gene regulation. It contains short regions of conserved nucleotides, a double helix, called helix II, which connects the two conserved regions, and flanking arms of variable sequence, which hybridize the ribozyme to its specific target. The flanking arms can be extended to form so called antisense ribozymes (5). The target sequence is cleaved at position 3' to specific GUX triplet, where X can be C,U or A, producing 2',3'-cyclic phosphate groups. The kinetics of the reverse reaction (re-ligation) of the ends by HR is negligible (6), but the ends can be potentially re-ligated in vivo by protein ligases, especially in uni-target RNA. This could be the case if monomeric viroid is targeted. In addition to well characterized elements of secondary structures of HR catalytic core, some recent results show that ribozymes adopt also tertiary structures (e.g.7-10) and that in vivo some ribozyme-protein interactions could be important for the ribozyme activity and stability (e.g. 11,12). HLVd as other viroids is presumed to replicate by an asymmetric pathway involving the rolling circle mechanism to produce minus-sense RNA oligomeric forms, which serve as templates for the multimers of plus-sense polarity. The later are processed to unit-length viroid progeny (1). Therefore, ribozymes can be, in principle, targeted either against minus-sense oligomeric replication intermediates of HLVd [(-)HLVdRI] or against plus-sense polymeric intermediates, as well as monomeric viroid RNA. In this study we selected HR targeted exclusively against (-)HLVdRI. Such targeting could be more effective for viroid eradication than targeting of chains of plus polarity for two reasons: 1.] there is much lower amount of (-)HLVdRI in infected plant cell than the amount of plus-sense viroid progeny (e.g.13) 2.] (-)HLVdRI form the oligotarget RNAs, as they are of oligomeric nature; HR could break these oligomers into monomeric molecules which cannot serve as templates for HLVd replication, while linear monomers of plus polarity are infectious (14).

In this study we investigated activity in vitro of HR targeted against GUC sites close to computer predicted loops in minus dimeric transcripts simulating (-)HLVdRI. The main aim was to verify a possibility to prepare ribozymes having ability to cleave stable (-)HLVdRI at physiological temperatures close to thermotherapy and heat shock conditions. Such ribozymes could participate as the additional factors in thermotherapy-mediated eradication of HLVd from hops in vivo. Thermotherapy conditions were selected for two basic reasons: Firstly, temperature close to heat shock is favorable for the formation of specific complexes between HR and (-)HLVdRI structures. Secondly, heat treatment of infected plants itself causes a significant reduction of viroid level (unpublished) and, therefore, it could mediate much favorable molar ratio between HR target RNA for an effective complex formation and viroid degradation.

Material and Methods

Ribozymes were synthesized by phosphoramidite method and cloned in transcription vector pBluescript IIKS(+/-). Restriction sites BamH1,HindIII and ClaI,HindIII were used for cloning of HR47 and HR57, respectively. HR47 was transcribed by T3 polymerase and HR57 by T7 polymerase. The transcription reactions were carried out according to manufacturer's recommendations. Final length of in vitro transcripts with an

additional vector sequences was 98 and 172 bases for HR47 and HR57, respectively. Secondary structure prediction using computer programme DNASIS (LKB), confirmed formation of stable catalytical core for both these ribozyme transcripts with free energy -57.2 and -134.3 kJ/mol for HR47 and HR57, respectively. For the calculations, the arm-covering substrate sequence was attached (in cis) to the 5'end of HR transcript.

Ribozyme reactions were carried out in 60 µl reaction mixtures containing 50mM Tris-HCl pH 7.5 as a standard ribozyme buffer (15). Ribozyme and [α-^{32}P]UTP- labelled substrate were hybridized in this buffer at different temperatures, depending on experiment. If not stated otherwise, we used 50nM truncated substrate S117 or 33nM (1 pMol) dimeric substrate having 555 bases. The ratio of binding sites S:HR was always close to 1:1. 10mM $MgCl_2$ (final concentration) was added to initiate the reactions. Reactions were stopped by addition of sequencing dye 1:1 V/V containing 80 % formamide, 10 mM EDTA and 0.001%.bromphenol blue. The samples were heat denatured (80^{o} C for 5 min) and analyzed in 6% sequencing gel. Autoradiograms were scanned using an ULTROCSAN (LKB) and quantified using the Gelscan 2D-spots computer program version 2.1 (LKB).

Results

As the (-)HLVdRI target is unusual in the respect that this RNA can adopt stable secondary structures (e.g.16), we concentrated in this study mainly to two unique GUC target sites at positions corresponding to nucleotide number 19 and 111 in plus momomeric RNA. Both target sites localized close to big internal loops on viroid dimeric secondary structure predicted at conditions approximated to ribozyme reaction et low temperatures. The initial interaction of ribozyme with this internal loop(s) could facilitate complex formation between ribozyme and target sequence. We designed two types of HR: HR47 and HR57 for the left (25 base, position 19) and right (50 base, position 111) loop, respectively. Helices II described for a wild type of HR by McCall (15) and by Haseloff and Symons (17) were selected for HR47 and HR57, respectively. Non identical II helices were selected for future possible identification of ribozymes in vivo in double transformants. In this study we selected right-handed hybridizing arms covering partly the internal loops predicted. Left-handed arm was of the same length in HR47 forming a symmetric ribozyme, but in the case of HR57 the arm was shorter in order to prevent formation of secondary structures (Fig.1). We assumed that rather short hybridizing arms in this particular case are important to prevent possible silencing of HR genes by RNA-RNA directed methylation, which has been observed in full-length viroid cDNAs by Wassenegger (18). The final design of these two ribozymes is shown in Fig.1.

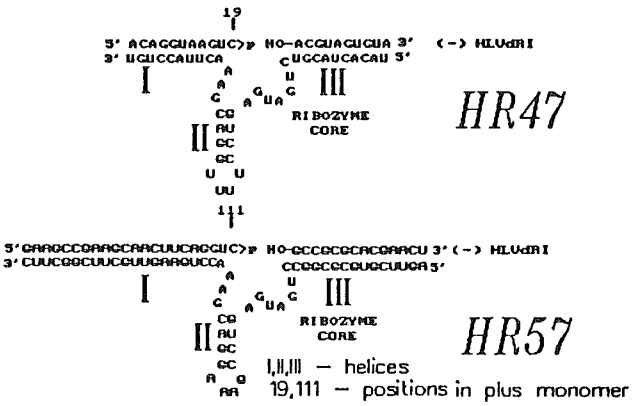

Fig.1 The detailed structures of HRs

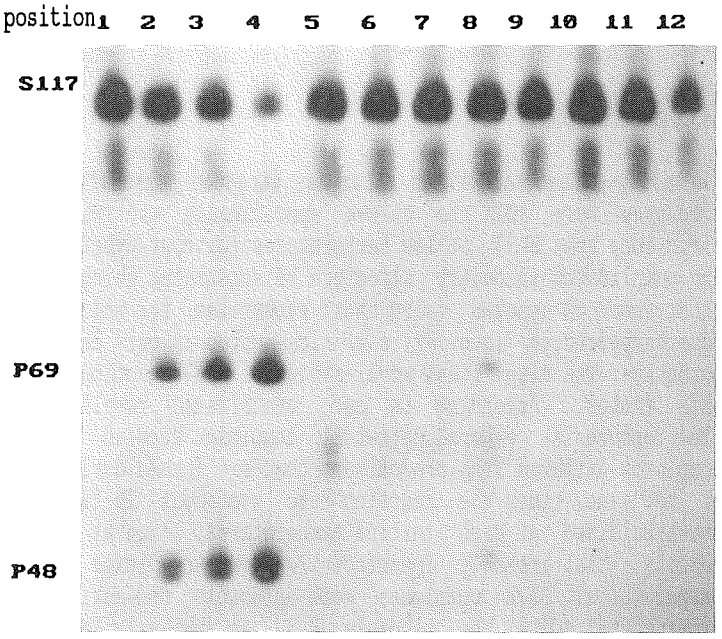

Fig.2 Reaction of HR47 with truncated S117. 1-4- 0,10,30 and 60 min of reaction in the presence of Mg^{2+}; 5-8- absence of Mg^{2+}; 9-12- control reaction without HR47

In order to avoid the possible strong influence of (-)HLVdRI, and to test the reaction conditions, in the first experiments we assayed HR47 activity and specificity with truncated substrate having 117 bases and one target site (Fig.2). The ribozyme was in this case pre-hybridized to the substrate at high initial temperature (94^{o}C). S117 has been cleaved as expected, in exact position generating two predicted products

P69 and P48. No significant cleavage occurred in the reactions without Mg^{2+}. S555 (two target sites) has been cleaved by HR47 with much lower rate under similar conditions (Fig.3). The most interesting question, which we wanted to test, was the ability of HRs to cleave HLVd at low temperatures around 40 C^{o} (heat shock). Both S117 and dimeric S555 were cleaved also at 40°C , but S555 with very low rate (Fig.3). Only about 10 % of S555 was cleaved out even after long incubation period (20 h). These results suggest strong inhibitory effect of substrate secondary structure on HR47 reaction. This assumption is in agreement with the significant influence of temperature on S555 cleavage (not shown). Apart from the HR47 stimulation by increasing temperature, the reaction was much slower than reaction with ribozyme targeted against bigger loop predicted in (-)HLVdRI (Fig.1) and, therefore, we concentrated further mainly on this ribozyme. HR57 cleaved S555 specifically in two target sites generating finally three products P256,P232 and P67. Although the influence of temperature on reaction rate of HR57 was obviously lower than in the case of HR47 (not shown), the ribozyme HR57 showed ability to cleave S555 in a significant extent at low temperatures (Fig.3). The reaction at low temperature was approximately 10 times slower than reaction with preformed complexes at high temperature. However, the most important finding consists in the fact that HR57 cleaved nearly 100 % of the substrate, suggesting its ability to form RNA-RNA complexes at temperature close to the heat shock conditions. The main difference between HR47 and HR57 reaction rate could be predominantly caused by the influence of S555 structure, because HR47 cleavage of S117 occurred at similar rate as S555 by HR57 (Fig.3). The difference in the length of hybridizing arms has to be also considered as important factor.

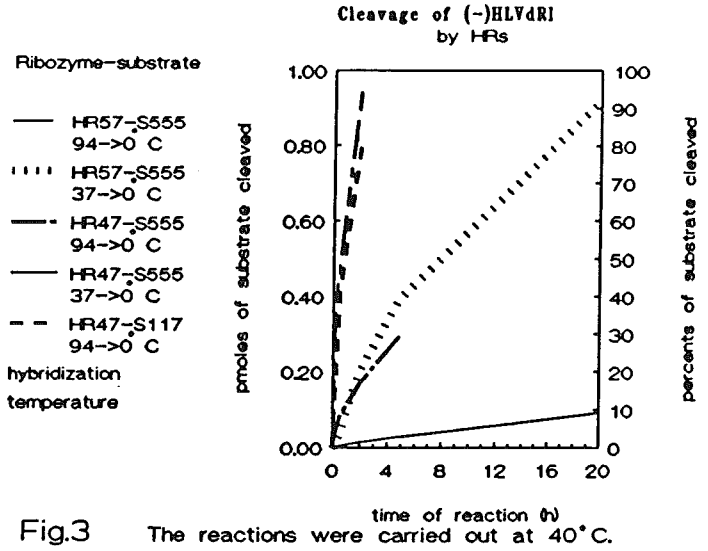

Fig.3 The reactions were carried out at 40°C.

Discussion

We assayed two hammerhead ribozymes targeted against hop latent viroid minus-sense intermediates. Both ribozymes were able to cleave specifically the viroid structures. HR47 showed much lower cleavage efficiency than HR57 at low temperatures (without hybridization). Such difference is obviously caused by the complicated secondary structure of substrate, which is not easily accessible for pairing. Unlike to other short HR assayed using short substrates, for which a chemical cleavage step is rate-determining at room temperatures (e.g.19), a critical step in our case could be the ribozyme binding and RNA complex formation. The significant acceleration of HR47 reaction at higher temperature could be explained just by this factor. According to our unpublished results, we found using temperature gradient gel electrophoresis, several different complexes between HR57 and viroid RNA at low temperatures. These complexes differed from predominant complex formed during heat-denaturation and subsequent hybridization. At the same time, the reaction rate was about 10-times higher when the ribozyme and substrate were pre-hybridised at high initial temperatures, than at low temperatures. It can be deduced from these results, that probably imperfect transient complexes (with partly bound ribozyme) were formed at low temperatures. Such complexes were probably non-active ones. Although our predictions need more experimental data, the ribozyme HR57 is obviously a good candidate for study of ribozyme mediated inhibition of hop latent viroid in vivo. This ribozyme has relatively short antisense arms, but at the same time, the complete substrate cleavage by this ribozyme has been observed at temperatures close to physiological conditions. It is possible that complexes between HR57 and target RNA are initiated by the "kissing" interaction within the loop.

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3′ TERMINAL SEQUENCE ANALYSIS AND HOMOLOGIES FOR TWO MEMBERS OF GENUS *RYMOVIRUS (POTYVIRIDAE)*

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Introduction. Potyviruses (family *Potyviridae)* are the largest and economically the most important family of plant viruses. More than 300 members of that family are known to date, and their molecular structures are well studied. Most potyviruses are transmitted from plant to plant by aphids, and the data on the molecular mechanisms of aphid transmission are accumulating. Members of the other genus, genus *Rymovirus*, have other vectors for transmission - several species of eriophyid gall mites (1). Little is known about the genomic structure of these viruses, the 3′ terminal sequences of only two rymoviruses, the brome streak mosaic virus (BrSMV) and the wheat streak mosaic virus (WSMV) have been published(2). Their putative coat protein (CP) sequence is located at the C-terminus of their RNA genome-encoded polyprotein. BrSMV and WSMV CP sequences have an unusually low (for potyviruses) percentage of similarity with aphid-transmissible potyviruses and also with each other. This is supported by serological experiments that showed no cross-reactions between BrSMV, WSMV and viruses from genus *Potyvirus.* Recently sequencing and serological data have revealed significant homology between two members of genus *Rymovirus*: the hordeum mosaic virus and the agropyron mosaic virus.

Our interest was focused on two molecularly uncharacterized members of genus *Rymovirus*, the ryegrass mosaic virus (RYMV) (type member of genus *Rymovirus*) and the oat necrotic mottle virus (ONMV).

Material and Methods. The 3′ terminal parts of RYMV (Denmark isolate) and ONMV were cloned using lambda ZAP vector based cDNA synthesis and cloning system by Stratagene. Positive clones were selected by hybridization with virus-specific labelled cDNAs and bluescript plasmids, carrying virus-specific inserts. Plasmid clones were sequenced in both directions and the sequence data was analysed using PCGENE software.

Results and Discussion. Our previous serological research revealed a significant serological relationship between RYMV coat protein and coat proteins of aphid-transmissible potyviruses. We also found a strong serological relationship between ONMV and WSMV. The obtained sequence data confirm these results.

The 3′ terminal regions of RYMV and ONMV are encoding the C-terminal part of one large ORF, coding a viral polyprotein. The putative coat protein sequences were identified by homology with other known potyviral CP-s.

The CP sequence of ONMV appears to have very high homology with that of WSMV (up to 93% in C-terminus of CP) and lower homology with other known potyviruses; WSMV and BrSMV from genus *Rymovirus* and plum pox virus (PPV)(3), and potato A potyvirus (PVA) from genus *Potyvirus* (4) (Fig.1). The 3′

untranslated sequence (UTR) of ONMV has 77% identity with the 3'-UTR of WSMV, but no significant homology with 3'-UTRs of other potyviruses.

Fig. 1. An alignment of coat protein C-terminal sequences of 6 potyviruses and a phylogenetic tree, generated on the basis of that alignment. Character to show that position in the alignment is perfectly conserved: "*"; character to show that position is well conserved: "#".

```
PPV      PLLD----HAKPTFRRIVARFSDVAEACVEKR-NYEKAYMPRYGIQRNLT
PVA      PMLD----HAKPSLRQIMRHFSALAEAYIEMR-SREKPYMPRYGLQRNLR
RYMV     YTLEPSCRHAQPTLRSIMAHFSDAATAYVVLR-NQKSRYMPRYGLKRGLN
ONMV     FKIEPMYKSARPSMRSIMRHFGEGARVMIEESVRIGRPIIPRGFDEAGVL
WSMV     FKIEPMYKAAKPSLRAIMRHFGEGARVMIEESVRIGKPIIPRGFDKAGVL
BrSMV    YDEIPMYSAANPTMRAIMRHFSDLAGLVIAESFKQGRPLIPKGYIKAGVL
                  *#*### *# #*## *   #  # #     #*#   # ##

PPV      DYSLARYAFDFYEMTSTTPVRAREAHIQMKAAALRNVQNRLFGLDGNVGT
PVA      DQSLARYAFDFYEITATTPIRAKEAHLQMKAAALKNSNTNMFGLDGNVTT
RYMV     DYSLAPYAFDFYEITSTSPLRAREAHAQMKAAAIRGKASRMFGLDGNVSA
ONMV     SVNNIVAACDFIMRNADDTPNFVQVQNSVAVNRLRGIQNKLFAQARLSAG
WSMV     SINILWAACDFIMRGADDTPNFVQVQNSVAVNRLRGIQNKLFAQARLSAG
BrSMV    DASSAARACDFVVRDRHDTATFVQVQNQVLVNRVSGITNWLFAQQCLALV
         # #    * **   #  ##    ###  # ## ###  # #*# #

PPV      QKQD------TERHTDGDVNRNMHTFLGVRGV-----------
PVA      SEED------TERHTATDVNRNMHHLLGVKGV-----------
RYMV     QSEN------TERHTVEDVNTRVHSLSGANML-----------
ONMV     TNED------NSRHDADDVRENTHTFAGVNA-------LA---
WSMV     TNED------NSRHDADDVRENTHSFNGVNA-------LA---
BrSMV    LTRTWRAMTLRCRRGHPQPRLSA-SFLEVSTSCRARRRLGVQR
          # #        *##  # # #   # ###
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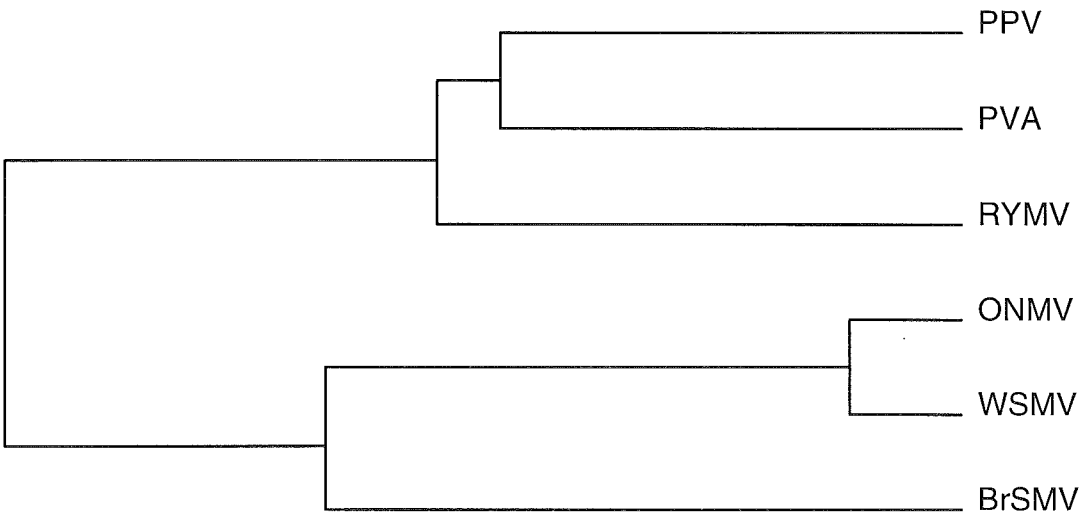

RYMV, on the other hand, has significant homology with aphid-transmissible potyviruses (PPV, PVA), both in the non-structural region (NIb) and in the coat protein region (Fig. 2). The homology was the most significant in the NIb gene region and in the CP "core" sequence. However, the CP sequence of RYMV shows only limited homology with other rymoviruses (Fig. 1). The highest degree of homology with aphid-transmissible potyviruses (up to 54%) was observed in the "core" part of RYMV CP with respect to PVA, while no obvious

homology was observed between CP-s in their N-terminal regions. This sequence difference might reflect the different nature of the virus vectors for transmission. Thus, according to our opinion, RYMV might be an evolutionary link between aphid-transmissible potyviruses and mite-transmissible rymoviruses. 3'-UTR of RYMV has no significant homology with an analogous region of any of the known potyvirus.

Fig. 2. Alingment of amino acid sequences of RyMV and PVA polyprotein C-terminal parts.

```
RYMV   DKVNYGTMDLTSNQPSGAFKTTKLLEDLL-EAVSHQSQEYTWLTKYCGAN -  49
PVA    NNVCWGSFHLQDSKPTKEFKTVKLVTDLLGEAVYTQGCDSKWLFNAAHTN -  50
         *  *   *    *   *** **  *** ***  *      **       *
RYMV   LLVIGKCPGNLITKHVIKGKSPTFDLFLSVDAQASDFFKPLMGDYAPSRL -  99
PVA    IQAVAQLESNLVTKHTVKGKCKLFETYLNVDKAAHDFFSKYMGFYKPSKL - 100
                ** ***  ***   *   * **  * ***   ** * ** *
RYMV   NREAFVKDITKYDTEIPIGNLSITDFENAV-------------------- - 129
PVA    NREAYTQDLMKYSKVIQVGEVDCGVFESALTGLLHNLGRWGFTTACYTTD - 150
       ****   *  **   *  *      ** *
RYMV   EDTYYILKDSGIEQCNYITDAIPIFDSMNMKAATGALYGGKKKDYFENYT - 179
PVA    EDS--------------------IYTALNMKAAVGALYRGKKRDYFDAMS - 180
       **                     *    ***** **** *** ***
RYMV   DDMKQNILKESYIRLREGKMGIWNGSLKAELRSKEKVEANKTRVFTAAPL - 229
PVA    PSEREHLLFLSCKRLYFGQLGVWNGSLKAELRPKEKVDLNKTRTFTAAPI - 230
             *  *  **  *  * **********  ****  **** *****
RYMV   DTLLAGKGCVDDFNNQFYAAHLKGPWTEGITKLFGRWNDFLSELPPSWDY - 279
PVA    ETLLGGKVCVDDFNNMFYSLHLKAPWSVGMTKFYGTWNQLMCKLPDDWVY - 280
        *** ** ******* **  **** **  * **  * **     ** * *
RYMV   FDADGSRFDSSLTPFLLNAVLNIRKKFMINWAFGQRCLENLYTEIIYTPI - 329
PVA    CDADGSQFDSSISPYMINAVLRIRLHFMEDWDIGSQMLQNLYTEI----- - 325
        ***** ****  *   **** **  **  *  *   * ******
RYMV   ATPDGS---------VVKKMRGNNSGQPSTVVDNSIMVIIAMQYAISKAE - 370
PVA    ----GTHQSQHQMAQLLKKFKGNNSGQPSTVVDNTLLVVLALHYALLKSG - 371
           *           **  ************   *  *  **  *
RYMV   FP----------AGRLRDQIRYFANGDDLVVAVEPSLSDKISSFSASFAE - 410
PVA    IPLEEQDSVCAYG----------VNGDDLLIAIRPDMEHKLDGFQALFSE - 411
        *                      *****  *  *    *   * * * *
RYMV   LGLSYDFSNKVNDRSELQFMSHTGKLIDGMYIPMLERERICAILEWSRSD - 460
PVA    LGLNYEFNSRSKDKKDLWFMSHKAIQCGEILIPKLEEERIVSILEWDRSH - 461
       *** * *     *   * ****          ** ** **    **** **
RYMV   EPQFQLDAISAAMIEAWGDDELLYQIRRYYSWLLEQEPYKSIAELGHAPY - 510
PVA    EPIHRLEAICASMVESWGYPELTHEIRRFYAWVLEQSPYNALATTGLAPY - 511
       **   * ** * * * **  **   *** *** *** **   *  * ***
RYMV   LAEAALKALYTGKDPDAELIAIYERAMLNTPPTEDRPTKVVHEANVTAAS - 560
PVA    IAESALKTLYTNVHP----------------------------------- - 526
        ** *** ***   *
RYMV   SAATQTST---------------------------TSPTVTSTSGASTS - 582
PVA    -----TSTELEKYSIQFDEQMDEEDDMVYFQAETLDASEALAQKSEGRKK - 571
            ***                              *      *
RYMV   TSSGTTSAPLASTTPPVSATTTPSTGTTAPTTPTVRAANLPDIAGHRKAK - 632
PVA    ERESNSSKAVA------------------------------------- - 582
             *   *
RYMV   ANRESQLNVRGENVDEDVPAASEFALPRLPTLGAKIRVPKFKGAIVLNKD - 682
PVA    ----------VKDKDVDLGTAGTHSVPRLKSMTSKLTLPMLKGKSVVNLD - 622
                     * *   *     ***     *   *  **  * * *
RYMV   HLIKYTPDQRDLSNTRATQEQFEKWYSGVRNEVEKTDEEMALLLNGFMVW - 732
PVA    HLLSYKPKQVDLSNARATHEQFQNWYDGVMASYELEESSMEIILNGFMVW - 672
       **  * * * **** *** ***  ** **    *     *   *******
RYMV   CMENGTSPDLSGSWTMMEGEEQIAYTLEPSCRHAQPTLRSIMAHFSDAAT - 782
PVA    CIENGTSPDINGVWTMMDNEEQVSYPLKPMLDHAKPSLRQIMRHFSALAE - 722
       * *******  * ****  ***  * * *   ** * ** ** ***  *
RYMV   AYVVLRNQKSRYMPRYGLKRGLNDYSLAPYAFDFYEITSTSPLRAREAHA - 832
PVA    AYIEMRSREKPYMPRYGLQRNLRDQSLARYAFDFYEITATTPIRAKEAHL - 772
       **   *     ******* * * * *** ********* * * ** ***
RYMV   QMKAAAIRGKASRMFGLDGNVSAQSENTERHTVEDVNTRVHSLSGANML - 881
PVA    QMKAAALKNSNTNMFGLDGNVTTSEEDTERHTATDVNRNMHHLLGVKGV - 821
       ******       ********    * *****  ***   * * *
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Our sequence data on ONMV and RYMV, together with the available sequence data on BrSMV and WSMV show, that genus *Rymovirus* of family *Potyviridae* is quite heterogenic in their CP sequences. This family could be divided into subgroups in the future, when more sequences and molecular biology data will be available.

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A POTYVIRUS INDUCED LEAF CURL DISEASE OF CELOSIA ARGENTEA IN NIGERIA

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Introduction

Celosia argentea L. (Amaranthaceae) is an important vegetable crop in South Western Nigeria. The leaves and the young succulent stems are good sources of vitamins C and B complex (1). A severe leaf curl disease on the crop was first observed in a commercial vegetable farm in 1989 during a survey for viruses of leafy vegetables in Lagos, Nigeria. Other symptoms induced on infected plants included leaf malformation mosaic and dwarfing. This paper presents some of the properties of the etiological agent of the disease.

Materials and methods

Host range and symptomatology

Crude sap obtained by triturating infected leaves of *Chenopodium quinoa* in 0.03M sodium phosphate buffer, pH 8.0, in precooled sterilized pestle and mortar was used to mechanically inoculate carborundum dusted leaves of some diagnostic plants. Inoculated leaves were rinsed with water and observed for about 4 weeks for symptom development in the greenhouse with temperature of between 20 - 22 °C.

Purification

After 3 successive local lesion transfers to *C. quinoa* the virus was multiplied in *N. benthamiana* and was purified by three cycles of low and high speed centrifugation from frozen leaves of *N. benthamiana.* Briefly, the grinding buffer used was 0.5M sodium borate buffer, pH 7.4, containing 0.005M EDTA and 0.015M DIECA. After low speed clarification and stirring for 30 min with 5 % triton-100 at 5°C, the homogenate was given high speed centrifugation and the pellets suspended in 0.01 M sodium citrate buffer, pH 7.4, containing 1 M urea and 0.1 % 2-mercaptoethanol. The supernatant after low speed running was laid over 20 % sucrose "cushion" and centrifuged at high speed. Further low speed centrifugation was followed by overnight run in caesium chloride at 33,000 rpm. Virus band was collected and diluted ten fold by with borate buffer, pH 8.0 and pelleted at high speed. The preparation was resuspended in borate buffer.

Insect transmission

The ability of *Myzus persicae, Aphid craccivora, A. faba, Brevicoryne brassicae* and *Rhopalosiphon padi* to transmit the virus was tested. The aphid species were starved for 2 h and allowed aquisition access feeding period of 2 1/2 to 5 min on *Celosia* virus infected leaves of *C. argentea, N. clevelandii* or *N. benthamiana.* The aphids were then transferred to 10 young seedlings of these plants and left overnight after which they were killed by Pirimor.

Serological tests

Serological relationship of the *Celosia* virus was tested with antisera to some potyviruses in DAS- and PTA-ELISAs, western blot and immunosorbent electron microscopy plus decoration (ISEM-D). DAS-ELISA was performed according to the method of (1), PTA-ELISA as described by (3) western blot (4) and ISEM-D was carried out as described by (5).The virus was tested against polyclonal antisera to *Asparagus* virus-1(AV-1) soybean mosaic virus (SoyMV), watermelon

mosaic virus-2 (WMV-2), bean common mosaic virus (BCMV), bean yellow mosaic virus (BYMV), maize dwarf mosaic virus (MDMV), plum pox virus (PPV), potato virus V (PVV), leek yellow stripe virus (LYSV), turnip yellow mosaic virus (TYMV) and pea seed-borne mosaic virus (PsbMV) and with group specific monoclonal antibody P-3-3H8. ELISA readings were considered positive if values exceeded twice the values for healthy controls (6)

Results

*Host range and symtomatology*The virus had a rather narrow host range infecting a few species in Amaranthaceae, Chenopodiaceae and Solanaceae families. *Chenopodium amaranticolor*, *C. quinoa*, *C. murale*, *C. rubrum* and *C. urbicum* were local lesion hosts. Beside local lesion induction, *C. quinoa* and *C. amaranticolor* were systemically infected by the virus. Other susceptible plants were *C. foetidum*, *Celosia argentea*, *N. bentamiana*, *N. clevilandii and N. occidentalis* showing symptoms ranging from mild mottle, vein-yellowing, green vein-banding, mosaic to leaf malformation (figs. 1a - c)

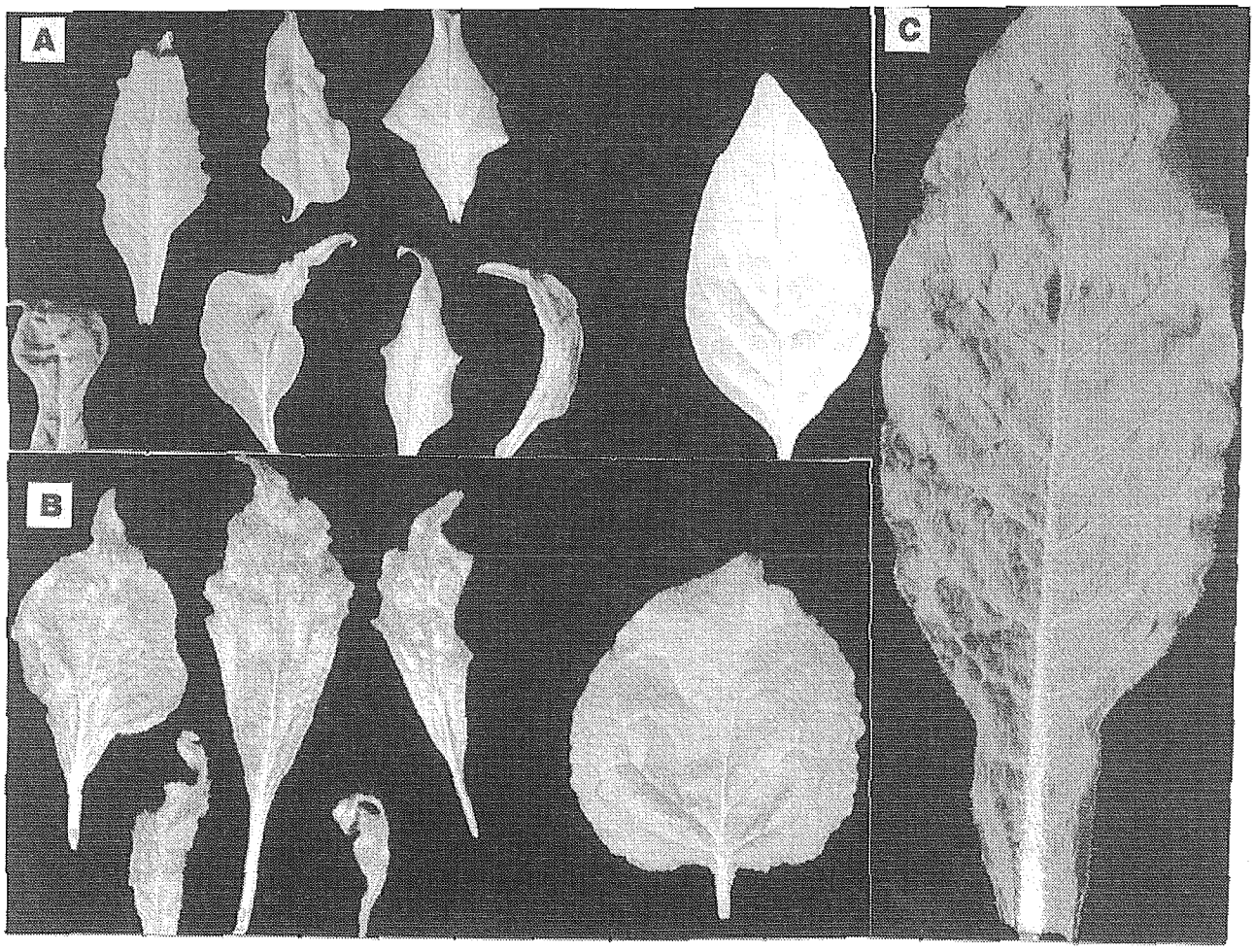

Fig. 1. Various forms of leaf malformation induced on *Celosia argentea* (A), *Nicotiana benthamiana* (B) and mosaic on *N. occidentalis* when mechanically inoculated with Celosia virus.

Purification and electron microscopy

The purification procedure described above yielded pure purified preparation. Flexuous rod-shaped particles were observed when examined under JEOL JEM 100 electron microscope (fig.2).

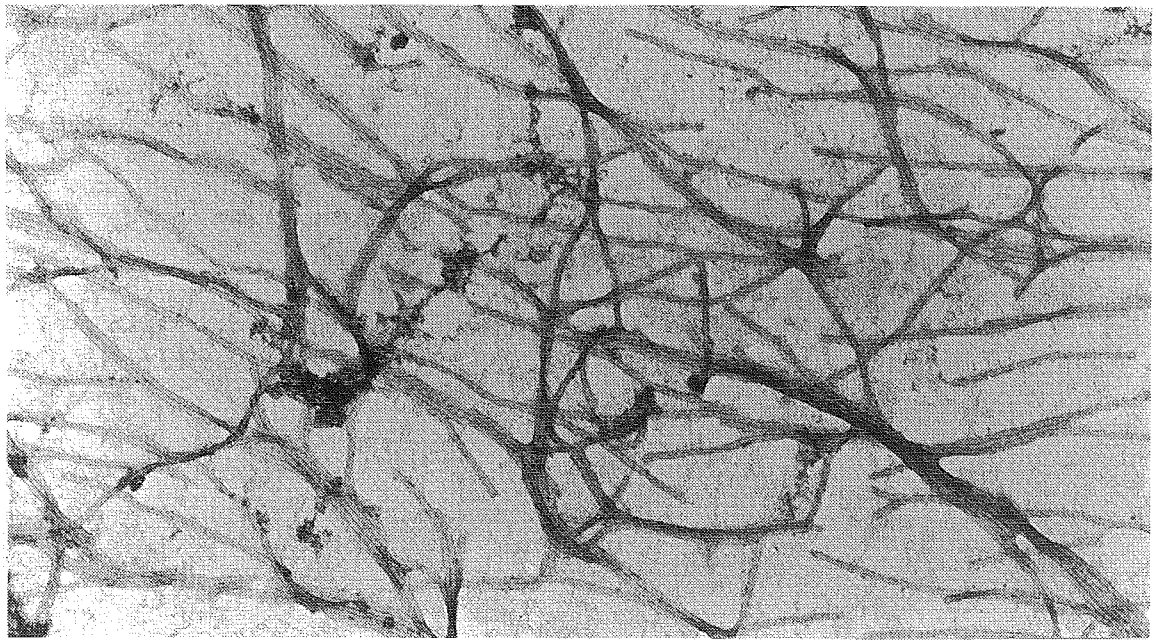

Fig. 2. Electron micrograph of purified preparation of Celosia virus showing flexuous rod-shaped particles (x 28,900).

Serology

The virus reacted positively with the potyvirus group specific monoclonal antibody P-3-3H8. The results with polyclonal antibodies tested are summarized in table 1.

Table 1: Serological reaction of *Celosia* virus with antisera to some potyviruses

	Serological tests			
Virus antisera	DAS	PTA	western blot	ISEM-D
AV-1	+	+	+	+
BCMV	-	-	+	-
BYMV	-	-	-	-
MDMV	-	+	NS	+
LYSV	NS	NS	NS	-
SoyMV	-	-	-	(+)
PPV	-	-	+	-
PVV	-	-	-	-
TuMV*	-	+	+	-
WMV-2	-	+	-	-

\+ Positive reaction - reaction not positive
NS not tested (+) Weak decoration of particle. * broad reacting antiserum (4)

Insect transmission test

Attempts to transmit the virus by *M. persicae, A. crassivora, A. fabae, Brevicoryne brassicae* and *R. padi* were not successful as non of the plants inoculated developed symtoms after 4 weeks of observation.

Discussion

The restricted host range of the *Celosia* virus, the flexuous rod-shaped particles and its positive reaction with the potyvirus group specific monoclonal antibody P-3-3H8 indicate its membership of the potyviridea family. Non of the aphid species tested was able to transmit the virus. The results of immunological studies showed that the virus is serologically related to AV-1, TuMV, MDMV-2, PVV. It seems, however, that the *Celosia* virus is more closely related to AV-1 than to the other viruses tested as there was consistency of reactivity of its antiserum with the virus in all the serological tests. The cross reactivity of the antisera to these viruses with the *Celosia* virus resulted probably because their induction was directed against the core regions of their coat proteins (CPs) which for most potyviruses have been shown to possess extensive sequence homology (7). Antiserum produced against this region have demonstrated to detect most potyviruses differing in biological properties (4, 7, 8, 9). The relationship of the *Celosia* virus with AV-1 requires further clarification as there indications that both viruses are not one and the same virus despite the positive reation observed in DAS-ELISA.

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PROBLEMS IN EVALUATION OF APRICOTS AND PEACHES FOR RESISTANCE TO PLUM POX VIRUSES

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Introduction

Breeding of cultivars of plums, apricots and peaches resistant to plum pox virus (PPV) is of basic importance for countries and areas with surface distribution of sharka disease. PPV is not possible to eliminate by production and growing of healthy trees of susceptible cultivars. PPV is transmitted by aphids and trees become quickly reinfected. In countries with most damaged fruit tree cultures breeding of resistant or tolerant cultivars to PPV started: in early sixties breeding of plums in Yugoslavia and Bulgaria whereas breeding of apricots and peaches in the middle of seventies in Greece. Resistance of apricot and nectarine cultivars was verified in conditions of spontaneous and artificial PPV infection (1), (2). Systematic approach on investigation of resistance to PPV was developed in France. Since the beginning of nineties investigators in France used for PPV transmissions aphid vectors and for the tests on resistance of cultivars a method of budding of susceptible peach variety GF-305 (3), (4).

Several cultivars (Stark Early Orange, Stella, Sunglo, Veecot, Harlayne, Goldrich, Henderson) of apricots were proved in Greece and France as resistant to PPV. On the other hand no peach cultivar with higher level of resistance to PPV was reported. Rankovic' and Šutic'(5) showed the low reliability of PPV detection by ELISA in artificially infected peach trees during summer.

We tried to verify the level of resistance of several mentioned apricot cultivars and to recommend apricot and peach cultivars with highest level of resistance for breeding. Our aim is to develop fast and reliable method for evaluation of resistance of apricots and peaches to PPV. The optimal time for detection of PPV in trees and its parts, leaves, flowers, fruits as well as suitable method for determination of PPV concentration in plant tissue is necessary to establish for this purpose.

Material and Methods

Plant material and infection

Stark Early Orange, Marii de Cenad, Harlayne cultivars of apricots evaluated in Greece and France as resistant to PPV and several other cultivars supposed to be resistant to PPV were grafted onto five years old apricot trees cv. Vegama susceptible to PPV being naturally infected with PPV and showing severe symptoms on leaves. PPV symptoms on resistant cultivars were evaluated in the year of grafting and in the next two years.

PPV naturally infected cultivars of peaches grown in orchard were used for evaluation of resistance to PPV. PPV was easily and reliably detected in flowers from the beginning to the end of flowering and in leaves during May (6).

Determination of relative concentration of PPV by ELISA

The determination relative concentration of PPV by ELISA in flowers and leaves of apricots and peaches was used for evaluation of resistance. PPV titer was established as dilution of investigated sample with last positive ELISA value.
Commercial or own prepared PPV antiserum was used in DAS-ELISA. Samples were prepared by grinding of 0,2 g of leaf or flower tissue in 4 ml of phosphate buffered saline-PBS pH 7,4 with 2 % of Polyvinylpyrrolidon - PVP and 0,2 % of egg albumin. ELISA value was considered positive when the mean absorbance at 405 nm was at least 0.04 (approx. three standard deviation units above negative control). Five branches of investigated apricot cultivar grafted on cv. Vegama or five PPV infected trees of investigated peach cultivar were used for determination of relative concentration of PPV. Petals from five flowers or five leaves from different sites of a branch or a tree were taken for average sample.

Results and Discussion

Apricot cultivar Veecot showed PPV symptoms already in the year of grafting. In the second year, PPV was detected by ELISA in cultivar Stark Early Orange but in very low concentration. PPV was not detected in cultivars Harlayne and Marii de Cenad. Results of three years evaluation of resistance in several apricot cultivars to PPV are given in Table 1. We proved that some of cultivars of apricot presented in literature as resistant are not fully resistant to PPV. No symptoms were observed and no PPV was detected in cultivar Harlayne and Marii de Cenad which could be considered as immune to PPV. Lower concentration of PPV was detected in flowers of cultivar Leronda in the third year after grafting. Cultivars Stark Early Orange, San Castrese and Harval showed very mild PPV symptoms in the second year after grafting and their leaves exhibited low concentration of PPV. Other tested cultivars, Mai Chua Sin and Chuang Zhi Hong without symptoms in the year of grafting developed PPV symptoms in the second year after grafting and the virus was proved by ELISA in leaves in the second year and in flowers in the third year in higher concentration. These cultivars were classified as medium susceptible. Cultivar Vegama with regard to symptoms and virus concentration in leaves and flowers was evaluated as very susceptible. The optimal time for PPV detection in apricot trees is during flowering in petals or in June and July in leaves. The relative concentration of PPV in flowers and in leaves of apricots is similar.

Peach trees infected with PPV strains occurring in the Czech Republic expressed during short period after flowering (May, June) almost no symptoms on leaves or vein clearing and mild

mosaic only. Determination of relative concentration of PPV by ELISA is therefore very important for evaluation of resistance from that point of view. We already proved the highest concentration of PPV in flowers of peaches (6).

We proved differences among PPV naturally infected peach cultivars in relative concentration of PPV determined by ELISA. These differences are supposed to be in relation to variability of resistance to PPV among peach cultivars. We devided peach cultivars with regard to the relative concentration of PPV in flowers into three groups. Peach cultivars Harmony, Envoy, Favorita Moretini 3 and NJC 102 with lowest concentration of PPV (5.10^{-2}; $2,5.10^{-2}$) were preliminary classified medium resistant. Cultivars Suncrest, Harcrest, Sunhaven, Springrest, Harson, Veteran and Canadien Harmony ($6,25.10^{-3}$; $3,12.10^{-3}$) were classified medium susceptible. Cultivars Harbinger, Harbrite, Harken, Redhaven, Rosired 1, peach seedling (6) and Maria Emilia ($1,56.10^{-3}$; $7,81.10^{-4}$) were classified susceptible to PPV. Leaf symptoms of PPV were observed on the all classified cultivars. Intensity of symptoms is in correlation with the relative concentration of PPV in most cultivars. The mildest symptoms were observed on leaves of cvs. Harmony, Envoy, NJC 102 and Favorita Moretini 3. Symptoms of vein clearing appeared on the first or second developed leaf of some branches only. Mosaic symptoms appeared also on the third or forth leaf in case of cultivars classified as susceptible to PPV. No symptoms were observed in the middle of May on later developed leaves.

Our results indicating low relative concentration of PPV in peach cultivars Envoy and Favorita Moretini 3 correspond to the observations of the Czech breeder Mr. Oukropec (personal communication) who find no leaf symptoms and no or very mild fruit symptoms on those peach cultivars artificially infected with PPV. Further studies are necessary to make evaluation of resistance to PPV in peach cultivars precise.

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Table 1

Evaluation of resistance of apricot cultivars to plum pox virus on infected cultivar Vegama

Cultivar	Symptoms on leaves			Relative concentration of PPV by ELISA		Evaluation
	In the year of grafting	2nd year after grafting (20.6.94)	3rd year after grafting (9.5.95)	2nd year after grafting in leaves	3rd year after grafting in flowers	
Stark Early Orange	none	very mild mosaic	very mild mosaic	5.10^{-2}	?	resistant
Marii de Cenad	none	none	none	0	0	immune
Harlayne	none	none	none	0	0	immune
San Castrese	none	mild oak mosaic	mild oak mosaic	5.10^{-2}	0 $3,12.10^{-3}$ (in leaves)	medium resistant
Harval	none	oak mosaic	none	$1,56.10^{-3}$	5.10^{-2}	medium resistant
Mai Chua Sin	none	oak mosaic	mild oak mosaic	$1,56.10^{-3}$	$1,56.10^{-3}$	medium susceptible
Chuang Zhi Hong	none	oak mosaic, vein banding	mild oak mosaic	$2,5.10^{-2}$	$2,5.10^{-2}$	medium susceptible
Leronda	none	none	none	0	$1,25.10^{-2}$	resistant
Vegama	severe diffuse spots, oak mosaic	oak mosaic, diffuse spots	oak mosaic, diffuse spots	$7,81.10^{-4}$	$3,9.10^{-4}$	very susceptible

Table 2
Preliminary evaluation of susceptibility of peach cultivars to plum pox virus

Cultivar	Symptoms of leaves	Relative concentration of PPV in flowers by ELISA	Evaluation
Harmony	occidentally vein clearing	5.10^{-2}	medium resistant
Envoy	mild vein clearing	$2,5.10^{-2}$	
Favorita Moretini 3	mild vein clearing, mosaic	$2,5.10^{-2}$	
NJC 102	mild vein clearing	$2,5.10^{-2}$	
Suncrest	mild vein clearing, mild oak mosaic	$6,25.10^{-3}$	medium susceptible
Harcrest	vein clearing, interveinal mosaic	$6,25.10^{-3}$	
Sunhaven	vein clearing, interveinal mosaic	$6,25.10^{-3}$	
Springrest	vein clearing, oak mosaic	$3,12.10^{-3}$	
Harson	vein clearing, mild interveinal mosaic	$3,12.10^{-3}$	
Veteran	vein clearing, oak mosaic	$3,12.10^{-3}$	
Canadien Harmony	mild vein clearing, mosaic	$3,12.10^{-3}$	
Harbinger	severe vein clearing, mosaic	$1,56.10^{-3}$	susceptible
Harbrite	vein clearing, ring and oak mosaic	$1,56.10^{-3}$	
Harken	vein clearing, occidentally inter-veinal mosaic	$1,56.10^{-3}$	
Redhaven	vein clearing	$1,56.10^{-3}$	
Rosired 1	mild vein clearing, mosaic	$1,56.10^{-3}$	
peach seedling (Polák,1995)	vein clearing, mosaic	$1,56.10^{-3}$	
Maria Emilia	veinal mosaic, brittleness, thickeness, and leaf rolling	$7,81.10^{-4}$	

CHARACTERIZATION OF THE INFECTIOUS *in vitro* TRANSCRIPTS FROM CLONED cDNA OF THE POTATO A POTYVIRUS

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INTRODUCTION

PVA is a member of the potyvirus group. It has an RNA genome and filamentous and flexuous particles about 730 nm long and 15 nm in diameter, which consists of a single repeated coat protein with estimated M_r of 30257 (1). The genome of PVA is monopartite, and consists of a single-stranded, positive-sense RNA molecule of 9565 nucleotides with a 5'-terminal genome linked protein (Vpg) and a poly(A) at its 3'-end (2). PVA is widely distributed in potato growing areas and can decrease the yield of infected potato plants up to 40%.

The complete nucleotide sequence of (at least) ten different potyviruses has been documented: tobacco etch virus (TEV), tobacco vein mottling virus (TVMV), plum pox virus (PPV), the necrotic strain of potato virus Y (PVY^N), pepper mottle virus (PepMoV), papaya ringspot virus (PRSV), turnip mosaic potyvirus (TuMV), pea seed-borne mosaic virus (PSbMV), soybean mosaic virus (SbMV) and potato virus A (PVA) (2). The sequence analysis of potyviruses and *in vitro* translation studies of potyvirus genomic RNAs have revealed a single open ORF encoding a large polyprotein. This polyprotein is proteolytically processed into functionally active viral proteins by virus-encoded proteases. Sequence similarities between the potyviruses analysed indicate that their polyproteins are cleaved at similar sites, yielding eight virus-encoded proteins in each. The potyviruses also contain conserved protein motifs, which may have functional importance for all members of the potyvirus group.

The *in vitro* synthesis of biologically active RNAs from full-length cDNA clones have been reported for seven (at least) potyviruses: TVMV, PPV, zucchini yellow mosaic virus (ZYMV), TEV, PRSV, SbMV and PSbMV. Generation of infectious virus from cloned cDNA of a potyvirus opens the possibility to study their biological functions

at the molecular level. Events of the virus life cycle such as replication and movement and the functions of gene products such as coat protein and helper component have been tested *in vivo* using infectious potyvirus cDNAs (3-5).

MATERIALS AND METHODS

PVA B11 was purified from *Nicotiana occidentalis*. RNA was extracted after incubation with 1% SDS for 5 min at room temperature, followed by two extractions with phenol/chloroform and one with chloroform. RNA was precipitated with ethanol.
Two types of cDNAs were synthesized, one with oligo(dT) primers and the other with random primers. Both cDNAs were cloned in lambda gt11 vector using *Eco*RI adaptors. At least 1000 clones were immunoscreened using commercial alkaline phosphatase-conjugated sheep polyclonal antibodies to PVA. Clone A-ASL dT0.1 was selected for further analysis. The subsequent clones were found by hybridization with the 5'-end DNA fragment of the previous clone. Five independent clones were chosen and were subcloned into plasmid vectors. Nested unidirectional deletions with exonuclease III were generated from both ends. The sequences were determined from both strands using the dideoxynucleotide chain-dermination method manually or automatically, or by both methods. The overlapped sequences were combined. The 5'-end terminal sequence was determined using PCR (2).
Full-length cDNA copy of PVA B11 RNA was constructed downstream from bacteriophage T7 RNA polymerase promoter. Because T7 RNA polymerase initiates transcription primarily at guanosine residues and because PVA RNA begins with an adenosine residue, a single extra guanosine residue not present in PVA RNA was added to the 5'-end by using polymerase chain reaction (PCR) and an oligonucleotide which was designed to create *in vitro* mutagenesis at the 5'-end of the PVA cDNA clone. The 5'-end primer contained an *Sph*I recognition site, a bacteriophage T7 RNA polymerase promoter, an extra guanosine residue and the first 25 nucleotides of the PVA sequence. The opposite primer was complementary to nucleotides 192 to 211 of the PVA sequence. The amplified DNA product was cut with *Eco*RI and *Sph*I, cloned and sequenced. The cloning of this *Eco*RI-*Sph*I fragment (213 bp) was the initial step in the construction of full-length cDNA copy of PVA RNA. The other clones used for the construction of the full-length cDNA were those ones which were used to obtain the complete nucleotide sequence of PVA. Fragments from overlapping clones sharing common restriction endonuclease sites were joined by ligation. A *Pin*AI site was introduced at the end of the poly(A) tail of the PVA 3'-end region by oligonucleotide-directed mutagenesis. The resulted plasmid was named pPVAcDNA1 (6).
The *in vitro* transcripts were made from *Sph*I-*Pin*AI linearized pPVAcDNA1 plasmid. *Nicotiana tabacum* mesophyll protoplasts were inoculated by electroporation with 100 µl of *in vitro* transcription mixture. After incubation of the protoplasts for 72 hr at

room temperature, extracts were assayed for PVA coat protein both by immunoblot assay and ELISA.
Carborundum-dusted leaves of tobacco plants were inoculated with an inoculum prepared by mixing 100 µl of *in vitro* transcription reaction mixture with 100 µl phosphate-buffered saline (PBS, pH 8.0). One leaf of each plant was mechanically inoculated with 100 µl of inoculum and was rinsed with tapwater 5 minutes after inoculation. Systemically infected leaves were assayed for PVA coat protein both by immunoblot assay and ELISA.

RESULTS AND DISCUSSION

Full-length cDNA copy of PVA RNA was constructed downstream from bacteriophage T7 RNA polymerase promoter. Introduction of capped transcripts from the cDNA or PVA RNA into tobacco mesophyll protoplasts resulted in the accumulation of PVA coat protein, whereas no accumulation of coat protein was observed in protoplasts inoculated with linearized cDNA, or uncapped RNA transcripts. PVA was detected by ELISA in 13 of the total of 16 plants inoculated with the capped *in vitro* transcripts in three experiments (6). Western analysis revealed coat protein, which was of identical size compared to the coat protein derived from plants infected with parental virus (6).
Current evidence suggests that successful transmission of potyviruses by their aphid vectors depends upon the interaction of two potyviral proteins, the coat protein and helper component. A triplet of amino acids, DAG, is conserved near the N-terminus of the coat protein of aphid-transmissible potyvirus isolates, and site-directed mutagenesis has shown that certain substitutions in the DAG region result in loss or drastic reduction in aphid transmissibility of TVMV (7). PVA strain B11 is a non-aphid-transmissible isolate, and has the DAS sequence at the N-terminal region of the coat protein (1,2). Using site-directed mutagenesis and infectious cDNA of PVA B11 we have shown that a single change of Ser to Gly in the DAS motif of the PVA B11 coat protein converts the non-aphid transmissible PVA isolate B11 into aphid-transmissible Puurand and Rabenstein, unpublished).

CONCLUDING REMARKS

The complete nucleotide sequence of potato virus A (PVA) was determined from six independent cDNA clones. The RNA genome of PVA is 9565 nucleotides long and contains one open reading frame (ORF) of 9177 bases encoding a large polyprotein of 3059 amino acids with calculated M_r of 340 kDa. Five potential proteinase NIa and one HC-pro, as well as one P1 proteinase recognition sites were found in PVA polyprotein by searching for cleavage site consensus sequences in the potyvirus group. The non-coding region preceding the ORF is 161 nucleotides long, has a very

high A content and very low G content. The termination codon is followed by a 227 nucleotide sequence, which is A/U rich (59%), and the poly(A) tail. Overall nucleotide sequence homology compared to several completely sequenced potyvirus genomes is about 53-58%, with overall amono acid sequence homology at about 65-71%. Potyviruses show relatively little homology to each other in their 5'- and 3'-untranslated regions (UTR). When the individual proteins of PVA were compared to the corresponding proteins of other potyviruses, P1 and P3 appeared at least conserved while the other proteins were in most cases from 65-80% homologous to each other.

Full-length cDNA copy of PVA RNA was constructed downstream from bacteriophage T7 RNA polymerase promoter. For technical reasons a single extra guanosine residue not present in PVA RNA was added to the 5'-end. The capped *in vitro* transcripts from cloned cDNA were infectious in the mesophyll protoplasts and intact plants of *Nicotiana tabacum*. PVA coat protein accumulated in transcript-inoculated tobacco protoplasts and plants according to the ELISA and immunoblot assay.

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DETECTION OF A SECOND DISTINCT STRAIN OF BEET WESTERN YELLOWS LUTEOVIRUS IN OILSEED RAPE USING MONOCLONAL ANTIBODIES

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Introduction

Beet western yellows luteovirus (BWYV) is commonly found in brassica crops such as oilseed rape (1). It is very closely related to beet mild yellowing luteovirus (BMYV), which is beside beet yellows closterovirus the main cause of yellowing of sugar beet in Europe. Both viruses (BWYV and BMVY) cannot be serologicaly distinguished using polyclonal antisera.
In order to distinguish BMYV from BWYV, two approaches have been used recently, one involving monoclonal antibodies (MAbs) (2) and the other nucleic acid probes (3), (4).
A MAb PAV-IL 1 produced against barley yellow dwarf luteovirus (BYDV) isolate PAV distinguished BMYV from BWYV (5), and is now used in epidemiological studies (6). Other MAbs did not distinguish between BMYV and BWYV (3),(7).
We describe here studies on the variability among isolates of BWYV collected from rape (*Brassica napus*) using a panel of monoclonal antibodies in triple antibody sandwich (TAS) ELISA.

Materials and Methods

Virus isolates
The isolates used in our studies, which were obtained from different regions of Germany, from Great Britain, France, New Zealand and the USA are listed in table 1.
Two isolates of BWYV (BN 5 ASL and BN LP 2/8) and one isolate of BMYV (2 ITB) were purified (4) and used for further investigations, where known amounts of antigens were necessary.

Transmission studies
Virus transmission studies were carried out as described by GRAICHEN and RABENSTEIN (1). Virus-free cultures of *Myzus persicae* Sulz., which have been continuously maintained on Chinese kale (*Brassica rapa* L. ssp. *pekinensis*) or pepper (*Capsicum annum* L.) were used for transmission experiments.

Antisera, monoclonal antibodies and serological tests
Polyclonal antiserum raised against BWYV and other luteoviruses were from the stock collection of Aschersleben. Eight MAbs against a German isolate of BWYV were produced as described by RABENSTEIN *et al.* (8). Four other MAbs against barley yellow dwarf virus (PAV-IL-1) (5), BWYV (510H) (7), BMYV (MAFF-24) and bean leafroll luteovirus (BLRV-5G4) (9) were provided by Drs C. D'Arcy (Illinois, USA), P. Ellis (Vancouver, Canada), I. Barker (Harpenden, U. K.) and L. Katul (Braunschweig, Germany), respectively.
Polyclonal IgG's and conjugates were used for performing DAS ELISA and western blotting according to standard procedures. MAbs were used in a triple antibody sandwich (TAS) ELISA according to the method described by D'Arcy *et al.* (5). The assay was performed using polyclonal IgG's for trapping virus proteins (purified preparations at the concentration of 500

ng/ml or leaf material diluted five folds) and different MAbs as detecting antibodies. The assay was developed by adding of anti-mouse or anti-rat alkaline phosphate conjugates to detect the MAbs binding to the specific epitopes.

Results and Discussion

The "common" isolate of BWYV, which occurs throughout the rape growing area in Germany, showed strong reaction with MAb G4C10 (Table 1). In no case, we found any reaction of BWYV isolates from rape with MAb BYDV-PAV-IL-1 used in TAS-ELISA. This MAb recognizes most of the BMYV isolates from sugar beet, but never gave a reaction with British isolates from rape (10).
A second serotype of BWYV, which failed to react with MAb G4C10 (Table 1) was identified in different rates of infected oilseed rape plants sampled in Germany and France.

Table 1. Reactivity of 3 distinct antibodies used in DAS- and TAS-ELISA towards different isolates of BWYV and BMYV

Isolates	Host	Geographic origin	polycl. DAS	MAb G4C10 TAS	MAb PAV-IL-1 TAS
BWYV type					
BN 2 EF	oilseed rape	Erfurt, D	+++*	+++	-
BN 5 ASL	oilseed rape	Aschersleben, D	++	+++	-
BN 16 EUT	oilseed rape	Eutingen/Stuttgart, D	++	-	-
BN 17 WH	oilseed rape	Quedlinburg, D	+++	-	-
BN LP 2/8	oilseed rape	Lippstadt, D	+++	-	-
BN LP 3/5	oilseed rape	Lippstadt, D	+++	++	-
BN HL 5/2	oilseed rape	Hohenlieth, D	++	+++	-
BN HL 9/3	oilseed rape	Hohenlieth, D	+++	-	-
BN MA 42	oilseed rape	Malchow/Poe, D	+++	-	-
BN MA 43	oilseed rape	Malchow/Poel, D	++	+++	-
BN Colza	oilseed rape	Colmar, F	++	-	-
BN GB1	oilseed rape	Broom's Barn, GB	++	+++	-
BO SIP	cow cabbage	Siptenfelde, D	++	++	-
BO GEM 8	Brussels sprout	Aschersleben, D	++	++	-
SO NZ	spinach	New Zealand	+++	++	-
BMYV type					
BM 1 ASL	sugar beet	Aschersleben, D	++	-	++
BM 2 GA	sugar beet	Gatersleben, D	+	-	++
BM 3 HL	sugar beet	Hohenlieth, D	+	-	++
BM 4 LB	sugar beet	Lauterbach, D	+	-	++
BM 5 TÜ	sugar beet	Tübingen, D	++	-	++
BM 6 OR	sugar beet	Corvallis, USA	+	-	++
BM 7 J	sugar beet	Colmar, F	+	-	++
BM 8 2ITB	sugar beet	Colmar, F	+	-	++
BM 9 GB	sugar beet	Norwich, GB	++	-	++
BM 10 MA	sugar beet	Malchow/Poe, D	+	-	++

*Reaction after 1 h incubation with substrate: +++ (absorbance value ›1.0). ++ (0.5-1.0), + (0.49-0.01), - (‹0.1).

The reactivity of nine MAbs with oilseed rape (cv. 'Sollux') infected with these two different serotypes of BWYV and with sugar beet infecting BMYV is presented in table 2.

Table 2: Reaction of MAbs in TAS-ELISA with oilseed rape (cv. 'Sollux') infected with two serotypes of BWYV and one isolate of BMYV

	Monoclonal antibodies								
Virus isolates	BWYV G4C10	BWYV 3F4	BWYV HE8	BWYV AG10	BWYV 9A10	BWYV 9B3	BWYV BC-510H	BLRV 5G4	BYDV PAV-IL-1
BN LP 2/8	0.04*	0.62	1.66	0.15	0.37	0.19	1.33	0.26	0.01
BN 5 ASL	1.62	0.49	0.96	0.12	0.25	0.27	0.72	0.40	0.01
BM 2 ASL	0.05	0.35	0.07	0.10	0.36	0.11	0.21	0.38	2.05
healthy controls	0.02	0.03	0.01	0.01	0.01	0.02	0.05	0.06	0.01

* Absorbance values were recorded after 1 h.

The binding properties of a panel of MAbs were further characterized by using purified quantified virus preparations of BWYV (BN 5 ASL and LP 2/8), a sugar beet infecting isolate (2-ITB) of BMYV, and German isolates of potato leafroll virus (PLRV ASL) and BYDV (PAV-1 ASL), respectively (Table 3).

Table 3: Reactivity of MAbs in TAS-ELISA with purified antigens (50 ng/well)

	Virus preparations				
Monoclonal antibodies	BWYV BN-5	BWYV LP 2/8	BMYV 2-ITB	PLRV ASL	BYDV PAV-1ASL
BWYV-G4C10	1.36*	0.06	0.02	0.02	0.03
BWYV-3F4	0.46	0.46	0.39	0.28	0.15
BWYV-HE8	1.35	1.38	0.38	0.03	0.03
BWYV-AG10	0.26	0.25	0.29	0.18	0.03
BWYV-AG8	0.39	0.24	0.39	0.46	0.03
BWYV-9A9	0.12	0.11	0.13	0.17	0.09
BWYV-9A10	0.15	0.15	0.16	0.29	0.03
BWYV-9B3	0.24	0.34	0.09	0.35	0.05
BYDV-PAV-IL-1	0.04	0.04	1.75	0.52	2.33
BLRV-5G4	0.27	0.24	0.48	0.16	0.03
BWYV-BC-510H	1.12	1.13	1.07	0.02	0.04
BMYV-MAFF-24	1.09	1.05	1.31	0.02	0.03

* Absorbance values were recorded after 1 h.

BWYV and BMYV reacted both with the non-differentiating MAbs 3F4, HE8, AG10, 9A9, 9B3, BLRV-5G4, BC-510H, and BMYV-MAFF-24 with various absorbance values. Furthermore, some of these common epitopes recognizing MAbs, showed also cross-reactivity with PLRV or BYDV-PAV (Table 3). No reaction of MAb G4C10 generated against a German isolate of BWYV with BWYV-LP 2/8 and the other luteovirus preparations was observed. On the other hand, MAb PAV-IL-1 produced against an American PAV-like isolate of BYDV gave a strong cross-reaction with BMVY-2-ITB and PLRV and failed to react with BWYV purified virions (Table 3).

The results of TAS-ELISA using MAb G4C10 were confirmed by Western blotting experiments and tissue print immunoassay (data not shown).
The host range of BWYV isolate BN LP 2/8 (a G4C10 negative serotype) is now under investigation. Like the "common" BWYV strain, this distinct strain was transmitted in a persistent manner by *M. persicae* to the indicator host plant *Capsella bursa-pastoris* (L.) Medic. and *Nicotiana* species.
The epidemiology of BMYV and BWYV seems to be very complex, involving several vectors, several strains and serotypes, BWYV showing a wider host range than BMYV. The MAbs BYDV-PAV-IL-1 and BWYV-G4C10 have considerable potential for distinguishing between BMYV and BWYV serotypes in epidemiological studies.

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DEVELOPMENT OF HIGH SENSITIVE AND RAPID TEST SYSTEM FOR SIMULTANEOUS QUANTITATIVE AND QUALITATIVE DETECTION OF PLANT VIRUSES

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Introduction

Among of 32 described virus groups a lot of differencies concerning virion shape (rod-shaped, bacilliformic and icosahedral), size and upbuilding, and also genome structure, organization and replication strategy is published. The majority of plant viruses have positive strand (ss)RNA genome, with large variety of replication mechanisms, genome structure and virion architecture. Likewise were described plant viruses with minus (ss)RNA, (ds)RNA, (ss)DNA and (ds)DNA genome (1, 2).

In spite the number of enzyme linked immuno sorbent assays (ELISA based) on effective and sensitive commercial kits for the plant virus detection, using of these systems requieres high amouts of labour, time and laboratory chemicals and plasticware (3). Lot of plant viruses are poorly detected by ELISA methodics, because their coat proteins (CP) reveal very low immunogenic activity or there can occur a large number of serologically different variants. Also many of plant viruses are very labile and number of viruses (*Cryptovirus* group) exist in extremely low concentrations in plant tissues. These reasons make the use of ELISA methodics for the detection of plant viruses limited or even impossible and alternative plant virus tests based on the properties of the nucleic acid should be used: time resolved fluoro-immuno assay (TRFIA), PCR amplification methods, hybridizaton with the labeled probes of the viral genome etc. (3, 4, 5, 6, 7, 8)

The aim of the present investigation is the following: develop methods for the simultaneous quantitative and qualitative detection (SQQD) of plant viruses from plant tissues using modern methods for rapid and efficient purification of nucleic acid macromolecules, followed by very sensitive detection systems (up to 200 pg) of them. Requirement for such kind of test methods is motivated as in field conditions agronomic crops frequently coinfect with some of plant viruses. In experimental plant virology, while investigated plant viruses and their host transactions there, is a need for routine simultaneous control of TMV contamination.

The objects selected for the project are: potato X potexvirus (PVX), potato leafroll luteovirus (PLRV), potato A and Y potyviruses (PVA, PVY), potato M and S carlaviruses (PVM, PVS), cocksfoot mottle sobemovirus (CfMV), and tobacco mosaic tobamovirus (TMV). This selection is based: at first - on our long time experience of handling seven of named viruses, TMV is selected as an extraordinarily stabile virus which is quite often a coinfectional inadvised agent; second - we have purified different isolates of all named above viruses; third - full-length sequences of all of these viruses are published and available.

Material and Methods

Virus preparates

All viruses (CfMV, PVX, PVM, PVS, PLRV, PVY, TMV) is isolated and purified using methodics of Sõber *et al.*, (9), Järvekülg *et al.*, (10), and Andreeva *et al.*, (11).

Oligonucleotides synthezising

Synthetic oligonucleotides antisesnse for PVA RNA genome helper component region and PVX coat protein region were synthezised using Applied Biosystems chemicals, protocols and apparatus.

Biotin labeling of synthetic oligonucleotides

The enzyme terminal deoxynucleotidyl transferase (TdT) (Promega) was been used for labeling with biotin of synthetic oligonucleotides. This procedure is described in the "Nonradioactive Labeling and Detection of Biomolecules" (12). The biotin labeled biotin-14-dUTP (Fermentas) was used in labeling procedures.

Viral RNA separation

For separation of PVA and PVX RNA from the specially prepared mix of multiple viral RNAs (RNAs from PVA, PVM, PVS, PVX, PVY and TMV) experiment protocols, dynabeads and magnetic stand from "Dynal" corporation were used.

Results and Discussion

Our preliminary experimental data show i principally that developed assay is suitable for the separation and following detection for plant viruses, but need more detail optimization procedures. Our data shows that the sensitivity of the method depends exceedingly from RNA quality. It means that a special attention should be put to the standard level of purified RNA.

In the present time there is synthesizing of a panel of oligonucleotides antisense for selected conservative area of viral nucleotide sequences is in progress and parallel optimization for finding out of the best oligos for virus RNA isolation from different virus RNAs mix is carried out. This is the basis for optimization of very fast (up to 30 min) isolation of genomic material of different viruses from crude plant sap *via* annealing to plant viral RNA-s biotinylated oligonucleotides and successive sedimentation by streptavidine coupled paramagnetic particles after that isolated and electrophoretically separated virus derived polynucleotides will be stained for determination (sensitivity 200 pg of RNA).

Anyway our main purpose is to develop high sensitive detection method for routine certification and carantine analyses of plant viruses in different agricultural activities. This SQQD method improved with PCR could also be applied in human healthcare and so for the detection of important cattle infecting diseases such as foot and mouth disease, leukemia and other virus derived infections.

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SUSCEPTIBILITY, RESISTANCE AND TOLERANCE OF POTATO CULTIVARS TO TOBACCO RATTLE TOBRAVIRUS INFECTION AND SPRAING DISEASE

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Introduction

Tobacco rattle tobravirus (TRV) is transmitted by soil-inhabiting *Trichodorus* and *Paratrichodorus* (trichodorid) nematodes, but different trichodorid species are associated with serologically distinct TRV isolates (1,2). The genome of TRV consists of two RNA segments, contained in separate rod-shaped particles. RNA-1 contains genes for putative RNA replicase components and can replicate in infected plants that do not contain RNA-2 . Virus isolates that contain only RNA-1 are common in nature, and are known as NM-type. RNA-2, which contains the particle protein gene, replicates only in plants that also contain RNA-1. Isolates that contain both RNA species are known as M-type, and these are the only ones that produce virus particles (3). The nucleotide sequences of RNA-2 can differ greatly among isolates (4,5), and variation in the particle protein gene gives rise to substantial antigenic diversity (6). Serologically similar isolates are grouped into serotypes.

In potato, TRV is the cause of one type of spraing disease, in which arcs of corky tissue form in the tuber flesh. These symptoms disfigure the tubers and thereby substantially decrease the value of the crop as ware. When planted, some symptom-bearing tubers produce foliage showing symptoms that typically are confined to one or a few of the shoots, which are the only ones to be infected. In British conditions, nearly all plants with these symptoms, known as stem-mottle, contain NM-type isolates (7). Potato cultivars differ in their susceptibility to the disease. Some cultivars do not develop tuber symptoms even when grown in soil containing viruliferous nematodes and are considered resistant to the disease. In some cultivars a small proportion of tubers develop mild symptoms, and some cultivars are more severely affected (8).

We have re-examined the reaction of selected potato cultivars to vector-mediated inoculation with TRV, and have identified some in which infection can occur without the expression of disease symptoms, i.e. they are tolerant of infection. Moreover, infected plants of at least two tolerant cultivars can serve as sources from which trichodorids can acquire the virus for further transmission.

Materials and Methods

Potato inoculation tests

Virus-free stocks of tubers of selected cultivars were planted individually in 12.5cm square pots containing sandy soil from a site at Tentsmuir, near Tayport, Fife. This soil was known to contain a high concentration of viruliferous *Paratrichodorus pachydermus*, able to transmit an isolate of the PRN serotype of TRV . The plants were grown to maturity in a glasshouse at ambient temperatures. After the haulm had died down, the progeny tubers were harvested from each pot and stored in the dark at $4^{o}C$ until required for analysis.

Virus acquisition by nematodes

Progeny tubers of cvs Wilja and King Edward produced by plants exposed to viruliferous trichodorids as above were planted in pots of sterile compost in a glasshouse. Plants in which the presence of TRV was confirmed by ELISA of leaf samples were removed

from their pots and as much of the compost removed as possible without damaging the root system. They were replanted in pots containing soil from Woodhill, Tayside, which contains a virus-free population of *P. pachydermus* (9). After senescence of the foliage, the nematodes were recovered from the soil and tested for their ability to transmit TRV to *Petunia hybrida* bait plants (9).

Virus detection methods

Infectivity tests. Tissue was either ground up with water in a mortar, or extracted with phenol. The infectivity of extracts was tested by manual inoculation of corundum-dusted *Chenopodium amaranticolor*.

Polymerase chain reaction (PCR). Reverse transcription and PCR were used to detect TRV in extracts from leaf tissue (10). Tests on RNA extracted from dormant tuber tissue (11) were similar except that 27 PCR cycles were done with an annealing temperature of 60^{o}C.

Results

Reactions of potato cultivars to infection

Progeny tubers from each of 13 cvs. grown in soil containing viruliferous nematodes were cut and examined for spraing symptoms, and extracts were made for virus detection by PCR and/or infectivity tests. Cultivars were chosen to include ones that are considered moderately resistant to spraing disease (NIAB ratings 5, 6 or 7) (12), together with some that are considered very resistant (NIAB rating 9) or sensitive (NIAB rating 1 or 2). On the basis of the results, the cvs. could be divided into three groups (Table 1).Spraing symptoms were not observed in cvs. in group 1, and virus was not detected in them. Most of the cultivars in this group are rated as highly resistant, and the results tend to confirm these ratings. The cultivars in group 2 are those rated susceptible to spraing disease and about half the tubers examined showed spraing symptoms and contained detectable amounts of virus. Typical spraing symptoms were not observed in any tubers of the cvs. in group 3, although some contained a few brown flecks. However, virus was detected in a proportion of the symptomless tubers, ranging from 20% of Home Guard to 60% of King Edward. The cultivars in group 3 are rated as moderately resistant to the disease, but our results suggest that they are susceptible to infection with TRV although they rarely show symptoms of spraing, i.e. they are tolerant of infection.

Table 1

Group 1 (Resistant)	Group 2 (Sensitive)	Group 3 (Tolerant)
Arran Pilot (9)*	Pentland Dell (1)	Wilja (5)
Bintje (7)	Maris Bard (2)	Romano (7)
Record (9)		King Edward (6)
Saturna (7)		Sante (6)
Fronika (9)		Arran Consul (6)
		Home Guard (7)

* Numbers in brackets are NIAB ratings for resistance to spraing disease (12)

Propagation from symptomlessly-infected tubers

When TRV-infected but symptomless Wilja tubers from the previous experiment were planted in sterile compost in the following season, transient yellow chevron symptoms were observed on leaflets on many of the plants and virus was detected in leaves, roots and daughter tubers. Moreover, when sap extracted from leaves was subjected to two cycles of freezing and thawing, it remained infective, and TRV nucleoprotein particles were observed by electron microscopy in extracts of the inoculated test plants. Thus, the virus isolates in these Wilja plants were M-type. This systemic infection was perpetuated through two further generations of tuber propagation.

M-type TRV was also detected by ELISA in leaves of plants of cvs King Edward, Home Guard and Romano grown from symptomless but infected tubers, suggesting that in these cvs too the infection could be fully systemic.

Potatoes as sources for virus acquisition by nematodes

When virus-free *P pachydermus* were allowed access to systemically infected cv. Wilja or King Edward plants grown from symptomless tubers, the nematodes recovered from 8 out of 21 pots containing cv. Wilja and 6 out of 25 containing cv. King Edward transmitted the virus to *P. hybrida* bait plants. No virus was transmitted by nematodes that had been allowed access to healthy control potato plants.

Discussion

Assessment of the susceptibility of potato cvs. to spraing disease is routinely done by growing tubers in soil known to contain viruliferous nematodes, either in the field or in pots in the glasshouse (13), and examining them for symptoms. The classical work, which demonstrated the causal relationship between TRV infection and disease incidence, concentrated on the detection of virus in symptom-bearing tubers (14,15). However, our tests show that symptomless infection occurs readily in some cvs.; these cvs. should be regarded as tolerant of infection. They include at least one cv., King Edward, which was previously recognized as having a low incidence and producing only mild symptoms of spraing (8). Interestingly, in plants grown from symptomlessly-infected tubers, the infection was with an M-type isolate, was perpetuated through four generations and was fully systemic. This is in contrast to the isolation of predominantly NM-type isolates from symptom-bearing tubers (16), and their limitation to one or a few stems and tubers in plants produced from such tubers.

From our limited tests with pot grown plants, we cannot predict the frequency with which symptomless infection might occur under field conditions. However, the results do indicate that, in addition to the recognized spraing-sensitive and spraing-resistant categories, there is a third group of TRV-tolerant cultivars. So far, we have only examined the reaction of cvs. to infection with one isolate of TRV, and they may behave differently to challenge with other isolates. From a practical breeding point of view, the results suggest that resistance or susceptibility to spraing disease may involve both genes that confer immunity to virus infection and genes that determine whether or not symptoms are produced in tubers of non-immune cvs. Symptom production may be an expression of a form of resistance to virus multiplication.

It has been considered that planting spraing-affected tubers is unlikely to introduce TRV to a virus-free site even though trichodorids are present (17). However, we have shown that tubers of two tolerant cvs., infected with an M-type isolate, can serve as sources from

which nematodes can acquire virus, and thus may be an important means of dissemination of the virus to new sites.

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MOVEMENT OF BYMOVIRUSES

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Introduction

Cereal viruses such as barley yellow mosaic virus (BaYMV), barley mild mosaic virus (BaMMV) or wheat spindle streak mosaic virus (WSSMV) are of increasing agronomic importance in many European and East Asian countries with yield losses of 30-70 % (1), (2), (3). All are members of the bymovirus group which was recently proposed to be a subgroup of the Potyviridae. Symptoms consist of yellow stripes and spots on the leaves. Infected cells usually contain rod-shaped virus particles and several inclusions such as crystal-like cytoplasmic inclusions and pinwheel structures that are typically found for potyviruses. However, unlike potyviruses, but similar to furoviruses, bymoviruses are transmitted by the soil-borne fungus *Polymyxa graminis* and their genome comprises two positive sense single-stranded RNAs.
In order to better understand the processes that damage infected plants and cause yield losses, we studied the movement of bymoviruses in several winter barley and wheat cultivars. Inoculation experiments carried out with BaMMV on roots (inoculation with viruliferous zoospores of *Polymyxa graminis*) and on leaves (mechanical inoculation) suggested a phloem-based pathway for bymoviruses in the plant (4). In order to obtain more data on other bymoviruses, we examined the symptom development of wheat plants that were mechanically inoculated with WSSMV and of winter barley plants that were inoculated with root powder containing viruliferous resting spores of *Polymyxa graminis*. We present a model for the long-distance movement of bymoviruses and its possible role during the bymovirus infection cycle.

Material and Methods

Plant material and antisera against BaYMV proteins

BaYMV-infected plants were taken from a field in Cologne-Vogelsang, FRG, and WSSMV-infected leaf material was kindly provided by Prof. Signoret (U.F.R. de Biologie et Pathologie Végétale, E.N.S. Agronomique, Montpellier, France).
Antiserum against the capsid protein of BaYMV was taken from a rabbit after immunisation with purified capsid protein that was produced in *Escherichia coli* by using the overexpressing pET3 vector (5) and the ^{arg}tRNA-encoding plasmid pSB161, a derivative of pUBS520 (6). Antiserum against RNA2-encoded proteins of BaYMV (28kD and 70kD) were produced as previously described (7).

Mechanical inoculation

For mechanical inoculation of leaves, plants were grown in sand (5/pot) and inoculated at the 3-leaf stage (2-3 wk after sowing) using an airbrush, as described by Adams *et al.* (8). The leaves were then rinsed with water to remove carborundum and excess inoculum.

Root powder inoculation

Root powder containing viruliferous resting spores of *Polymyxa graminis* was prepared from infected plants from the field by cutting, drying and mortering of the roots until a fine powder was obtained. Seeds were then germinated in pots containing soil mixed with root powder (one table spoon/pot). After two weeks pots with young seedlings were transferred to a cold room and were vernalized for 4-6 weeks at 4 °C.

Assimilation of $^{14}CO_2$

Plants were inoculated with $^{14}CO_2$ by using an air-tight chamber where small parts of several leaves can be kept under controlled gaz conditions. $^{14}CO_2$ was emitted into this chamber using ^{14}C-labelled $NaHCO_3$. Leaf and root samples of 2 cm length were taken at different times after incubation and subsequently dried in an exsikkator. Assimilates were extracted with methanol and aliquots of each sample were measured in a liquid scintillation counter.

Western blot analysis

For the detection of viral proteins by western blot analysis, soluble proteins were extracted by grinding frozen leaf and root samples in PBS-Tween (200-400µl for 100 mg tissue). After centrifugation, 16µl of each supernatant was denatured with 8µl 3x concentrated sample preparation buffer and applied to SDS-polyacrylamide gels (12% w/v) as described by Laemmli (9). Proteins resolved by SDS-PAGE were equilibrated in transfer buffer (48 mM Tris, 39 mM glycine, 0.05% (w/v) SDS, 20% (w/v) methanol) and electrophoretically transferred to a nitrocellulose filter. After 2 h blocking with PBS-Tween containing 5% non-fat milk powder, the filters were incubated with antiserum at 1:600 or 1:1000 in PBS for 1 h and subsequently washed (3 x 10 min) with PBS-Tween. Antibody-binding reactions were visualised by incubating the filter with goat anti-rabbit conjugates that were coupled with peroxidase and by using enhanced chemiluminescence reagents (Amersham).

Results

Symptom development and detection of viral proteins in winter barley after root powder inoculation with BaYMV

Seeds of the susceptible winter barley cultivar Rubina and the resistant cultivar Gaulois were germinated (5 seeds/pot) and inoculated with dried root powder of BaYMV-infected plants from a field. No other virus was detected in the plants that were used to produce the root powder. The number of infected plants could be significantly increased when plants were cut 3 weeks after inoculation (after 1 week of vernalisation). Weekly samples were taken from the youngest leaves of five plants (2 cm of a leaf/sample) and stored at -70°C.
After 6-7 weeks of growing in the greenhouse at 15 °C, symptoms appeared at the lower part of the youngest leaf (compare Fig. 2 B). After that all new growing leaves developed BaYMV symptoms. Antisera directed against viral proteins of BaYMV such as the capsid protein (RNA1) and the 28 kD and the 70 kD protein (both RNA2) were used for Western blot analyses of leaf extracts (data not shown). Positive signals of Western blots correlated with the presence of symptoms and all three proteins were detected in leaves with symptoms at 6-7 weeks after growing in the greenhouse. None of these proteins was detected in leaves or parts of leaves without symptoms. After 50-60 days of growing in the greenhouse stem extension began and the lower part of the youngest leaf was free of symptoms (compare Fig. 2 C). All other new growing leaves including the flag leaf were free of symptoms and no viral protein was detected.

Symptom development and detection of viral proteins in wheat after mechanical inoculation with WSSMV

Wheat plants (cultivar Aramon) were mechanically inoculated at the leaves with WSSMV. 10-14 days after inoculation symptoms appeared at the lower part of the youngest leaf and subsequently all new growing leaves showed symptoms (compare Fig. 2 B). The antisera directed against BaYMV proteins that were mentioned above were also used for the detection of WSSMV. Proteins of similar sizes were detected by Western blot analyses of leaves showing symptoms due to amino acid sequence homologies of BaYMV and WSSMV proteins (10). After stem extension progressed new leaves were free of symptoms as observed for BaMMV and BaYMV (4).

Assimilation of $^{14}CO_2$ at a small area of a winter barley leaf and its spread in the plant

In order to compare the pathway of virus movement after mechanical inoculation with assimilate transport, winter barley plants with three leaves were incubated with $^{14}CO_2$ at the upper part of the third leaf. Leaf and root samples of 2 cm length were collected from eight plants at different times after incubation (5, 10, 30 and 60 min). As depicted in Fig. 1 assimilates moved through the plant at an average speed of 30 cm/h and could be detected in the roots and in the lower part of the second leaf after 60 min.

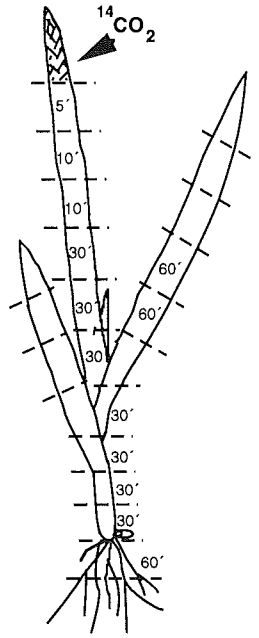

Fig. 1 Scheme for assimilate transport at different times after incubation of the third leaf of a winter barley plant with $^{14}CO_2$. Assimilates could be detected in the roots and in the lower part of the second leaf after 60 min.

Discussion

Symptom development of plants infected with BaYMV (after root powder inoculation) or WSSMV (after mechanical inoculation) was very similar and is comparable to symptom development of BaMMV-infected plants which was described previously (4). It appears therefore that all bymoviruses use the same pathway in the plant and may share a common transport mechanism. Based on the following results it can be assumed that long distance movement of bymoviruses is carried out in the phloem: Most infected cells are situated in and around vascular bundles (7), where chloroplasts are severly damaged and may result in symtoms on the leaves (yellow stripes). Inoculation experiments using BaMMV have shown that movement from leaves to roots is much faster (detection at 5 days in the roots after mechanical inoculation of leaves) than movement from roots to shoots (detection in the leaves at 5-6 weeks after inoculation of roots; (4)). In addition, movement from roots to shoots could be stimulated by treatments that lead to a temporary reversal of the principal phloem flux (vernalization, cold damage or cutting of leaves). Inoculation experiments with a small part of a leaf showed that movement within a leaf is directed downwards and towards new growing leaves and roots. This is comparable to the pathway that took ^{14}C-labelled assimilates in our experiments. However, assimilate transport was much faster (30cm/h) than viral proteins could be detected. Viruses may have to traverse cells and replicate first until a sufficient amount of viral proteins is available for detection. Short distance movement of bymoviruses outside occurs most likely via plasmodesmata from cell to cell. Filamentous structures were found to be associated with plasmodesmata in BaYMV- and WSSMV-infected cells (data not shown).

According to our model two replication cycles may occur in the plant (Fig. 2 A and B). Under natural conditions the pathway of a bymovirus starts in the root hairs after infection with viruliferous zoospores of *Polymyxa graminis*. It can then spread from cell to cell and replicate in other root cells until it is taken up again by new resting spores. This small replication cycle may occur many times during one season. However, in order to reach new secondary roots, viruses have to move close to the apex of the plants. Since many cells have to be traversed for this it is likely that the phloem tissue is used at times of a temporary reversal of the principal phloem flux. Once the apex is reached viruses can also be transported to new growing leaves following the phloem flux. Replication in the leaves may occur in large numbers as assimilates become available. Following the phloem flux, viruses move then rapidly to new growing leaves and to roots (detection within a few days; see above). This second replication cycle may take a bit longer in the beginning (movement from roots to shoots within weeks), but once plants are growing it may contribute to a larger number of viruliferous resting spores. However, more experiments have to be done to confirm this hypothesis.

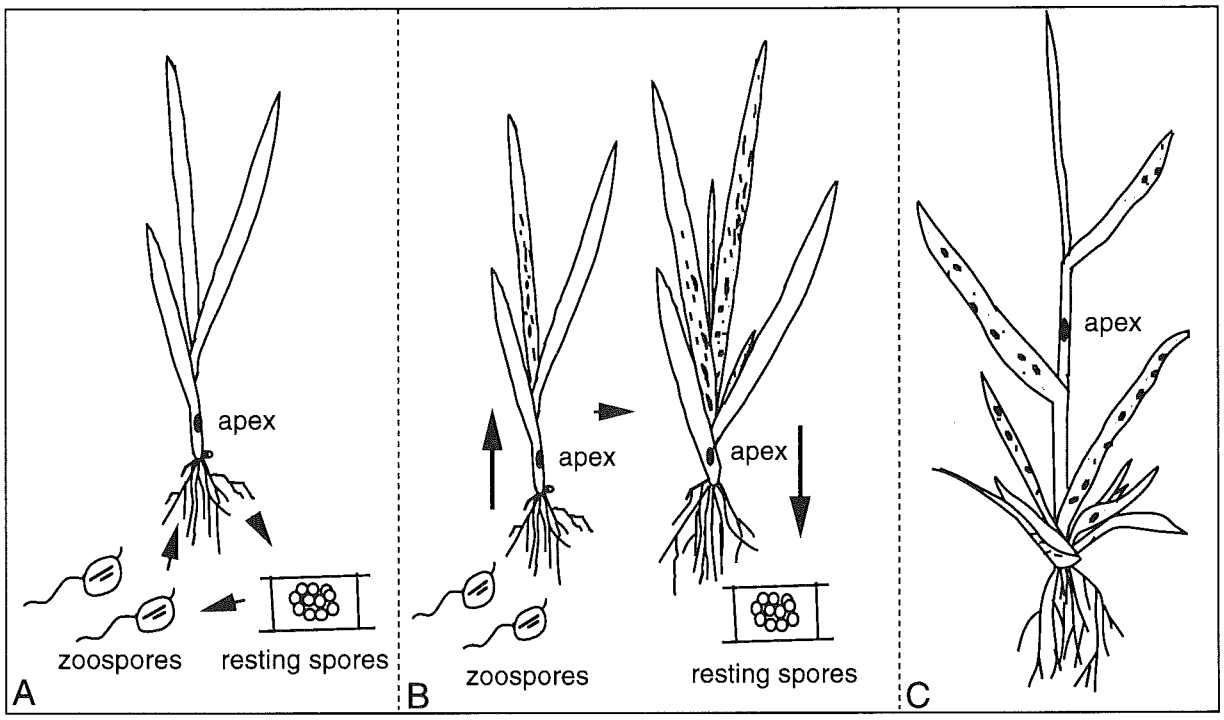

Fig. 2 Different stages of plant development during the bymovirus infection cycle
A Small bymovirus infection cycle
B Large bymovirus infection cycle involving movement to the leaves
B and C Symptom development on leaves at different stages

Once stem extension began and plants have reached a certain size, the apex is located at a higher position in the stem which may be less accessable for viruses (Fig. 2 C). Young leaves are now free of symptoms and no virus could be detected by Western blot analyses. At that stage plant growth may be too fast and distances may be too long for viruses to move to the apex.

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CHARACTERIZATION OF BARLEY GERMPLASMS RESISTANT TO SOIL-BORNE MOSAIC INDUCING VIRUSES BY OLIGO AND PCR FINGERPRINTING

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Introduction

Soil-borne mosaic inducing viruses, i.e., barley mild mosaic virus (BaMMV), barley yellow mosaic virus (BaYMV), and BaYMV-2, cause one of the major diseases of winter barley in Europe. In extensive screening programmes different types of resistance to these soil-borne viruses were observed and different genes conferring resistance at least to BaMMV were identified by segregation analysis. Resistances to these viruses are mainly derived from exotic barley germplasms from East Asia (1), (2) but have also been found in accessions of *Hordeum spontaneum* Koch derived from Turkey (3). Attempts to characterize these exotic germplasms by isozyme-electrophoresis were not very efficient, since the level of polymorphism was rather low within exotic resistant germplasms on the one hand, and in comparison to German commercial cultivars on the other hand (4). Therefore, a characterization of resistant exotic germplasms and resistant genotypes of *H. spontaneum* Koch as well as of resistant and susceptible German cultivars was carried out on the molecular level by generating oligo fingerprints and PCR fingerprints (RAPDs) in order to estimate the degree of genetic similarity between barley varieties resistant to soil-borne mosaic inducing viruses derived from different parts of the world. Preliminary results of theses studies are presented.

Material and Methods

Nineteen barley cultivars (*Hordeum vulgare*) derived from Germany (Alraune, Corona, Gerbel, Brunhild, Colambo, Jana) and East Asia (Kanto Nijo 19, Mihori Hadaka 3, Misato Golden, Mokusekko 3, Muju covered 2, Namji Milyang Native, Ou 1, Res. Ym No. 1, Taihoku A, Chikurin Ibaraki 1, Ea 52, Rokkaku 1, Zairai Rokkaku) as well as five Turkish varieties of *Hordeum spontaneum* (Candarli, Iceneler, Kupalan, Menemen, Pinarbasi) were assayed for their oligo fingerprint and RAPD patterns (Fig.1). Equal quantities of leaf tissue from ten plants of each variety grown in the greenhouse were used for DNA isolation. Isolation of DNA was carried out according to (5).

Oligo-fingerprints: 10µg of genomic DNA were digested with restriction enzymes *Alu*I, *Bcl*II, *Bsp143*I, *Dra*I *Hinf*I, and *Tru*I. After electrophoretical separation in agarose gels, DNA fragments were transferred to nylon membranes and hybridized with the following simple repetitive sequence probes (microsatellite probes): $(GATA)_4$, $(GGAT)_5$, $(ACA)_6$, $(CAT)_6$ and $(GACA)_4$. Detection was carried out non-radioactively by chemiluminescence.

PCR-fingerprints: PCR reaction mixtures (25µl) contained 25ng genomic DNA, 0.4 mM dNTPs, 6mM $MgCl_2$, 0.3µM of a random 10mer primer (Operon Technologies Inc.) and 0.2U

Taq DNA-polymerase (Red Goldstar, Eurogentec Inc.) with the corresponding reaction buffer. The mixture was overlaid with mineral oil and amplification was carried out in a Perkin Elmer DNA thermocycler 480 using the following temperature profile: an initial denaturation step (94°C/4min) was followed by 45 cycles of 94°C/1min, 36°C/1min, 72°C/2min. The polymerization step was extended for 3sec/cycle at 72°C. Fragments were separated on a 2% agarose gel (NuSieve agarose, FMC), stained in ethidium bromide and visualized on an UV-screen (286nm).

Statistical analysis: Oligo and RAPD patterns were scored using the software package RFLP-SCAN. Genetic similarity (GS) between two particular cultivars, *i* and *j*, was estimated according to (6). Principial coordinate analysis and UPGMA cluster analysis was carried out with these data using the software package SPSS (Statistical Package for the Social Sciences).

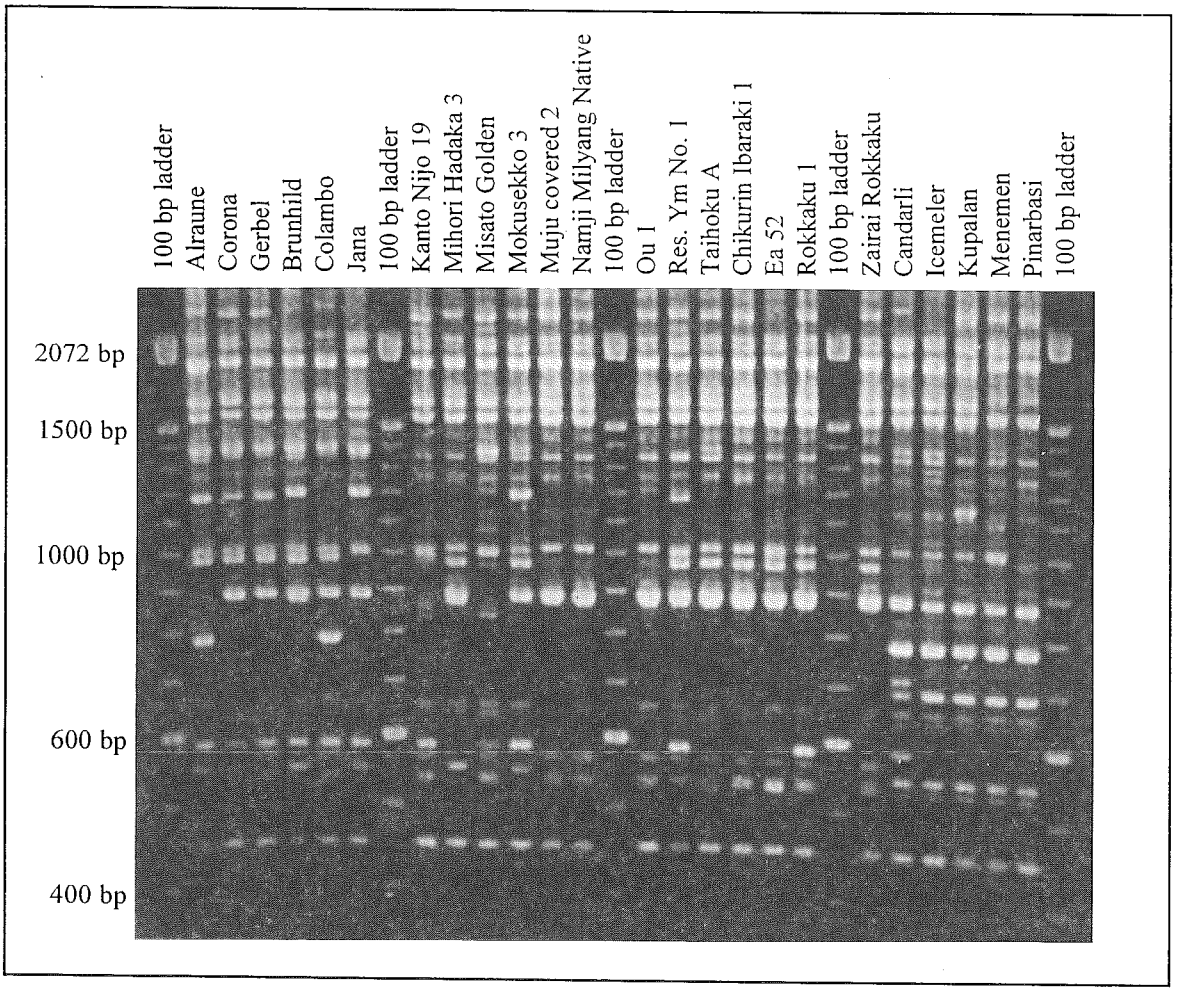

Fig. 1. PCR-fingerprints of susceptible and resistant German winter barley cultivars and exotic barley varieties resistant to yellow mosaic inducing viruses using RAPD-primer OP-K12

Results

Oligo fingerprints: Out of the enzyme/probe combinations tested only $(GATA)_4$ and $(ACA)_6$ revealed clear polymorphism between the varieties studied. However, although the bands are specific for each genotype, the patterns are very complex and extremely difficult to analyze,

therefore. For this reason and due to the fact that only in a very limited number of enzyme/probe combinations clearly recognizable patterns were obtained, no statistical analysis was carried out on oligo-fingerprints.

PCR fingerprints: As can be seen in Figure 1 patterns generated by using RAPD-primers were distinct and polymorphic. Based on the analysis of 15 primers corresponding to 476 bands ranging from 2590 bp to 280 bp the degree of genetic similarity (GS) was estimated in a range of 0.67 to 0.89, revealing a large genetic diversity between barley varieties resistant to yellow mosaic inducing viruses. Associations among the 24 genotypes revealed by principial coordinate analysis are presented in Figure 2. The first and the second principial coordinate (PC 1, PC 2) account for 39.3% and 23.6% of the total variation in RAPD-based GS estimates, respectively. PC 1 clearly separates Turkish varieties of *H. spontaneum* and resistant German cultivars from exotic germplasms derived from East-Asia. It is interesting to note, that Japanese malting barley varieties like 'Misato Golden', 'Kanto Nijo 19' and 'Res. Ym No.1', which derived their malting quality in a way from German spring barley and their virus resistance from 'Mokusekko 3' are more closely related to German cultivars than the other East-Asian varieties. PC 2 clearly separates German resistant cultivars from *H. spontaneum* varieties. Comparable results were obtained by using UPGMA clustering (data not shown).

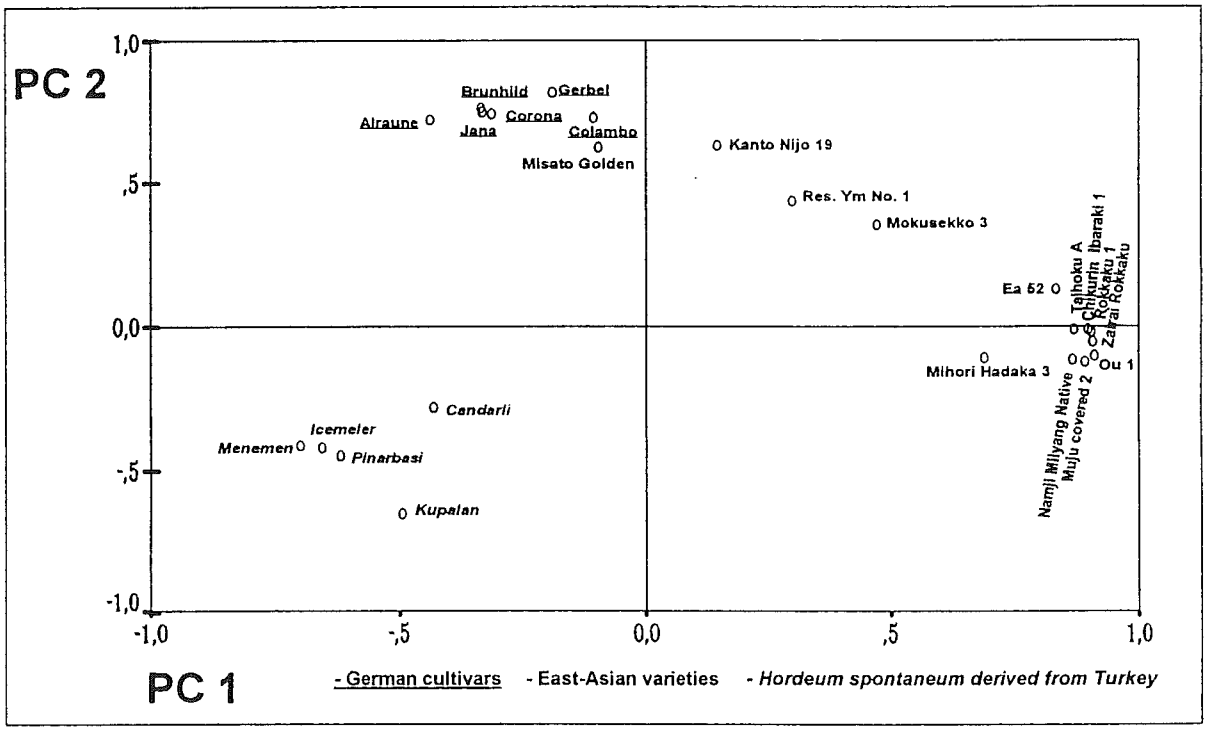

Fig. 2. Association among resistant and susceptible German barley cultivars, resistant varieties derived from East-Asia and resistant accessions of *Hordeum spontaneum* Koch derived from Turkey revealed by principal coordinate analysis performed on genetic similarity (GS) estimates calculated from PCR data of 15 RAPD-primer

Discussion

Although results are preliminary, genetic similarity observed in this study by RAPDs is in the range of those reported for other inbreeding species by RFLPs, e.g. oats (7) and barley (8), elucidating that RAPDs are very well suited to describe genetic diversity on the DNA-level. Concerning resistance to the yellow mosaic inducing viruses it turned out that varieties

derived from the same origin are quite closely related but still different from each other, as expected. Regarding application in practical plant breeding it may be concluded that the rapid introgression of different resistance genes, i.e. combining these genes with those for superior agronomic performance, may be easier by using exotic varieties more closely related to German barley cultivars, e.g. 'Misato Golden'. On the other hand distantly related resistant varieties may be better suited to be used in recurrent selection programmes, in order to exploit their resistance. In further studies resistant varieties derived from the Eastern part of Europe as well as *H. spontaneum* varieties derived from Israel are included.
In the end we intend to provide a detailled genetic characterization of exotic barley germplasms resistant to soil-borne mosaic inducing viruses derived from different parts of the world. This will finally enable the breeder - in combination with information on agronomical traits - to choose different well characterized genotypes in order to incorporate resistance to barley yellow mosaic virus disease into adapted breeding lines of winter barley.

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Detection of leek yellow stripe potyvirus in leek plants using an antiserum obtained against the recombinant coat protein*

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Introduction

Allium species are infected by several viruses which mainly belong to poty- and carlaviruses (14). As they cause, especially in mixed infections, heavy yield and quality losses (4, 1) resistance breeding programs are carried out. For testing resistance specific antisera are required. As viruses of *Allium* species are difficult to separate by differential hosts and local lesion hosts are missing it is problematic to get pure virus isolates and as a conse-quence specific antisera. We tried to solve this problem by molecular biological techniques: the cloned and expressed viral coat protein gene should be used to produce specific antisera. Here we describe the production and application of such antisera for detection of leek yellow stripe potyvirus (LYSV). This virus is widely distributed in leek (*Allium ampeloprasum* L.) and causes considerable yield losses. Donors of resistance seem to be available.

Material and Methods

The virus was purified from field grown leek plants showing symptoms characteristically for LYSV infection by differential and buoyant density gradient centrifugation in CsCl. The final preparation contained flexous, filamentous particles typically for poty- and carlaviruses. From the preparation 3 µg of RNA were isolated. cDNA synthesis with AMV reverse transcriptase was primed with dT_{15}. The ds cDNA was generated with Klenow enzyme and RNAse H, blunted with T_4 DNA polymerase and ligated into pUC 18/Sma I (5). The Plasmid was transformed in *Escherichia coli* XL-2 blue. White colonies were used for hybridization with radioactive labeled cDNA of a glasshouse isolate of LYSV which is thought to be pure. Positive reacting clones were tested for sizes of inserts (11). Two of them were chosen for sequencing. Purification of the fusion protein LYSV-cp/glutathione-S transferase (GST) was performed according to the manufacturers instructions (Pharmacia) by column affinity chromatography. For this purpose an fresh bacterial culture was grown to $OD_{260\,nm}$ 1.0, and the protein synthesis induced by the addition of IPTG to 0,1 mM. After 3 h growth at 37 °C the bacteria were collected by centrifugation and disrupted in 10 ml PBS/l culture medium with an ultrasonifier (Hilscher). After centrifugation the supernatant was used for chromatography. The purity and concentration of the fusion protein was checked electrophoretically by SDS-PAGE (10 % gel) and Coomassie staining. ELISA experiments were performed according to (2) and (9). For Western blotting we used a semidry method (Bio-Rad, 1 h, 10 V), nitrocellulose membranes (Bio-Rad) and buffers according to the manufacturers instructions.

Results

Clone L 1 contained the larger of both sequenced inserts. It consists from 1857 nucleotides excluding the polyadenylated tract. This sequence coded for one large polyprotein which is typically for potyviruses. The none coding region of the sequence amounts to 595 nt. This unusual long noncoding region shows a pronounced secondary structure including repeats and inverted repeats. The deduced amino acid sequence shows a high degree of homology with garlic virus 2 (GV-2) (7) and aphid transmissible potyviruses of the genus *Potyvirus* (6). The

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cDNA of clone L1 represents the whole coat protein (cp) gene and a part of the NIb gene of the virus. The protease cleavage site NIb/cp is probably FVFQ/A what results in a cp of 31,4 kD. This size is in accordance with the size of the cp estimated by Western blots.
To obtain a sufficient amount of cp for immunization of rabbits it was expressed in the overexpression plasmid pGEX4-T (Pharmacia): the insert of L 1 was recloned in this plasmid via some recloning steps so that it was in frame with GST, the gene for the fusion protein of the vector. The cp, including a part of NIb, could be successfully expressed in *E. coli*. In Western blot experiments the protein showed the correct size and reacted with a potyvirus group-specific polyclonal antiserum (AS). The fusion protein was purified by immuno affinity chromatography on glutathione S-Sepharose (Pharmacia) and used for intramuscular and intravenous immunizations of rabbits according to (5). AS with highest titer were obtained from the intramuscularly injected animals. One of the AS (8/2 rec) was used for detection of LYSV in plant material utilizing Western blot and ELISA techniques. In Western blot the AS did not show nonspecific bands (results not shown). It was not possible to produce alkaline phosphatase conjugates with IgG from any of the tested antisera for carrying out DAS-ELISA. In plate trapped (PTA)-ELISA the AS showed high specificity if compared with a potyvirus group specific monoclonal antibody and twoat this time available LYSV antisera. The results are summarized in table 1.

antibodies / viruses (N° of different ones)	MAb 3H8	8/2 rec	V	G
Rymo (4)	only RgMV +	-	n.s.	n.s.
Poty (13)	+	only LYSV +	n.s.	n.s.
Carla (3)	-	-	n.s.	n.s.
Tymo (1)	-	-	n.s.	n.s.
healthy (3)	-	-	n.s.	n.s.

Tab. 1: Reaction of different antisera with viruses belonging to different genera.
+ - positive reaction, - - non reaction, n.s. - nonspecific reaction; MAb 3H8 - group specific monoclonal antibody, detecting most of aphid transmitted potyviruses, 8/2 rec - antiserum against recombinant LYSV cp, V, G - different polyclonal AS, at this time available.

In a first experiment the antiserum was used for the detection of LYSV in material from the collection of *Allium* species of the gene bank Gatersleben using Western blots and in further experiments for resistance evaluations of leek by PTA-ELISA. In the latter experiment a newly produced polyclonal antiserum was included.
By Western blots it was shown that 20 % of the tested field grown accession numbers (n=40) from the gene bank were naturally infected with LYSV. In all these cases a cp of the same size was detected though the degradation products of the cp differed (results not shown). In these experiments we noticed that it is not possible to use frosted plant material for testing as the cp seems to be degraded.
Testing 62 numbers of breeding material for presence of LYSV by PTA-ELISA, 20 were found to by healthy with both antisera. 37 samples reacted positive with both AS but 5 numbers reacted only with the polyclonal antiserum showing high extinction values.

Discussion

According to the criteria developed by Shukla and coworkers (3; 13) GV-2 seems to be a garlic isolate of LYSV: the degree of homology between the cp of both viruses reaches 87 %, for the 3' NTR 93 %. The overall homology of both cp with the cp of PVY (10), the type mem-

ber of the genus *Potyvirus*, is approximately 59 %. That means, that both viruses belong to the genus *Potyvirus*.
Serological differences between GV-2 and LYSV could be explained by some differences in the amino acid sequence of the N-terminal region of the cp of both viruses (fig. 1). At least one of these regions of differences is located at the outer surface of the virus particle and should be involved in its antigenic determinants.

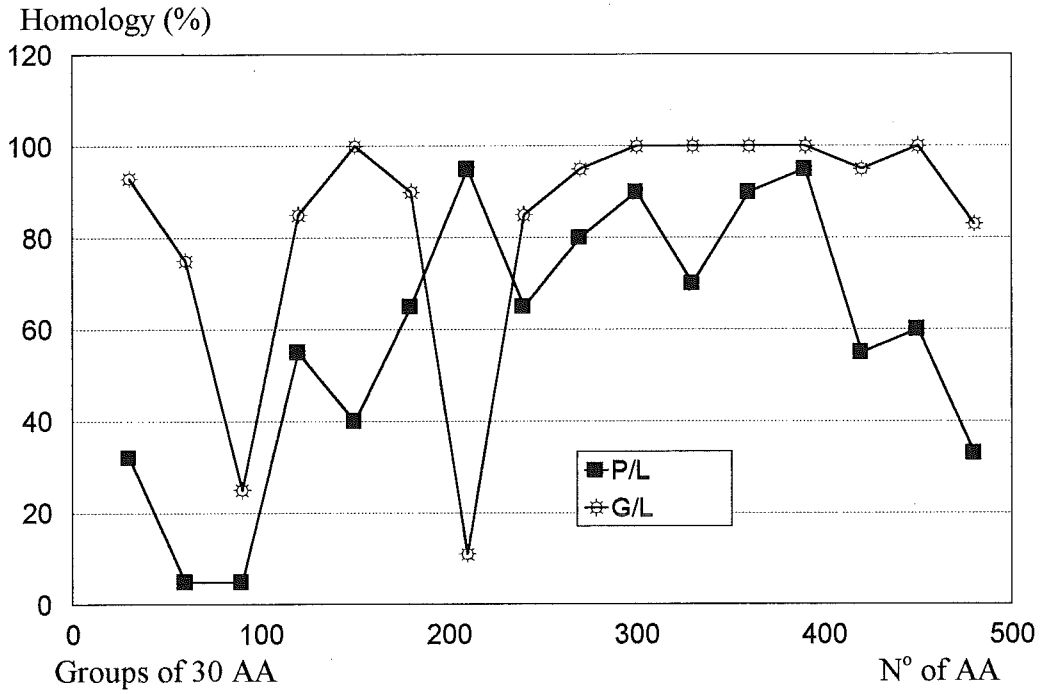

Fig. 1: Comparison of the degree of homology between PVY/LYSV and GV-2/LYSV in the different regions of their cp. P- PVY; L - LYSV; G - GV-2.

The deduced aa sequence of the cp of LYSV revealed that it is an typical potyvirus. An exclusion is the slightly differing protease cleavage site NIb/cp. Comparable different sites were found for tobacco etch virus (TEV) and tobacco vein mottling virus (TVMV). They are shown in table 2. It seems that phenylalanin (F) in some cases can play an important roll in recognition of the cleavage sites by proteases.

Protease cleavage site NIb/cp	**Virus**
VxHQ/A,S	consensus sequence
FVFQ/A	LYSV
LYFQ/S	TEV
VRFQ/S	TVMV

Tab. 2: Differing from consensus motif protease cleavage sites NIb/cp of potyviruses.

A DAG motif, which is typical for aphid transmitted potyviruses, is present. A further peculiarity of the viral RNA is the long 3' NTR. It is possibly necessary for RNA stabilization in *Allium* species.
The obtained antiserum against the recombinant cp reacted only with the homologous virus (tab. 1) despite of the high degree of homology with other potyviruses especially in the core region of the cp. A possible explanation for this fact could be that the C-terminal part of the cp in the fusion protein is folded in a manner that new neotopes appear and other epitopes are not

accessible. The corresponding antibodies can not detect the intact virus particle, which lacks such neotopes.
Unsolved stays the question why it is not possible to produce conjugates with IgG from antisera against the recombinant cp.
The differing results for the detection of viruses in field material by PTA-ELISA could reflect the problem mentioned in the introduction: the polyclonal antiserum was raised against a mixture of nearly identical leek potyviruses. As the AS against the recombinant cp seems to be directed against the C-terminal part, which shows pronounced differences between isolates, it will not react with a other virus isolate, for instance GV-2. This hypothesis raises up the question if resistance of leek against one isolate of LYSV is active against another isolate of this virus.
The results of our work show that it is possible to use cloned and expressed viral sequences for the production of highly specific antisera which can be used for PTA-ELISA. Comparable results we obtained with an other virus of leek - garlic common latent carlavirus (12).

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STRUCTURE-FUNCTION RELATIONSHIPS OF DEFINED REGIONS OF THE COAT PROTEIN OF POTYVIRUSES

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Introduction

The potyvirus genome consists of a single stranded, positive sense RNA. The aphid-, mite- and whitefly-transmitted potyviruses have a single molecule of RNA of approximately 10,000 bases, while the fungus-transmitted potyvirus genome is distributed across two molecules of RNA. Potyviruses belong to picorno-like supergroup of viruses whose RNAs have a protein, VPg, covalently bound to the 5'-end, a poly (A) tail at the 3'-end, and are expressed as a large polyprotein which is subsequently cleaved by proteases to yield several functional proteins, including a conserved ordered gene set of nonstructural proteins that are involved in RNA replication. The order of these products in the polyprotein of aphid-transmitted potyviruses is: first protein, helper component, third protein, cylindrical inclusion protein, small nuclear inclusion protein which includes VPg at its N terminus, large inclusion protein and coat protein. Coat protein and Vpg are the only potyvirus proteins present in the virion, whereas other proteins are found in the host cell (1). The following description of the structure-function relationships of potyvirus coat protein is from information obtained from the aphid-transmitted potyviruses.

Structure of Potyvirus Coat Protein

The coat protein is the most characterised gene product of potyviruses. Coat protein sequences of more than 100 strains of about 40 different potyviruses are currently known. Comparison of these sequences and particle assembly properties suggest the presence of three different domains in the coat protein molecules of potyviruses: (i) a surface-exposed N-terminus, which varies in length (19 to 97 amino acid residues) and sequence; (ii) a highly conserved core of 215-227 amino acids; and (ii) a surface-exposed C-terminus of 18-20 amino acids (2).

The flexuous particles of potyviruses are 11-12 nm wide, made of approximately 2000 coat protein subunits arranged in a helical manner around a single copy of the viral RNA. The basic pitch of the potyvirus nucleocapsid helix is 3.3 nm with 7-9 coat protein subunit per ring (3). Structural predictions for the coat protein of potato virus Y suggest the presence of seven helical regions, features similar to those known to occur in the rod-shaped tobacco mosaic virus. On the basis of these observations, Shukla *et al.* (4) suggested that potyviruses and other rod-shaped plant viruses may share common structural features of polypeptide folding and subunit packing with well studied tobacco mosaic virus particles.

Functions of the Potyvirus Coat Proteins

Functional studies and sequence comparisons suggest that the three distinct domains, namely the N-terminus, core and C-terminus, of the potyvirus coat protein identified on the basis of sequence comparisons and particle assembly properties are involved in different biological and other functions such as virion assembly, serology, cross-protection, aphid-transmission, virion transport and host range.

Virion Assembly: Until recently the only function established for the coat protein of potyviruses was that it encapsidates the viral RNA. However, it was not known if the whole coat protein was involved in the assembly of particles. In 1988 we showed that the N- and C- termini of the potyvirus coat protein are located on the virion surface and removal of these termini by trypsin digestion leaves a fully assembled virus particles composed of the coat protein core region, that appears indistinguishable from untreated native particles by electronmicroscopy, suggesting that the N- and C- termini are not required in particle assembly (4). *In-Vitro* assembly studies showed that flexuous virus-like particles can be produced using the dissociated core region of the potyvirus coat protein (5). Recombinant DNA methodologies were later used to express either full-length or truncated coat protein or coat protein with N-terminal region substituted with foreign peptides in the bacterium *Escherichia coli*, the yeasts *Sacchromyces cerevisiae* and *Pichia pastoris* and in the insect cells using the baculovirus system (5, 6). The expressed protein readily assembled to form potyvirus-like particles which were similar in their morphology (stacked-ring appearance) to particles obtained *in-vitro* reassembly of dissociated coat protein in the absence of RNA. Electron microscopic examination of ultrathin sections of microbial cells revealed that particle assembly was

occurring inside these surrogate host cells (7). The virus-like particles appeared in vast arrays of parallel strands, sometimes extending the length of the bacterial cell. In spontaneously lysed cells expressing very high levels of coat protein, potyvirus-like particles could sometimes be seen bursting out of the cell. At times the bacterial cells were strung together with bridges of virus-like particles appearing to connect individual cells in the process of separation. Results from all these studies confirmed our previous observation (4) that only the core region is involved in the assembly of potyvirus particles.

Serology: Antisera produced against potyvirus particles have been used extensively to study antigenic relationships between strains or distinct potyviruses. However, unlike many other plant virus groups, serology has proved most unsatisfactory when applied to potyviruses. There are three major problems associated with serology of potyviruses, namely variable cross-reactivity of potyvirus antisera, unexpected and inconsistent paired relationships between distinct potyviruses, and lack of cross reaction between some strains. As explained below, these problems are due to inherent complexities associated with potyvirus coat proteins and particles (8, 9). Recent biochemical and immunochemical investigations of particles, coat proteins and overlapping, synthetic octapeptides corresponding to entire coat protein sequences of potyviruses have demonstrated that the surface-exposed N-terminus constitutes the most immunodominant region of potyvirus particles (4, 8) and that virus-specific epitopes usually are located in the N-terminus, whereas cross-reacting potyvirus group-specific epitopes are contained in the highly homologous core region of coat proteins (4, 8, 9). Accordingly, antibodies produced against the virus-specific N-terminus were found to be virus-specific and those generated against the core region were found to react with many potyviruses (4, 8, 9). The epitopes for the paired serological relationships between distinct potyviruses were also found to be located in the surfaced-exposed N-terminal region (1, 4), whereas lack of cross-reaction between some strains has been shown to be due to very low sequence homology in the N-terminal region of the strains (1, 8). These observations show that the antigenicity of different domains of coat protein has different uses in the serology of potyviruses.

Cross-protection: It is now well established that the coat protein is involved in classical cross-protection of plant viruses (10). The coat protein domain involved in cross-protection of potyviruses is likely to be the surface-exposed, amino-terminal region since this is the only region in the entire coat protein that is relatively virus-specific while the core and surface-exposed C-terminal regions posses significant sequence identity (11). Although the amino-terminal regions of coat proteins of strains of one potyvirus are generally very similar in size and sequence, strains of some potyviruses, for example plum pox virus, passionfruit woodiness virus and sugarcane mosaic virus (SCMV), have been found to differ substantially in this region (1). This may be the reason why strains of some potyviruses do not cross-protect (12). For example, the strains of SCMV differ in the surface-exposed, N-terminal region spanning amino acid residues 27 to 70 (13), and display five different sequence motifs among seven strains examined (14). These sequence motifs were found to correlate with the cross-protection behaviours of some of the strains tested; strains with similar motifs cross-protected each other while those with different motifs did not (12, 14).

Aphid transmission: Coat proteins function in transmission by vectors in several plant virus groups (15) including potyviruses (16). The N-terminal domain, given its surface location on virus particles, is ideally placed to interact with helper component protein during aphid transmission. Examination of deduced amino acid sequences of aphid transmissible (AT) and non-aphid-transmissible (NAT) isolates of tobacco etch virus (TEV) led Harrison and Robinson (15) to predict that an amino acid triplet DAG, present at two locations in the N-terminal domain of the AT strain of TEV coat protein, may be involved in coat protein-helper component interactions since those DAG triplets were absent in the NAT strains of TEV due to amino acid substitutions in both regions. This hypothesis has recently been confirmed by Atreya *et al.* (17, 18) who observed that the coat proteins of the AT and NAT strains of tobacco vein mottling virus (TVMV) differ from each other by a single nucleotide (G-E) at position 8455 which changed the DAG triplet to DAE in the NAT strain. These authors further showed that replacement of the coat protein coding region of the full-length cDNA of the AT strain with the coat protein region of the NAT strain rendered the resultant hybrid virus non-aphid transmissible. Subsequently the authors made a series of mutations in the DAG triplet and neighbouring positions of TVMV and found that deletion of the triplet or mutation of the third residue G to E abolished the transmission. A search for the DAG sequence in the amino-terminal domains of the coat proteins of aphid-transmissible isolates revealed that the DAG sequence was present between positions 5-13 in 43 strains of 17 distinct potyviruses examined (11).

Virion transport: Movement of plant viruses between cells is an active process mediated by virus-encoded movement proteins which are believed to interact with host proteins and structures to facilitate passage of infectious entities through plasmodesmata (19). Unlike in some other plant virus groups, a potyviral protein with a dedicated movement function has not yet been identified. However, it has recently been demonstrated that different domains of the potyvirus coat protein may be involved in cell-to-cell and long distance movement of potyvirus particles. Using

viral mutants and nontransgenic and coat protein-expressing transgenic plants, Dolja *et al.* (19, 20) showed that the core domain of TEV coat protein provides a function essential during cell-to-cell movement and that the surface-exposed N- and C-terminal regions are necessary for long distance transport of potyvirus particles.

Host range: Circumstantial evidence suggests that the coat protein N-terminus of potyviruses may be a determinant for their host range. Comparison of coat protein sequences of two strains of SCMV showed that SCMV-SC exhibits high sequence identity (92%) with that of the SCMV-MDB except for the region between amino acid residues 27 and 70 in the amino-terminal domain. This region of SCMV-SC was smaller (44 residues) than the equivalent region in SCMV-MDB (59 residues), showed low sequence identity (22%) with the SCMV-MDB sequence and may have arisen by recombination (13). Comparison of the sequences of these two SCMV strains with five other strains of the virus showed that there were five different sequence patterns that could be grouped further into three subsets (14). The five different patterns correlated with host range with the strains infecting sugarcane (Brisbane, Isis and SC) forming one group while those that infect other graminaceous plant species and are non-transmissible to sugarcane (MDB/BC and Sabi/Bundaberg) formed the other two groups (14). It is interesting to observe that this grouping, based on the coat protein structure, correlated well with that achieved by reactivities of these strains on differential sorghum cultivars (21). This suggest that coat proteins of potyviruses may play a role in the determination of host specificity since the high sequence identity in the bulk of the coat protein coding region and the upstream NIb coding region of SCMV-SC and SCMV-MDB (13) implies that the remainder of their genomes are highly similar. This observation is further supported by the recent findings of Dolja *et al.* (19, 20) who showed that the surface-exposed N-terminal region of the coat protein mediates long distance movement of potyviruses. Since the movement proteins of plant viruses are believed to interact with host proteins to be functional this interaction would involve specific sequences of the virus and host proteins. Thus, it may be reasonable to suggest that the N-terminal sequences of distinct potyviruses are designed to interact with specific host proteins. Examination of sequences in the N-terminal domains of potyvirus coat proteins also show that most coat proteins that are 287 amino acid residues or longer contain partially repeated sequence motifs (1). This partial gene duplication has been suggested to be a frequent mechanism for generating diversity in the virus-specific, N-terminal domain of the coat protein of potyviruses, and the capacity to change the surface-exposed N-terminal domains of the coat protein without affecting its ability to form particles may have been an important factor in the evolution of new species of potyviruses with new host and vector specificity, and hence be the reason why there are so many potyviruses (1).

Conclusion

The foregoing discussion clearly suggests different functions for different domains of the potyvirus coat proteins. However, the precise mechanism how these domains mediate the implicated function is yet to be determined. For example, the nature of the molecular interactions involved in particle assembly is not known. The availability of recombinant expression systems will enable the process of particle assembly to be examined in detail (5, 7). Similarly, the mechanism by which the helper component protein interacts with the N-terminal domain of coat protein to mediate the aphid transmission of potyviruses is not understood. It is also not known at this stage whether the N-terminal domain is really involved in the classical cross-protection of potyviruses. This needs to be examined using infections cDNA transcripts with substitution of the N-terminal region by the corresponding region of different strains of a potyvirus where failed cross-protection has been observed, for example the strains of SCMV isolated from different plant species (12, 14). Use of infectious cDNA transcripts may also reveal if the N-terminal domain contains the host specificity determinant. Apart from its role in the long distance transport of virion (19), the surface-exposed C-terminal domain has not been implicated in any other function. Future studies may show additional functions for this and the other two domains of the potyvirus coat proteins.

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ANALYSIS OF WHEAT SPINDLE STREAK MOSAIC VIRUS (WSSMV)

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Introduction

Yellow mosaic disease is caused by soil-borne viruses and can lead to serious damage in wheat crops. Slykhius (1) identified a soil-borne virus in wheat grown in Ontario, Canada, which was designated as wheat spindle streak mosaic virus (WSSMV). It has also been reported in the USA, India, France, Italy and China. In Japan the causal agent of a yellow mosaic disease is called wheat yellow mosaic virus (WYMV). As WSSMV and WYMV show very similiar features, they were considered to be strains of the same virus (2). WSSMV is a bipartite virus consisting of two single-stranded, polyadenylated, plus-sense RNAs. It is transmitted by the funghus *Polymyxa graminis*.
Because different particle lengths for WSSMV and WYMV are reported in the literature we newly determined them. To understand the organisation of the genome and to obtain sequence information for the construction of vectors suitable for use with pathogen derived resistance strategies we cloned and sequenced the 3´terminal half of the RNA1 of WSSMV, which contains the capsid protein (CP) gene.

Material and Methods

Virus purification was done as described by Huth (3), *RNA isolation and cDNA synthesis* were described by Sohn et al. (4).

Virus particle length measurements
Plant sap of infected leaves were ground in potassiumphosphate buffer, pH 7.0 and then applied to pioloform coated grids for 5 minutes. After incubation with 2% uranylacetate, the grids were washed with 40 drops of distilled water. Measurements were made with a calibrated Morphomat 30 (Zeiss) using a transmission electron microscop (EM 109, Zeiss).

Cloning of the CP gene of WSSMV
For introduction of the sequences of a start codon and helpful restriction sites the following primers were synthesized (CP-A: 5´GGTGGTTGTGACATTGAACCCATGGCTGCGGACACACAAACTGAC3´; CP-B 5´GCCTTATCCATCTAGATACCATGGCGTACATCGCGTTTCACG3´) and used for *in vitro*-mutagenesis (USE mutagenesis kit, pharmacia) following the manufacturers instructions. The mutagenized regions were tested by restriction enzyme digestion and sequencing. All molecular cloning techniques were performed as described by Sambrook et al.(5). Both the vector pRT103 (6) and the mutagenized cDNA were digested with the restriction enzymes NcoI and XbaI before ligation. Next the NcoI-cut CP gene was ligated to the vector pRTexint (7) behind the cauliflower mosaic virus (CaMV) 35S promotor and exon1, intron1 sequences of the maize *shrunken1* gene.

Cultivation of the Triticum monococcum cell suspension and protoplast isolation from these cells was carried out as described by Lörz et al. (8). One million protoplasts were transfected with 25 µg plasmid DNA and 100 µg sonicated calf thymus DNA using polyethylenglycol according to the

protocol of Maas and Werr (9) . The protoplasts were harvested after cultivation for 48 h in the dark at 15 /26 °C .

ECL western blot analysis
Protein extracts of leaves and protoplasts were separated by SDS-PAGE on a 12% polyacrylamid gel (10). Blotting and ECL-western analysis were done as described by Schlichter et al. (11).

Results

Virus particles were purified from leaves of naturally infected wheat plants (*Triticum durum* L. *cv. Aramon*) collected from a field near Montpellier in Southern France. Virus particle length measurements were made with particles from plant sap and from a purified virus fraction after the CsCl gradient step. As an internal standard, tobacco mosaic virus (TMV) particles were used because they can be reproducibly measured as 300 nm. The result is shown in figure 1. Virus particle length measurements (115 particles) directly from plant sap showed two clear maxima between 600-650 nm and 300-350 nm. Measurements of the purified virus particles (137 particles) showed a larger number of particles of less than 300 nm and no clear maximum. With both the sap and purified fraction measurements obvious long particles (1325 nm, 1780 nm) were also found.

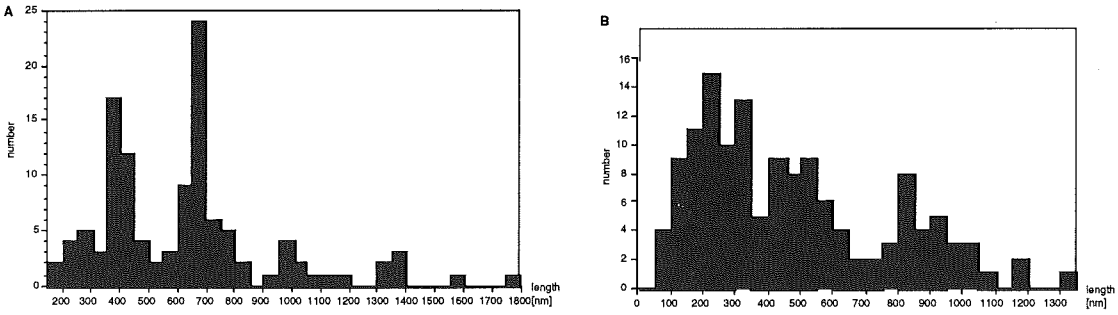

Fig. 1: Length measurements of WSSMV particles from plant sap (a) and from purified virus fraction (CsCl gradient, b)

After isolation of the viral RNA and cDNA synthesis, clones corresponding to two different RNAs were detected. A cDNA complementary to the 3´-terminal part of RNA1 of WSSMV has been sequenced (4). One large open reading frame of 4410 nucleotides and a non-translated region of 231 nucleotides at the 3´-terminal end were found. Following comparison to sequence databases (EMBL) the encoded polyprotein was comprised of a putative cytoplasmic inclusion protein, a putative proteinase, a RNA dependent RNA-polymerase and the capsid protein. The capsid protein shows 74% overall identity and 81% identity in the core region with the BaYMV capsid protein sequence. This high sequence homology with BaYMV, in addition to the significant identities with barley mild mosaic virus (BaMMV, 35%) and its marginal homology to capsid protein sequences of aphid- and mite-transmitted potyviruses (22-24%), supports the classification of WSSMV as a distinct member of the *Bymoviruses*, family *Potyviridae*.

For cloning the CP gene sequences, restriction sites (NcoI, XbaI) were introduced in the cDNA by *in vitro*-mutagenesis as well as sequences coding for an ATG start codon. This was necessary because the CP region is normally expressed as part of a processed viral polyprotein. The stop codon corresponds to the original stop

codon of the viral RNA1. The CP gene was cloned in the plant expression vector pRT103, which contains the 35S-promotor and also in vector pRTexint, which contains additionally the sequences of the untranslated exon 1 and intron1 of the *shrunken1* gene of maize between promotor and the coding region. Protoplast transfection of a suspension culture of *Triticum monococcum* with these plasmids were carried out with PEG-mediated uptake. Transfected protoplasts were cultured at different temperatures, 15°C and 26°C, which are optimal for virus or cell development respectively. ECL-western analysis of the cell extracts (1: pRTexint CPW, 2: pRT103CPW, 3: negative control) was done using a antiserum raised against in *E. coli* overexpressed, purified BaVMV-CP (1:1000), which detects the WSSMV-CP and gave low background in plants. Protein extracts from WSSMV-infected and non-infected leaves were used as controls (K+,-). Transient expression of the CP gene could be shown with western analysis after transfection of the plasmid containing enhancing exon and intron sequences, independent of the used temperature conditions.

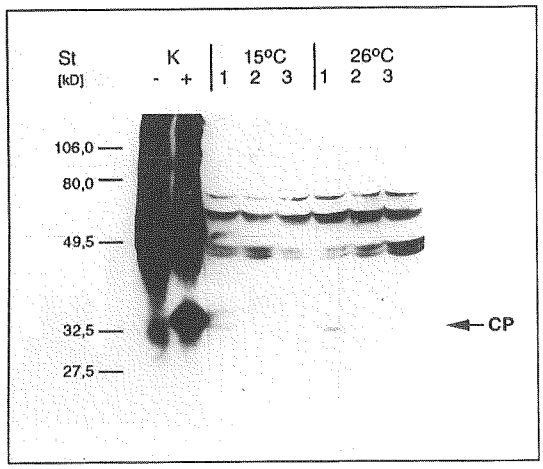

Fig. 2: Transient expression of the WSSMV capsid protein in *Triticum monococcum* protoplasts
ECL-western analysis of the cell extracts transfected with different plasmids (1: pRTexint CPW, 2: pRT103CPW, 3: negative control) was done using a antiserum raised against in *E. coli* overexpressed, purified BaVMV-CP (1:1000), which detects the WSSMV-CP Protein extracts from WSSMV-infected and non-infected leaves were used as controls (K+,-).

Discussion

Length measurements of virus particles from plant sap (Fig.1a) showed two clear maxima (300-350 nm and 600-650 nm), which demonstrates that the virus is bipartite. The relationship between the two length maxima of the virus particles (1,75) correspond to the relationship between the nucleotide number of the viral RNAs (1,71) estimated from a northern blot. Other peaks (around 900, 1200, 1500 and 1800 nm) have also been reported in the literature (12,13,14) and can be explained by aggregation of different combinations of these two particles. The tendency for particle aggregation explains the very long particles found in WSSMV-infected plant sap.
Measurements of particles after CsCl gradient purification (b) revealed no clear maximum and showed large numbers of particles with less than 350 nm. Fragmentation of the thin and long virus particles is probably promoted by

higher temperatures and by mechanical forces during the isolation procedure (15).

CP-mediated protection is the most common and one of the most successfull genetically engineered resistance strategies for RNA viruses (16). Cloning of the CP gene of WSSMV in a plant expression vector is the first step. As has been demonstrated, the expression of the cloned cDNA region led to the expression of the viral CP, if the enhancing exon1 and intron1 sequences of *shrunken1* gene of maize were included in the vector. These sequences are known to stimulate transient gene expression in monocotyledonous cells. Using the reporter gene chloramphenicoltransacetylase for which a sensitive enzyme assay exist it was shown that the CaMV 35S promotor is sufficient for transient gene expression (7). The level of transient expression and the sensitivity of the western blot analysis in the experiment reported here was not sufficient to detect the CP after expression under the control of the 35S promotor alone. However, there are several reports of stable transformation of monocots using the 35S promotor. Therefore transformation of wheat with both CP gene containing constructs is in progress in order to investigate CP-mediated protection in wheat.

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MONOCLONAL IMMUNOENZYME TEST SYSTEM FOR BEET NECROTIC YELLOW VEIN VIRUS DETECTION

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Introduction

Beet necrotic yellow vein virus (BNYVV) belongs to the furovirus group and may cause a significant damage on sugar beet crops. It is transmitted by the soil-borne fungus *Polymyxa betae*. BNYVV contains four types of virions, encapsulating four types of single-stranded RNA molecules of different length.

The monoclonal antibody (MAb) technology provides a means to produce a supply of highly specific uniform antibody which can be useful in the routine detection of plant viruses (1). In this paper we report the production and characterisation of MAbs to BNYVV and their use in the development of highly sensitive and reliable test system for BNYVV detection. We started with the development of monoclonal double-antibody sandwich-ELISA, whereupon the enzyme-labelled MAb was replaced by the combination of biotinylated MAb and streptavidin conjugated to either monomeric horseradish peroxidase (HRP) or HRP homopolymers as enhanced enzyme labels (2). The application of streptavidin conjugated to polymeric HRP at different polymerisation degrees including -20, -40 and -80 (Str-polyHRP20, Str-polyHRP40 and Str-polyHRP80) brought about a significant amplification of the enzymatic signal and the essential improving of the assay detection limit.

Materials and Methods

Virus and virus purification. BNYVV isolate K-88, obtained from the soil samples in Kirghizia, was purified by the method of Koenig (3).

Polyclonal antiserum production. Rabbit polyclonal antiserum to BNYVV was produced in All-Russian Research Institute for Plant Protection (St.Petersburg, Russia).

Immunisation of mice and production of hybridomas. Mice were immunised with 2 to 3 injection of purified BNYVV preparation in Freund's adjuvant. The last injection was done without adjuvant. Cell fusion was performed as described by Van Deusen (4).

Production of ascitic fluids. Ascitic fluid was produced by intraperitoneal inoculation of $5x10^6$ to 10^7 hybrid cells into syngenic BALB/c mice that had been injected 3 to 20 days before with 0,5 ml pristane. The ascitic fluid was harvested 10 to 14 days later.

Antibody purification. MAbs were purified from ascitic fluid using precipitation with 50% ammonium sulphate, followed by affinity chromatography on Protein-A Sepharose.

Biotinylation of antibodies. MAbs (3 mg/ml) were dialysed overnight against 0.1 M sodium bicarbonate pH 8.6 and 0.1 volume of fresh solution of N-oxysuccinamide ester aminocaproilbiotin 1.0 mg/ml in DMSO was added. The solution was incubated for 2 h at room temperature and dialysed overnight against PBS.

HRP-conjugate preparation was done according to the method of Tijssen and Kurstak (5).

Streptavidin-polymeric HRP conjugates were purchased from STD Inc. (Russia, Perm).

Enzyme-linked immunosorbent assay (ELISA). ELISA procedures were modified from Clark and Adams (6).

Results.

Production and characterisation of monoclonal antibodies to BNYVV

Two fusion experiments were performed and fusion products were plated into 800 culture wells. Growth of hybrid cells was observed in 95% of them. Supernatant fluids of 11 cell cultures reacted strongly to BNYVV-infected material, but not to uninfected plant tissue or plant material infected with the Tobacco mosaic virus. Seven hybrid cell cultures producing the highest antibody titre were selected for cloning and further propagation. Five hybrid cell lines were obtained after three cloning steps. Some characteristics of these lines are presented in Table 1.

Development of ELISA for BNYVV detection.

Different conditions for adsorption of coating MAb (pH and concentration) were compared. The optimal pH for MAbs adsorption was shown to be 9,6; the capture MAbs concentration was in the range of 1-2 mg/ml. Three MAbs (R3, R4 and R5) were conjugated to HRP. All possible combinations of capture and revealing antibodies for BNYVV detection in DAS-ELISA were compared. The most sensitive assay was with R4-HRP conjugate combined with either of R1, R3 or R4 as a capture antibodies (Table 2). BNYVV was detectable in plant extracts in dilutions down to 1/1000. The system R1 - R5-HRP was also adequate for BNYVV detection but the level of non-specific reactions was higher, so this system was less suitable for BNYVV detection. The Str-HRP and Str-polyHRP conjugates were used to improve the detection level of immunoassay for BNYVV. MAbs R1, R3 and R4 were used to capture the virus and MAb R4 was biotinylated. Compared to DAS-ELISA the detection level of BSMV increased as much as twice with conventional Str-HRP conjugate, up to 6 times with Str-polyHRP20 and Str-polyHRP80 conjugates and up to 12 times with Str-polyHRP40 conjugate (Table 3).

TABLE 1

Properties of mouse monoclonal antibodies from hybridoma cell lines prepared for BNYVV

Hybrid cell line	Antibody isotype	Titre by TAS-ELISA (reciprocal)	
		Ascitic fluid	Culture fluid
R1	IgG1	10^5	$4x10^3$
R2	IgG2a	10^6	$8x10^3$
R3	IgG1	10^6	10^4
R4	IgG1	10^7	10^4
R5	IgG2a	10^7	10^4

TABLE 2

Detection of BNYVV in extract from infected leaves in DAS-ELISA.

Capture MAb	Minimal detectable level, dilution of extract		
	MAb-HRP conjugate		
	R3	R4	R5
R1	1/320	1/1000	1/1000
R2	1/250	1/640	1/300
R3	1/200	1/1000	1/700
R4	1/300	1/1000	1/700
R5	1/200	1/640	1/320

TABLE 3

Comparison of different ELISAs for detection of BNYVV in extract from infected leaves

Conjugate	Minimal detectable level, dilution of extract
	Capture MAb R1 (or R3 or R4)
MAb-HRP	1/1000
Str-HRP[a]	1/2000
Str-polyHRP20[a]	1/6000
Str-polyHRP40[a]	1/12000
Str-polyHRP80[a]	1/6000

[a]- the second MAb was biotinylated MAb R4

Discussion

Serology provides a useful technique for the routine detection of plant viruses (7). In this study we developed highly sensitive immunoenzyme test-system based on the MAbs application for BNYVV detection. The availability of MAbs to BNYVV eliminates the whole number of problems, such as limited amounts of antiserum or variability among antiserum lots, associated with the use of polyclonal antibodies.. We started with the development of monoclonal double-antibody sandwich-ELISA, and the most sensitive Mab combination was found. BNYVV was detectable in plant extracts in dilutions down to 1/1000. The direct enzyme labelling of the revealing MAb may essentially decrease the MAb affinity (8, 9), so the system avidin (streptavidin) - biotin was used to improve the assay detection limit. Techniques based on the avidin-biotin system have been reported to be 2-100 times as sensitive as conventional procedures (10). Biotin can be coupled to macromolecules under mild conditions without affecting their biological activity. Due to it's neutral isoelectric point and the absence of carbohydrate residues streptavidin provides higher specificity of immunoassays as compared to avidin. PolyHRP conjugates were used to amplify the enzymatic signal and to improve the assay sensitivity. Conventional streptavidin-HRP conjugate was compared with the conjugates of streptavidin with HRP homopolymers. In the most sensitive MAb combination for BNYVV detection in DAS-ELISA the monoclonal enzyme conjugate was replaced by

the biotinylated MAb in combination with streptavidin coupled to either HRP or to HRP homopolymers. The best result was obtained with Str-polyHRP40 as enhanced enzyme label. Compared to DAS-ELISA the detection level of BNYVV increased as much as twice with conventional Str-HRP conjugate, up to 6 times with Str-polyHRP20 and Str-polyHRP80 conjugates and up to 12 times with Str-polyHRP40 conjugate Compared to other currently available technologies for high sensitive detection, poly-HRP conjugates have an advantage of genuine simplicity, requiring only standard lab equipment and minor protocol variations.

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THE APPLICATION OF MONOCLONAL ANTIBODIES FOR BARLEY STRIPE MOSAIC VIRUS DETECTION

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Introduction

Barley stripe mosaic virus (BSMV), a typical member of the Hordeivirus group, is a seed-borne, rod-shaped, positive-strand RNA virus with a multipartite genome. BSMV has a helical capsid composed of identical coat protein molecules. The BSMV infection of barley can cause serious economical losses but taking into account that seed transmission is the major factor in disease epidemiology and no biological vectors are known for BSMV, these losses can be greatly ameliorated by planting uninfected seeds.

Serology provides a useful technique for the routine detection of plant viruses (1). In this paper enzyme immunoassay based on the use of monoclonal antibodies (MAbs) were developed for the detection of BSMV. Assays employing conjugates of MAbs to horseradish peroxidase (HRP) were compared to systems with biotinylated MAbs and streptavidin conjugated either to monomeric HRP or to HRP homopolymers with different polymerisation degrees including those of -20, -40 and -80 (2). The system for BSMV detection with polymeric HRP was sufficiently sensitive to detect a single infected seed among more than 10^4 healthy ones. The assay detection limit was 1 ng/ml.

Materials and Methods

Virus and virus purification. BSMV three-component strains V-1 and North Dakota-18 were used in this work. Pure BSMV preparations were obtained as described by Agranovsky et al. (3)

Immunisation of mice and production of hybridomas. Mice were immunised with 2 to 3 injection of purified BSMV preparation in Freund's adjuvant. The last injection was done without adjuvant. Cell fusion was performed as described by Van Deusen (4).

Production of ascitic fluids. Ascitic fluid was produced by intraperitoneal inoculation of $3x10^6$ to $8x10^6$ hybrid cells into syngenic BALB/c mice that had been injected 3 to 20 days before with 0,5 ml pristane.

Antibody purification. MAbs were purified from ascitic fluid using precipitation with 50% ammonium sulphate, followed by affinity chromatography on Protein-A Sepharose.

Determination of binding constants of MAbs was done according to the method of Beatty et al. (5)

Biotinylation of antibodies. MAbs (3 mg/ml) were dialysed overnight against 0.1 M sodium bicarbonate pH 8.6 and 0.1 volume of fresh solution of N-oxysuccinamide ester aminocaproilbiotin 1.0 mg/ml in DMSO was added. The solution was incubated for 2 h at room temperature and dialysed overnight against PBS.

HRP-conjugate preparation was done according to the method of Tijssen and Kurstak (6).

Streptavidin-polymeric HRP conjugates were purchased from STD Inc. (Russia, Perm).

Enzyme-linked immunosorbent assay (ELISA). ELISA procedures were modified from Clark and Adams (7).

Results

Production and characterisation of monoclonal antibodies to BSMV

Four fusion experiments were performed. Supernatant fluids of 20 cell cultures reacted strongly to purified BSMV and to BSMV-infected plant material, while no reaction was observed with healthy plant material, the purified tobacco mosaic virus, the potato virus A, the cucumber mosaic virus, the arabis mosaic virus and the poa semilatent virus. Characteristics of six hybrid lines established after three cloning steps are summarised in Table 1.

Development of ELISA for BSMV detection

Five of six anti-BSMV MAbs were conjugated to HRP (we failed to prepare the conjugate B7-HRP). To determine the optimal conditions for the use of MAbs in ELISAs, we compared the detection level of BSMV under different assay conditions. The optimal coating condition was shown to be at pH 9,6 for IgG and at pH 7,4 for IgM; the concentration of the capture MAb was in the range of 1-4 µg/ml. All possible combinations of capture and revealing antibodies for BSMV detection were compared. Preliminary experiments showed a very high level of background with IgM-based HRP conjugates. So only MAbs B1, H9 and 2A11 were used as revealing antibodies (Table 2). All combinations were tested with purified BSMV preparation and with extracts from leaves and seeds of infected plants. The best signal to noise ratios were found with two systems, B1 - B1-HRP and B5 - H9-HRP, allowing the detection level of 26 ng/ml BSMV. The system B1 - B1-HRP was more suitable for the BSMV detection in the extracts from infected leaves and seeds because of the lower background level. With rabbit polyclonal antibodies as the capture, the detection level was only 65-125 ng/ml (Table 2). Taking into account the relative epitope specificity of MAbs to BSMV (8), the mixture of MAbs directed to overlapping (B1+B5) and to non-overlapping (B1+B6, B1+B7, B5+B6, B5+B7) epitopes on the surface of BSMV particles was used to capture the virus. The minimum detectable level of BSMV was not less than 30 ng/ml in all these systems.

The streptavidin-biotin system was used to amplify the enzymatic signal and to improve the BSMV detection level. The application of MAb B1 to capture the virus, and biotinylated MAb B1 with streptavidin conjugated to HRP to reveal the bound antigen, gave twice higher sensitivity as compared with monoclonal DAS-ELISA (13-14 ng/ml for purified BSMV). The application of streptavidin conjugated to polymeric HRP at different polymerisation degrees including -20, -40 and -80 (Str-polyHRP20, Str-polyHRP40 and Str-polyHRP80) brought about a significant amplification of the enzymatic signal (Table 3). Extracts of BSMV-infected leaves and seeds were tested in these assays. The most sensitive MAbs combinations, B1 - H9-Bi - Str-polyHRP40 and H9 - H9-Bi - Str-polyHRP40, were adequate for detecting BSMV in dilutions down to 1/15000 in seed extracts.

TABLE 1. Properties of mouse monoclonal antibodies from hybridoma cell lines prepared for BSMV.

Hybrid cell line	Antibody isotype	K_a, M^{-1}	Titre by TAS-ELISA (reciprocal)	
			Ascitic fluid	Culture fluid
B1	IgG2a	$2,8x10^{10}$	10^7	$5x10^4$
B7	IgG2a	$1,5x10^9$	10^5	10^3
H9	IgG1	$1,7x10^{10}$	10^7	10^4
B5	IgM	$2,4x10^7$	10^5	$5x10^2$
B6	IgM	$4,2x10^7$	10^5	10^3
2A11	IgG1	$4,4x10^6$	10^5	10^3

TABLE 2. Detection of BSMV in DAS-ELISA.

Capture MAb	Minimal detectable concentration, BSMV, ng/ml		
	MAb-HRP conjugate		
	B1-HRP	H9-HRP	2A11-HRP
B1	26	30	32
B7	68	70	125
H9	35	45	60
2A11	50	60	60
B5	30	26	60
B6	60	60	60
rabbit polyclonal antibodies	65	80-90	125

TABLE 3. Comparison of different ELISAs for BSMV detection.

Capture MAb	Biotinylated MAb	Minimal detectable concentration, BSMV, ng/ml			
		Conjugate			
		MAb-HRP[a]	Str-polyHRP20	Str-polyHRP40	Str-polyHRP80
B1	B1-Bi	26	4	2-3	4
	H9-Bi	35	2-3	1	2
H9	B1-Bi	30	2-3	1-2	2-3
	H9-Bi	45	2-3	1	2

[a] - in DAS-ELISA the biotinylated MAb was replaced with the same MAb, conjugated to HRP

Discussion

MAbs to BSMV described in this paper were used for BSMV epitope analysis in our previous communication (8). Three independent methods used in that work (additive ELISA, competitive ELISA and inhibition of latex agglutination) revealed at least three distinct antibody-binding sites on the surface of native BSMV particles: epitope MAb B6, epitope MAb B7 and immunodominant region, where overlapping epitopes of 4 other MAbs are localised. All known strains of BSMV are serologically related (8, 9), so all these MAbs were suitable for BSMV detection.

To develop the test-system for reliable BSMV detection, we compared all possible combinations of capture and revealing antibodies in DAS-ELISA. To cover microtiter plates we used rabbit polyclonal antibodies to BSMV, each of the MAb by itself and the mixture of MAbs, directed to overlapping or to non-overlapping epitopes. The best result was obtained with two MAbs combinations: B1 - B1-HRP and B5 - H9-HRP. The minimum quantity of BSMV detectable by each of these two systems was 26 ng/ml. The suitability of this sandwich ELISA for BSMV detection in extracts from infected leaves and seeds was demonstrated. It was interesting that the combination of low-affinity MAb B5, used as the capture antibody, with the high-affinity MAb H9, conjugated to HRP as a revealing antibody, had the same sensitivity as the system with the high-affinity MAb B1 as the capture and the revealing antibody. The sensitivity of the systems B1 - H9-HRP and B5 - B1-HRP was only slightly lower than that of the former two systems (Table 2). One can suppose that the binding of BSMV particles with immobilised MAb B1 or B5 involves the same part of the polypeptide chain (epitopes of MAb B1 and B5 are overlapping) and this binding provides conditions favourable for effective binding of high-affinity conjugates H9-HRP and B1-HRP in the next stage of the ELISA.

The minimum detectable level for some other plant viruses in ELISA was in the range of 1-10 ng/ml, so the BSMV detection limit was unsatisfactory. The relatively low sensitivity of all DAS-ELISA versions can be due to the direct enzyme labelling of the revealing MAb, which decreased the MAb affinity (10, 11).

To avoid the direct enzyme labelling of the revealing MAb, the system avidin (streptavidin) - biotin was used. Conventional streptavidin-HRP conjugate was compared with the conjugates of streptavidin with HRP homopolymers. The minimum detectable level of BSMV in the two most sensitive ELISA combinations with Str-polyHRP40 conjugate was 1 ng/ml. These systems detected BSMV (in some, but not in all studied cases) in one infected seed portion diluted up to the equivalent of 15000 healthy seed portions. Such dilutions allow quick screening of large seed lots at less expense.

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SPECIES-SPECIFIC PROPAGATION OF TGMV VECTORS

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Introduction

Replication of potato spindle tuber viroid (PSTVd) can be inhibited by antisense nucleic acids, as has recently been demonstrated by antisense RNA expression in transgenic plants (1). However, antisense RNA transcription can be blocked by RNA-RNA directed methylation of the antisense transgene (2) and by other mechanisms of gene silencing. Antisense DNA is another approach, and we have found that this inhibits viroid replication more efficiently than antisense RNA in infection tests in tissue culture. It would therefore be useful to develop a method of propagation of antisense DNA in vivo to achieve efficient viroid inhibition. This DNA would have to be single-stranded and accessible for pairing with the target viroid RNA, which replicates in plant nuclei. Geminiviruses are single-stranded viruses which replicate in the plant nucleus and are thus good candidates to propagate such antisense DNA in vivo. The bipartite geminivirus, tomato golden mosaic virus (TGMV) has been used as a vector for the propagation of foreign DNA (4). The TGMV coat protein gene encoded by component A (supporting virus replication) is not essential and can be replaced by DNA of choice. Recombinant TGMV-A can be introduced to plant tissue via an Agrobacterium tumefaciens Ti-plasmid vector. If component B, supporting virus movement, is also introduced separately the modified virus can spread throughout an infected plant. Here, we cloned a subgenomic portion of viroid cDNA, as an antisense DNA, into a NptII DNA sequence (replacing the coat protein gene) of a TGMV-A vector. The resulting vector was assayed for its ability to replicate in several plant species selected, which serve as the common hosts for TGMV and PSTVd.

Material and Methods

We constructed modified TGMV-A tandem repeats essentially as described (5,6) to retain two copies of the TGMV replication origin and two full length copies of the AL1, the viral replication gene. Most of the coat protein coding sequence was replaced by gene by NptII having a mutation at the 5' end and designated as neo- (7). The anti-viroid antisense sequence used covers positions 282-359/1-94 of the PSTVd genome. The cDNA insert was cleaved from vector pRH751 (8) and blunt end ligated into a SmaI unique site in neo-. The resulting cassette, of 4.792 kb, was excised by cleavage with BglII, treated with T4 polymerase and blunt end ligated to a filled in XbaI site in the plant binary vector pGA482, yielding VL-TGpGA. A control vector, TGpGA, without the viroid sequence. The wild type TGMV-A and -B constructs used are as described (9). All binary vectors were transferred to Agrobacterium tumefaciens strain LBA4404 by triparental mating (10). For inoculation we used two-week old seedlings of N.tabacum (cv.White burley); N.benthamiana; N.clevelandii and L.esculentum (cv.Rutgers). Inoculation was of young leaves, using carborundum, and also by injection into decapitated stem apexes. We used mixed cultures of Agrobacterium containing binary vectors carrying either modified or wild type TGMV-A plus wild type TGMV-B. Tomato plants were transformed by these vectors by inoculation of cotyledons and the first true leaf explants and regenerated on MS medium (pH 5.9) supplemented with vitamins, 3% sucrose, zeatin riboside (1.75 $mgxl^{-1}$) and indolyl-DL-aspartic acid (0.87 $mgxl^{-1}$). The medium was solidified with 0.8% agar. Transgenic plants were identified by their kanamycin-resistant phenotype.

Unless otherwise stated, TGMV DNA was isolated from infected material using an alkaline treatment protocol (11) three weeks after inoculation. TGMV-A was detected either using Southern blot analysis with a transcription probe specific for AL1 from vector β_3 (9) or by PCR. For PCR we prepared the following oligonucleotides: T: 5'TAGAGGATCCCCTCTACACC3', specific for TGMV at position from 1346 to 1365 and N: 5'TCTCATGCTGGAGTTCTTCG 3', specific for NptII gene at position from 1090 to 1110. PCR enabled specific detection of modified TGMV A and analysis of antisense gene, because these primers bordered the unique SmaI restriction site used for VL- cloning.

Results

The TGMV vector constructed is depicted in Fig.1. This vector contains conventional T-DNA borders from Ti plasmid and, in addition to neo-, one functional copy of NptII. We found some instability of this vector during cloning and appearance of small plasmids in some preparations. We selected, however, a vector of the proper size of 17.99Kb containing a complete modified TGMV cassette with the integrated antisense gene VL-. This vector was further assayed for its ability to infect several host species (Table 1). In most species, except for N.cleveleandii, we were able to detect this vector in infected plants only by PCR, suggesting a very low level of replication. No detectable signal was observed on Southern blots screened by a specific riboprobe, while a very strong signal was observed for the wild type of TGMV under our experimental procedure. PCR analysis performed using DNA extracts from N.benthamiana revealed also some instability of VL-, which was absent in several preparations and final product length thus reached about 400 instead of 600bp calculated. This was true also for N.clevelandii, but the level of the vector was, in this species, remarkably high and reached nearly 100% of the wild type control.

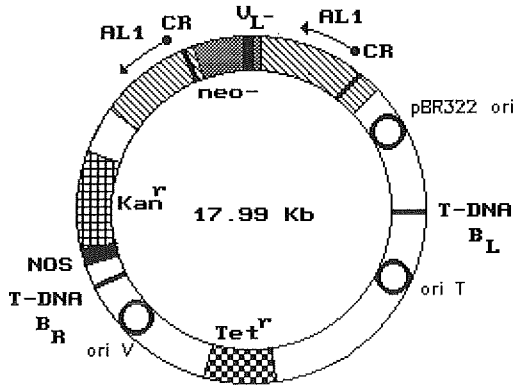

Fig.1 Vector V_L-in TGpGA. V_L antisense gene targeted against PSTVd CR common region; AL1, AL1 ORF; neo- mutated NptII gene. B_R, B_L- T-DNA borders.

A characteristic distribution of TGMV molecular forms was detected in N.clevelandii infected with VL-in TGpGA (Fig.2). We also observed some symptoms in N.clevelandii plants, but these symptoms like epinasty and leaf malformations and rugosity were much weaker and appeared later (about 2-3 weeks after inoculation) than those usually observed for wild type of TGMV. Essentially the same results we found for control TGpGA without viroid antisense gene, suggesting that low vector level in some species and high replication level in N.clevelandii was not due to PSTVd-specific sequence. In order to increase the vector level in L.esculentum, which is of special interest as plant species-tester of PSTVd infection, we used plant transformation. By Southern blotting we found a low but detectable level of this vector in primary kanamycin resistant tomato regenerants. The amount of TGMV detected in transgenic tomatoes was about 500-times lower than amount usually observed for wild type TGMV in tester-N.benthamiana. Surprisingly, the vector disappeared during subsequent vegetative cloning of tomato transformants, suggesting also some instability or induction of resistance to TGMV during this quenching infection.

Table: Vector levels in different plant species

Vector	Plant species	TGMV detection		
		Southern blot	Virus level	PCR
$V_L(-)$	N.benthamiana	-	n.d.	+
	N.clevelandii	+	100%	+
	N.tabacum cv. White burley	-	n.d.	+
	L.esculentum cv.Rutgers	- (+)*	(~)**	+ +
Wild type of TGMV	N.clevelandii	+	100%	-

*detection in transformed tomatoes ** quenching infection
n.d. not determined

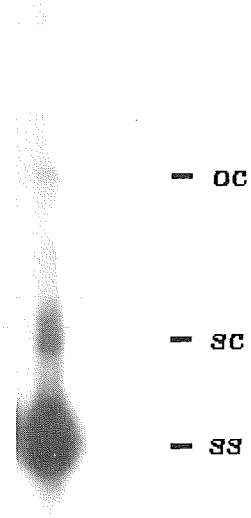

Fig.2 Molecular forms of TGMV vector isolated from N.clevelandii. ss-single--stranded;sc-supercoiled; oc-open-circular.

Discussion

We constructed a vector based on TGMV-A having replaced the coat protein gene with a mutated sequence of NptII. A similar vector has been successfully used with the aim of studying homologous recombination to yield a functional NptII in tobacco (4). In this work, integrated components A and B of TGMV were in cis in the same binary vector to ensure simultaneous incorporation of both virus components. In our experiments, we inoculated plants by mixture of separate A and B DNA. However, it is unlikely that low infectivity of our vector was due to this difference as others have used mixed infection successfully (e.g.5,9,12). The size of our vector was probably also not a critical factor, as the TGMV vector mentioned above (4) also tolerates some difference in length. On the other hand, we found surprisingly a high replication level of the TGMV vector in one species- N.clevelandii and also a quenching infection in tomato. These results suggest that in principle, the vector can replicate, but the replication is strongly host dependent. One possibility is that the vector sequence mutated during the cloning procedure and that this mutation affected its ability to replicate in some hosts. We can assume that this possible mutation did not affect the interaction between A and B components as we were unable to detect any significant vector level even after tobacco leaf disc agroinfection with modified component A alone (not shown). From our PCR analyses we often found an absence of the antisense gene and also some instability of the vector in bacteria. It is possible that some recombination events occurred also in plant cells causing a release of viable but modified vector sequence adapted to N.clevelandii. A possible adaptation of TGMV to different host species cannot be ruled out (12,13). Besides a replicational pathway of geminivirus release from tandem DNA copies, release by homologous recombination has also been identified (14,8). This latter mechanism could lead to accumulation of recombinant molecular forms of geminivirus (e.g. 8). The proposed mutation remains to be determined.

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INITIAL INTERACTIONS BETWEEN VIRUS AND PLANT CELLS

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Introduction

Plant viruses, unlike many animal and human viruses, can not enter cells directly by endocytosis. Because of the rigid outside skeleton of cuticula and cell wall, plant viruses have to be introduced by mechanical wounding (1). It is most likely that during the wounding process virus particles are introduced directly into the cytoplasm of damaged but still viable cells. So far no direct evidence has been found for fusion and endocytosis as a mechanism of entry (2,3). Both electroporation and polyethylene glycol enables virus and RNA to enter plant protoplasts isolated from mesophyl plant tissue. Wounding and PEG cause the protoplasts or protoplasmic extrusions (4,5) to swell and shrink causing disconti-

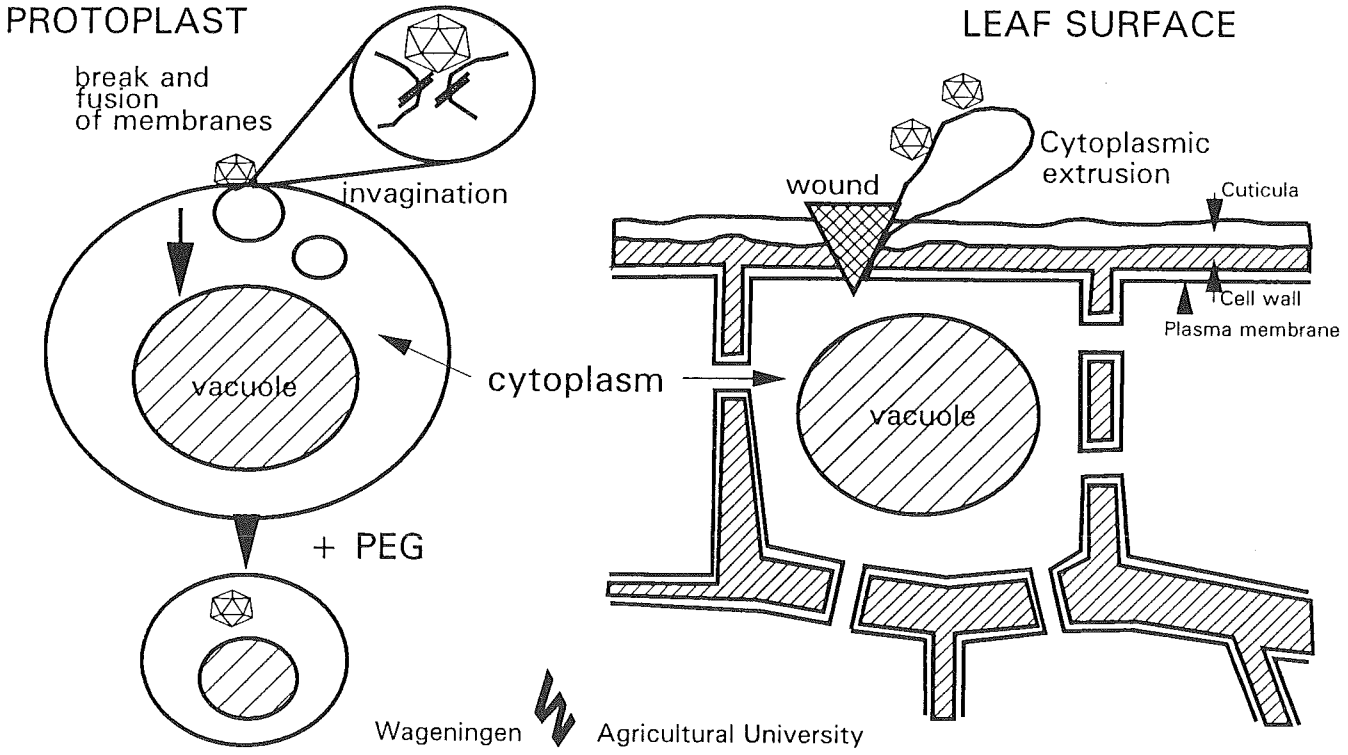

Figure 1. Model of virus entry in leaf protoplasts and in epidermal cells of leaf tissue. Protoplasts are inoculated by briefly mixing a suspension of protoplasts with virus or RNA and with a 40 % (w/v) polyethylene glycol (PEG, Mr 6000) followed by dilution of the mixture. The hydrophilic PEG competes with protoplasts and virus or RNA for hydration water causing the virus or RNA particles to come in close contact with the membrane of the protoplast. The osmotic changes of addition of PEG or dilution causes respectively shrinkage and swelling of the protoplasts. Invagination of the plasma membrane and fusion of lipid vesicles to the plasma membrane, necessary to remove or add the lipid bilayer, form the source of membrane discontinuities. Cytoplasmic extrusions have been demonstrated by Laidlaw (4). Careful contraction of the extrusion of the still viable cytoplast, with the concomitant invagination of the plasma membrane internalizes the virus through discontinuities of the plasma membrane. Both models may use the same mechanism of membrane discontinuity to allow virus or RNA particles to enter the cytoplasm.

nuities (breaks and fusions) in the plasma membrane. These discontinuities or lesions may be similar to the ones caused by electroporation (Fig. 1).

The virus deposited in the cytoplasm is then dissociated by ribosomes with concurrent translation of its exposed viral positive-sense genome. For at least four taxonomic groups this process of co-translational disassembly has been suggested and substantiated by experimental evidence in vitro. In all these cases, some destabilization of the virus particle prior to translation of the RNA by ribosomes has to be realized.

In this report we show that epidermal protoplasts of barley could be isolated and infected with brome mosaic virus (BMV)-RNA.

Materials and methods

Virus and barley were propagated as described before (2,3). Isolation of RNA and of mesophyl protoplasts from barley was also done as described (2,3). Isolation of epidermal protoplasts of barley was done according to Dietz et al. (6).

Results

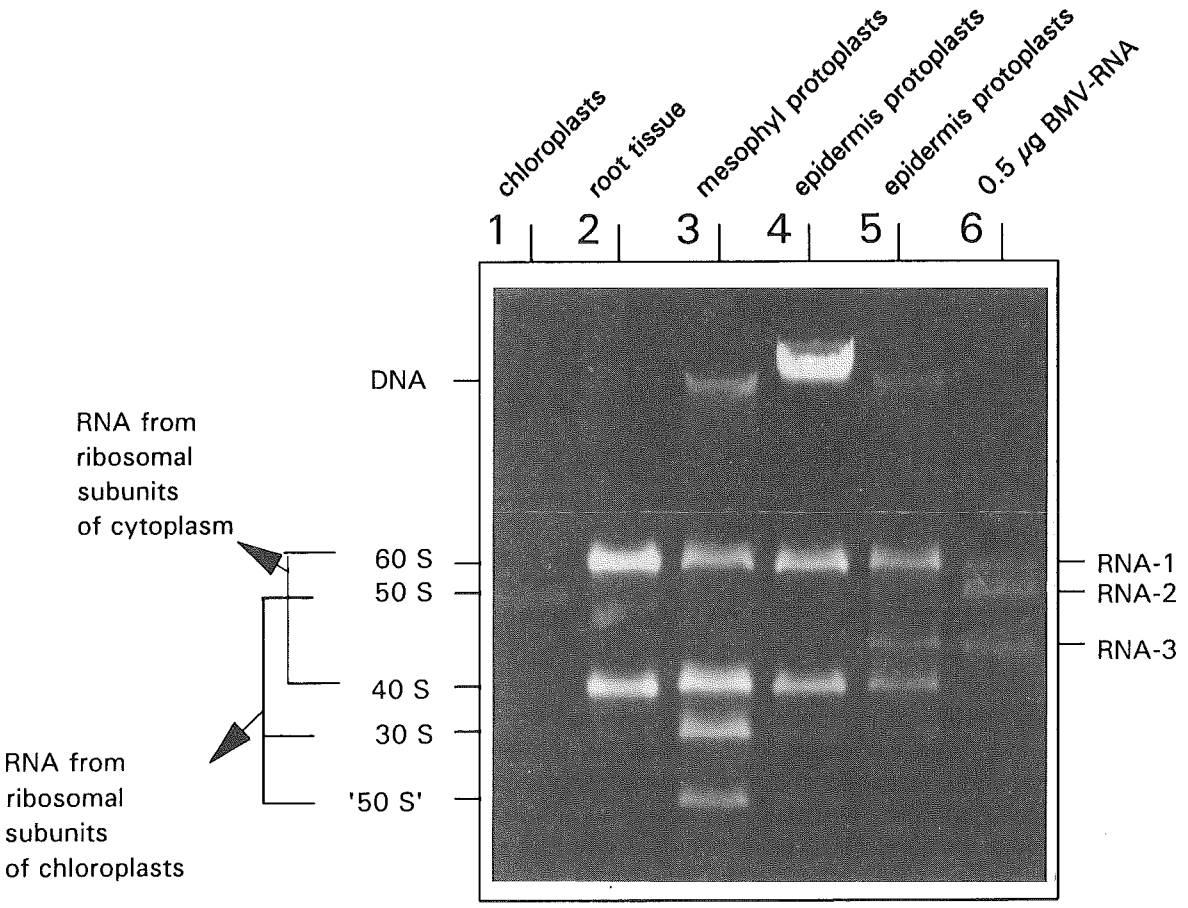

Figure 2. Ribonucleic acid contents of different tissues and organelles of barley and synthesis of viral RNA in epidermal protoplasts 20 hours post inoculation with brome mosaic virus (BMV)-RNA. The extracts of 50,000 protoplasts were loaded onto an 1 % (w/v) agarose gel. Lane 1: extract from chloroplasts, lane 2: extract from barley root tissue, lane 3: extract from barley mesophyl protoplasts, lane 4: extract from barley epidermal protoplasts, lane 5: extract from BMV-RNA-infected epidermal protoplasts, lane 6: 0.5 µg of marker BMV-RNA.

The enriched fraction of epidermal protoplasts from barley was still contaminated with a few percent of mesophyl protoplasts. This was demonstrated by the total nucleic acid extraction of mock infected epidermal protoplasts (Fig. 2, lane 4), where traces of nucleic acids from ribosomes of chloroplasts were seen. For comparison extracts of chloroplasts (lane 1) and root tissue without chloroplasts (lane 2) are given. Lane 3 shows the ribosomal nucleic acid composition of both prokaryotic (chloroplast) and eukaryotic (cytoplasm) extracts whereby the RNA from the 50-S-ribosomal subunit is specifically degraded ('50 S').

Inoculation with BMV-RNA resulted in synthesis of new viral RNA which is indicated by RNA-3 which can even be visualized in the ethidium-bromide-stained gel (Fig. 2, lane 5). Analysis of RNA synthesis at different times after inoculation by Northern blotting and of protein synthesis by Western blotting confirmed replication (results not shown).

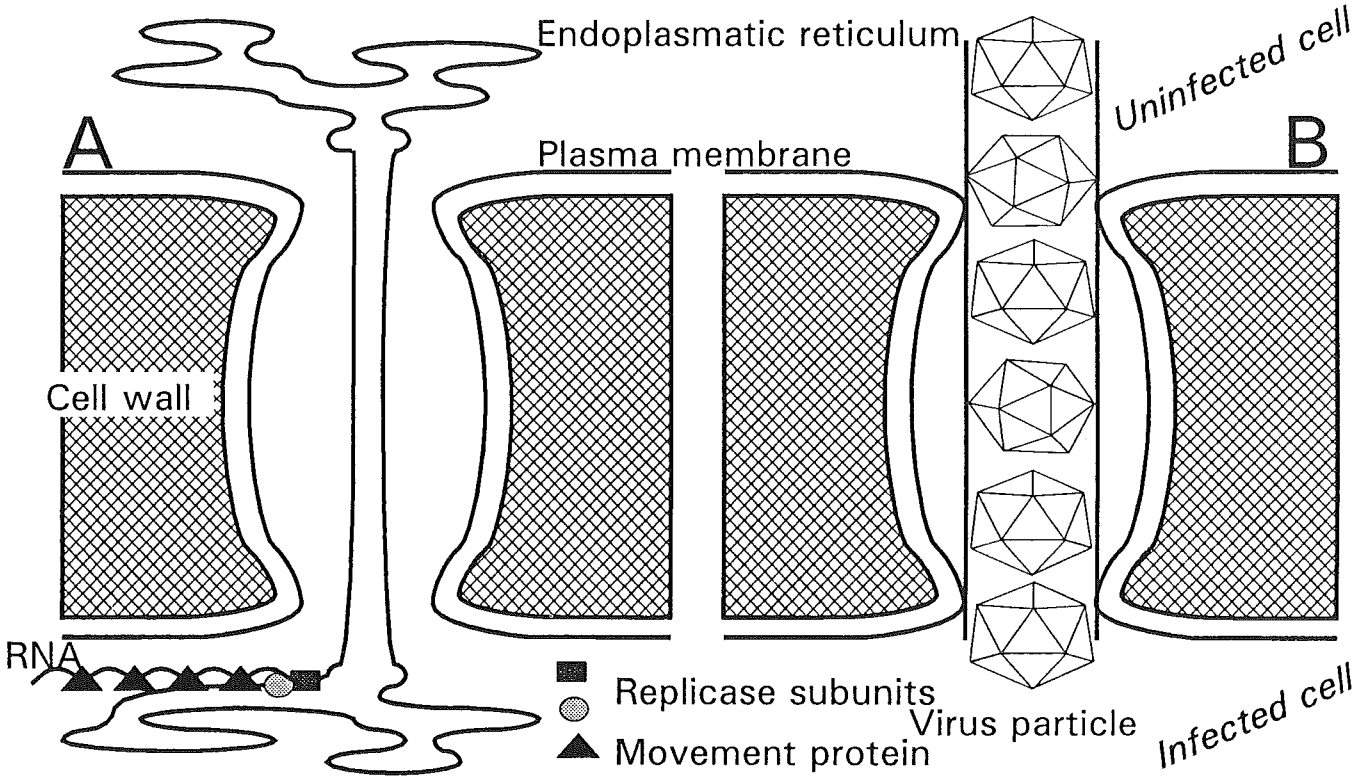

Figure 3. Two models of transport of viral genomes through plasmodesmata. Model A. The viral genome is transported as nucleic acid covered by movement protein. This protein binds specifically to single stranded and also widens the pore size of plasmodesmata. Specific targeting of the genomic RNA may be caused by subunits of the replicase complex, which is often associated with membranes. Model B. Complete virus particles are transported from the infected cell through viral coded tubule which run along the widened pore of the plasmodesmata and have removed the endoplasmatic reticulum.

Discussion

It was demonstrated that epidermal protoplasts of barley could be isolated and infected with BMV-RNA. Earlier Fannin and Shaw (7) had described the successful infection of isolated tobacco protoplasts. This supports the idea that virus particles deposited through wounds of the leaf surface and the plasma

membrane can potentially replicate in epidermal cells. After translation (possibly cotranslationally uncoated), transcription and replication either newly formed particles or nucleoprotein complexes might be involved in translocation of the viral genome through plasmodesmata from the infected epidermal cell to new healthy cells in plant tissue. For tobamoviruses (Fig 3 A) it is proposed that ribonucleic acid covered with at least the movement protein is transported along the endoplasmatic reticulum that runs parallel to the plasma membrane through the plasmodesmata. The movement protein binds specifically to single stranded nucleic acid and modifies the pore size of the plasmodesmata. Specific targeting of the genome-movement protein complex to the membrane may be caused by the replicase complex.

In another model (Figure 3 B) it is proposed that complete virus particles are transported through tubules which penetrate the widened plasmodesmata from the infected cell and extending into the healthy one. This model is proposed for como-, nepo- and geminiviruses (7). On the basis of amino acid sequence similarity of the movement protein of tobamovirus and the movement protein of bromovirus most likely follows the tobamovirus model.

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To the occurence of virus diseases in field and greenhouse crops in Saxonia

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Introduction

Since the end of seventies the laboratories of the Plant Protection Service in Dresden, Leipzig and Chemnitz carry out searches concerning the occurence of important virus diseases

The main fields are:
- pest control programm as a part of the official controlled program "Umweltgerechte Landwirtschaft"
- works in the sovereignty of the countries according to national rules
- submissions of the experts

the following crops are examined:

barley, wheat	BaYDV, BaYMV/BaMMV, WDV
sugar-beet	BYV, BMYV
rape	BWYV
pea	PEMV, PSbMV
potatoes	PLRV, PVY, PVX, PVS, PVM, PVA
plum, peach	PPV
cherry	PNRV, PDV
apple	virus and virus-like diseases as apple chlorotic leaf spot, apple mosaic, apple stem grooving, apple stem pitting, apple rubbery wood, apple flat limb, apple rough skin, apple star crack, (apple proliferation)
pear	pear ring pattern mosaic, rubbery wood, pear stony pit, pear vein yellows, (pear decline)
cucumber	CGMMV, CMV
tomato	ToMV
flower	TSWV/INSV in cut- and pot flowers Tobamo, CMV, PVY, TSWV in petunia and others

Results

Field crops. The barley yellow dwarf virus (BaYDV) is the most important virus of cereals in Saxonia. Searches concerning the occurence of BaYDV show the spreading of vector nonspecific PAV-strain in Saxonia with infection culminations in the year 1984 (barley), 1989 (wheat) and 1990 (barley).The administration district Leipzig belongs to the areas with a strong infection pressure (table 1). In the autumn of the last two years as well the vector occurence as the infection potential was low so that the barley was not endangered in the years 1994 and 1995.

To the most important viruses of cereals belongs also the barley yellow mosaic virus (BaYMV). After an inrease on the eighties the BaYMV-infections decrease considerably because of the cultivation of resistant varieties. HUTH (1991) refers to the spreading of a resistance refractive strain of BaYMV-2 in the old federal countries of germany. It is assumed that this strain also has spread in the new federal countries. Controls carried out in the last few years show no endangering in Saxonia.

In the year 1990 the wheat dwarf virus (WDV) has been detected first in germany near Dresden by the czech virologist Josef Vacke. Controls in the later years confirm the occurence especially in the region of Leipzig. But the virose was localized. Only in the year 1993 greater attacks have been detected near Leipzig and Hadmersleben.

The importance of sugar yellowing is greatly retrograded. This is due to the break of the infection chain especially because there was no cultivation of seed plants in the beginning of the nineties. Since 1992 especially the share of infection with beet yellows virus (table 2) is very low.

The spreading of the beet western yellows virus (BWYV) in winter rape may only be estimated as small to medium, although the culture has greatly spreaded in Saxonia (table 3).

Vegetables.In spite of all known hygienical provisions against cucumber green mottle mosaic virus there were again important losses in a specialized factory of cucumber production in the year 1993/94.

Flowers. Great problems give virus conditioned growth anomalies caused by Tobamo-Virus and CMV at petunias propagated by cuttings. In spite of searching suspicious samples of numerous greenhouse cultures concerning the tomato spotted wilt virus (TSWV) and impatients necrotic spot virus (INSV) both were not found in Saxonia up to now. The vector Frankliniella occidentalis has been detected in Saxonia since the end of eighties.

Fruit trees. Since 1992 tests of fruit viruses with woody indicators and ELISA at breeding materials (apple, pear, cherry) are carried out in Dresden. The acceptance of virus erradication by means of heat therapie and tip culture is started in 1995. Pillnitzer breedings as well as important wild forms, sorts and origins of the Gene Bank Fruit shall be stored in a Super Nuclear stock.

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Table 1. Infection incidence of barley with barley yellow dwarf virus (BaYDV) in administration district Leipzig, 1987-1994

date of sampling	testing sites	total	infected plants			vector
			%	max %	min %	
1/87	30	2400	0,6	2	0	Rh.m.
1/88	13	1300	1,9	6	0	Rh.m.
1/89	16	1560	1,9	6	0	Rh.m.
12/89	7	460	23,0	42	0	Rh.m.
4/90	100	3100	47,0	100	0	Rh.m.
1/91	10	410	13,0	20	5	Rh.p.; Rh.m.
4/91	4	210	17,0	25	0	Rh.p.; Rh.m.
4/92	20	700	17,0	25	0	Rh.p.; Rh.m.
4/93	12	220	2,3	10	0	Rh.m.
	4/94	170	0,6	5	0	S.a.

*Rh.m. = Rhopalosiphum maides
*Rh.p. = Rhopalosiphum padi
*S.a. = Sitobion avenae

Table 2. Infection incidence of sugar-beet with beet mild yellowing virus (BMYV) and beet yellows virus (BYV) in Saxonia, 1988 - 1995

date of sampling	district	testing sites	total	infected plants %	
				BMYV	BYV
9/88	L	3	74	84	57
6/89	C	3	120	8	0
	L	3	162	47	37
9/89	C	7	286	74	74
6/92	C*	4	265	25	10
	D*	7	140	95	0
	L*	4	270	28	0
9/92	C	4	53	45	26
	D	12	264	84	51
	L	4	318	14	0,3
6/93	C	5	317	53	0
	D	7	140	60	0
	L	1	80	0	0
9/93	C	5	236	26	0
	D	9	180	29	0
	L	5	400	0	0
6/94	C	4	158	63	0
	D	9	158	41	1
	L	2	75	0	0
9/94	C	5	136	22	12
	D	9	167	26	4
	L	8	160	0,1	0
6/95	C	5	100	1,0	0
	D	7	140	2,1	1,4
	L	2	100	3	1

* Chemnitz
* Dresden
* Leipzig

Table 3. Infection incidence of winter oil-seed rape with beet western yellowing virus (BWYV) in Saxonia, 1993 - 1995

date of sampling	district	testing sites	total	infected plants		
				%	max %	min %
11/93	C*	8	160	2,5	0,5	0
	D*	7	140	2,8	0,5	0
	L*	2	60	5,0	5	5
5/94	C	9	180	0,5	5	0
	D	4	80	5,0	20	0
	L	1	20	0	0	0
11/94	C	8	136	0,7	6	0
	D	9	157	0,6	6	0
	L	4	51	11,7	20	15
4/95	C	7	140	3,6		
	D	4	80	5,0	10	0
	L	4	80	26,3	60	1

* Chemnitz
* Dresden
* Leipzig

Contribution to testing and breeding for virus resistance in sour cherries (Investigations to natural infection of the PNRV by pollen in sour cherries and breeding for virus resistance)

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Abstract

In 1978 a program was started concerning Prunus Necrotic Ringspot (PNRV) in connection with testing and breeding for virus resistance in sour cherries. About 65 varieties, 40 clones and more than 2000 seedlings, have been investigated (WOLFRAM, B. et al., 1985). The virus status was tested at first in the Institute for Phytopathology in Aschersleben since 1991 in the Saxonian Centre for Agriculture. The extent of virus infection was ascertained. It was dependent on variety. Important results concerning virus transmission by seed and pollen were obtained. Crosses between virus free mothers an virus infected fathers yield more virus free progenies than those between virus free fathers and infected mothers. Virus infection by pollen transmission could be found to be delayed in some varieties and in some hybrids from our crossing experiments. On the other hand the tolerance of efficiency, that means a minimum decrease of growth and yield in the case of virus infection, is a further breeding goal. Some of these hybrids could be selected. First results on the heritability of this property are obtained and described.

Introduction

The Prunus Necrotic Ringspot Virus (PNRV) is the most important virus which is found especially in sour cherries. It can cause the following damages and symptoms :

- blossom buds are drying, mostly they have shorter stalks, it is possible that branches die or (in worst cases) the whole tree dies (shock symptom)
- growth, yield and selffertility can decrease
- less affinity of the graft budding in the nursery
- outgrowths on the bottom side of the leaf (enations)

PNRV can be also present without visible symptoms and damages
PNRV is transmitted by seed, pollen (dependent on variety), mechanically by graft budding and by nematodes.

There are different types of sensitivity in sour cherries caused by PNRV:

- **susceptible**: all damages and symptoms can appear
- **tolerant**: PNRV is present in the tree, but visible symptoms or decrease of yield, selffertility and growth do not occur
- **resistant**: this means pollenresistance; there are cultivars which are still virus free after ten or more years. This is a resistance to infection, which is quantitatively determined.

Our testing and breeding program was carried out with the following objectives:

1. establishment of the virus status and the type of virus sensitivity of cultivars and hybrids necessary for crossings
2. examination of several progenies concerning transmission and spreading of PNRV and to explore the heritability of pollenresistance
3. evaluation of a trial with six hybrids from 'Köröser' x 'Schattenmorelle'.

The following investigations and results refer to the Prunus Necrotic Ringspot Virus only disregarding other virusdiseases like Prune Dwarf Virus, Leaf Roll Virus or Little Cherry Virus. In 1978 a program was started concerning PNRV in connection with testing and breeding for virus resistance and virus tolerance in sour cherries. The first results were published in 1985 (WOLFRAM; B. et al.).

Material and methods

1. The virus status and phenomenological investigations in cultivars, hybrids and seedlings were continued. The transmission and the spreading of the PNRV were ascertained and lead to conclusions about the sensitivity types. About 40 sour cherry cultivars or hybrids (three trees per cultivar from the collection of the Genbank Obst, Dresden-Pillnitz, planted 1982/1983) and about 800 seedlings, progenies from 'Köröser', 'Schattenmorelle', 'Fanal', 'Leopoldskirsche' and hybrids 'Köröser' x 'Schattenmorelle' were investigated.
2. The behaviour of the trees concerning transmission and spreading of PNRV by pollen, yield and growth were investigated. The type of sensitivity of the progeny 'Köröser' x 'Schattenmorelle' was examined.
3. Within this program a trial was planted in 1980 (until 1990) with virus infected and virus free trees of the following cultivars and hybrids: 'Köröser' (15 trees virus free), 'Schattenmorelle' (27 trees virus free) and six hybrids - progenies from 'Köröser' x 'Schattenmorelle': 21,1 (17 trees virus free, 13 virus infected); 43,87 (20 trees virus free, 9 virus infected); 24,46 (14 trees virus free, 6 virus infected); 21,2 (14 trees virus free); 13,9 (22 trees virus free); 14,32 (10 trees virus free, 10 virus infected). 14,32 was infected by seed, made virus free by heat treatment and than on another site planted virus free and virus infected. 'Schattenmorelle' and 'Köröser' were virus free only and not virus infected at the time of planting.

The virus status was tested by ELISA (enzyme-linked immonosorbent assay) or Shirofugen. The testings were carried out at first in the Institute for Phytopathology in Aschersleben and since 1992 in the Saxonian Centre for Agriculture.

Results

1. About 40 sour cherry varieties and hybrids were tested for PNRV in 1985, 1990, 1993, 1994 and 1995. Most of the tested cultivars were infected by PRNV until eightst year after planting. These results were very similar to those published by WOLFRAM, B. et al. 1985 and by GRÜNTZIG, M. et al. 1989. Therefore the known cultivars were classifed into three types of sensitivity regarding of their behaviour in the collections (table 1). The classification refers only to trees which always have a high and constant yield or which are infected later without differences to yield. This classification is not definitive and variations are possible depending on pressure of infection, the virulence of the PNRV and the site of plantations. For example the variety 'Schattenmorelle', in the literature defined as susceptible, has a delayed infection by pollen

in most of the investigations. It reacts susceptible if infected. 'Röhrigs Weichsel' and 'Leopolds-kirsche' have also a very delayed infection by pollen. But they react tolerant to PNRV concerning yield and growth if infected. Some trees lost possibly due to hypersensitivity to PNRV

2. Transmission of PNRV by seed firstly depends on the virus status of the crossing parents. Crosses between virus free seed parents and virus infected pollen parents as a rule yield more virus free seedlings than those between virus infected mothers and virus free fathers (table 2). Secondly the transmission of PNRV by seed and pollen is conditioned genetically by the variety (table 2). For example crossings with 'Köröser' lead to increased rate of infected trees than crossings with 'Schattenmorelle'.

3. Testings and investigations concerning transmission, spreading of PNRV, yield and growth were carried out in the trial from 1980 until 1990 with six hybrids of the combination 'Köröser' x 'Schattenmorelle' and their parents. 'Schattenmorelle' and two hybrids (21,2 and 13,9) were infected in this time with less than 15%. 'Köröser' and especially the hybrids 43,87; 24,46 and 21,1 were infected to more than 80%.
The results concerning yield and growth of the hybrids 21,1; 43,87; 24,46 including 14,32 (virus free and virus infected) at the time of the planting can be seen in table 4.
The yield of the virus free trees was higher. The values (table 4) were calculated with the analysis of variance. There after significant differences concerning yield between virus free and virus infected trees could be found only disregarding the interrelations between hybrids and years. Differences concerning growth are contradictory. It is possible that as a result of missing PNRV a higher yield decreased the growth of the trees.

Conclusions

1. A lot of sour cherry cultivars are pollenresistant (s. Table 1), that means the natural infection by pollen is very delayed. Such varieties if infected can react to PNRV sensitive/hypersensitive or tolerant. For example: the pollenresistant 'Schattenmorelle' and 'Korund' react to PNRV sensitive or hypersensititve. The pollenresistant 'Röhrigs Weichsel' and 'Leopoldskirsche' react to PNRV tolerant.

2. Some varieties like 'Köröser' and 'Karneol' immediately are infected naturaly and react to PNRV tolerant.

3. In progenies ('Köröser' x 'Schattenmorelle') characteristics concerning types of susceptibility could be found.

4. The aim of breeding is the selection of pollenresistant and tolerant types. Pollenresistant types are to favor.

Table 1. Classification of the cultivars in the types of sensitivity to PNRV

susceptible (sensitive/hypersensitive)	**tolerant**	**resistant by pollen** (that means a very delayed infection)
Schattenmorelle	Köröser	Röhrigs Weichsel
Korund (Pi-Sa 14,32)	North Star	Fanal
	Kelleriis 16	Ujfehertoi fürtös
	Erdi bötermi	Königin Hortense
	Meteor (USA)	Eugenie Imperatrice
	Karneol (Pi-Sa 21,1)	Paljus
	Morina (Pi-Sa 5,4)	Schwäbische Weinweichsel
	Pi-Sa 7,55	Oblacinska
	K 27/2	Shirpotreb Tschornaja
	Leopoldskirsche	Stevnsbear
		Schöne von Montreuil
		Rivers Durchscheinende
		Leopoldskirsche
		Safir (Pi-Sa 6,38)
		Schukowskaja
		Schattenmorelle
		Korund

Table 2. Natural transmission and spreading of PNRV by pollen in several crossing combinations

Year of sowing	crossing combination	number (n) of seedlings	years of testing		
			1988 virusfree %	1991	1994/95
1985	Köröser$^-$ x Schattenmorelle$^-$	121	(100)	93	80
1980	Köröser$^-$ x Fanal$^-$	13	100	69	38
1985	Köröser $^-$ x Leopoldskirsche$^-$	32	-	63	-
1982	Köröser$^+$ x Pi-Sa 2,37 (Topas)$^-$	29	-	72	62
1980	Köröser$^+$ x Korund $^+$ (Pi-Sa 14.32)	12	66	58	-
1981	Köröser$^+$ x Pi-Sa 24,46$^-$	14	-	14	0
1980	Schattenmorelle$^-$ x Fanal$^-$	71	99	79	72
1985	Schattenmorelle$^-$ x Leopoldskirsche$^-$	12	-	100	100
1982	Schattenmorelle$^-$ x Pi-Sa 21,2$^-$	17	-	94	82
1980	Schattenmorelle$^-$ x Pi-Sa 17,5$^+$	12	-	83	67
1982	Schattenmorelle$^+$x Pi-Sa 14,32$^+$	20	-	20	10
1983	Pi-Sa 14,32$^+$ x Schattenmorelle$^-$	8	75	0	0
1983	Pi-Sa 43,87$^-$ x Schattenmorelle$^-$	15	100	67	-
1981	Pi-Sa 17,5$^+$ x Pi-Sa 21,1$^-$	15	20	20	13
1980	Pi-Sa 43,87$^-$ x Pi-Sa 21,2$^-$	14	100	100	50

- virusfree at time of the crossing; + virusinfected

Table 3. Natural transmission of PNRV by Pollen

Cultivar/hybrid	1980	1982	1986	1988	1990	number of virus infected trees after ten years	
	number of virus free trees					n	%
Schattenmorelle	27	27	27	27	25	2	7,4
Köröser	15	15	15	12	5	10	66,7
Pi-Sa 43,87	15	15	14	14	1	14	93,3
Pi-Sa 24,46	14	13	12	12	3	11	78,5
Pi-Sa 21,1 (Karneol)	17	17	12	8	1	16	94,1
Pi-Sa 21,2	14	14	14	14	12	2	14,3
Pi-Sa 13,9	22	22	21	21	21	1	4,5

Table 4. Yield and growth of virus free and virus infected trees of 4 progenies Köröser x Schatten-morelle

hybrid	number of trees	yield / kg per tree									crown vol. m^3
		1983	1984	1985	1986	1987	1988	1989	1990	mean	1990
21,1											
virus free	17	2,4	4,9	5,4	1,0	10,7	4,6	11,7	5,9	5,8	12,8
virus infected	13	1,5	1,4	5,3	1,4	7,4	2,0	7,7	4,0	3,8	14,4
43,87											
virus free	20	3,0	4,3	1,5	1,7	8,3	2,0	1,5	3,1	3,2	10,3
virus infected	9	0,8	2,4	0,8	1,1	4,3	0,6	0,8	2,3	1,6	6,7
24,46											
virus free	14	3,0	5,3	2,1	0,1	8,2	2,5	3,2	2,1	3,3	7,6
virus infected	6	1,2	1,6	1,4	0,1	10,0	0,5	3,0	1,3	2,4	6,9
		1988	**1989**	**1990**	**1991**	**1992**	**1993**	**1994**		**mean**	**1993**
14,32											
virus free	10	0,4	12,1	4,0	-	21,8	9,0	18,5		11,0	6,2
virus infected	10	0,3	2,7	1,5	-	16,3	4,5	15,0		6,7	7,6

Bacterial diseases of plants

STUDIES ON THE RESISTANCE OF CORIANDER AGAINST BACTERIAL UMBEL BLIGHT CAUSED BY *Pseudomonas syringae* pv. *coriandricola*

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Introduction

Bacterial blight of coriander is causing considerable yield losses all over the world. The disease has been reported in England (1), Hungary (2), Russia (3), Poland (4), Mexico (5), U.S.A (6) and South Australia (Hayward, pers. communication). In Germany the disease was reported for the first time in 1989 (7).
Characterization of the pathogen in Göttingen revealed that the disease is caused by a hitherto unnamed pathovar of *Pseudomonas syringae*. Therefore, the new name *Pseudomonas syringae* pv. *coriandricola* was proposed (8),(9).

The goal of our studies was to test a large number of coriander lines from many parts of the world for resistance against the pathogen.

Material and Methods

We screened 267 lines of coriander in the glasshouse for resistance. Pretests revealed that the coriander plants are susceptible to the pathogen in the umbel stage as well as in the rosette stage. Therefore, we tested all lines in the rosette stage in the glasshouse, in order to save time and space.
To determine the optimum inoculum concentration, we tested concentrations between 10^2 and 10^8 cfu/ml. The abaxial sides of young leaves were infiltrated with a bacterial suspension by an atomizer attached to a pressure pump. The plants were incubated in a glasshouse under optimal conditions for the pathogen (22-25 °C and 50-70% RH). Fourteen days after inoculation the plants were evaluated for disease development, especially water-soaked lesions. The plants were classified according to the percent of infected leaf area in highly resistant, resistant, weakly susceptible, susceptible and highly susceptible.
In order to check the inoculation conditions the susceptible line "Corry" was inoculated along with resistance screening. Pretests revealed that the line Corry reacts constantly susceptible to the pathogen (10).
Bacterial multiplication in different coriander lines was also studied. The abxial sides of young leaves of two resistant and two susceptible lines were inoculated with a bacterial concentration of 10^5 cfu/ml. In these experiments we estimated

the plants every day for symptom development. Bacterial concentrations were determined in intervals of 2, 4, 6, 8, 10, 17, 21 and 25 days.

Results

An inoculum concentration of 10^4 cfu/ml appeared to be best suited to screen coriander plants in the rosette stage for resistance. Under these conditions the leaves never developed necrotic spots (Table 1). The water-soaking was more intensive on young leaves than on mature leaves.

Table 1: The effect of bacterial concentration, leaf age, and leaf type on development of disease symptoms.

concentration of inoculum cfu/ml	Young leaves	Mature leaves	Pinnate leaves
control	-	-	-
10^2	-	-	-
10^3	+	-	-
10^4	++	+	-
10^5	+++ n	++ n	- n
10^6	n	n	n
10^7	n	n	n
10^8	n	n	n

-	no typical symptoms
+	water-soaked lesions
n	necrosis

Inocula with 10^5 cfu/ml caused water-soaked spots as well as necrosis, and bacterial concentrations higher than 10^5 cfu/ml produced necroses in all leaf stages. The pinnate leaves never developed water-soaked spots but only necroses by high bacterial concentrations. The necrotic spots caused by high bacterial concentrations also on rosette leaves could be falsely misinterpreted as a resistant reaction.

The resistent reaction differed from the susceptible reaction in that the water-soaked spots were smaller (about 0,5 mm) than on susceptible plants (about 2-4 mm). In addition, the bacteria needed 6 days to cause the first water-soaked spots on leaves of resistent lines, while only 4 days were necessery to cause spots on susceptible lines. On the other hand,the resistant plants developed necrosis within 14 days after inoculation, while the susceptible plants needed at least 21 days to develop necrosis.
The multiplication of the bacteria was four-fold higher in the leaves of the susceptible lines compared to resistant lines (Figure 1).

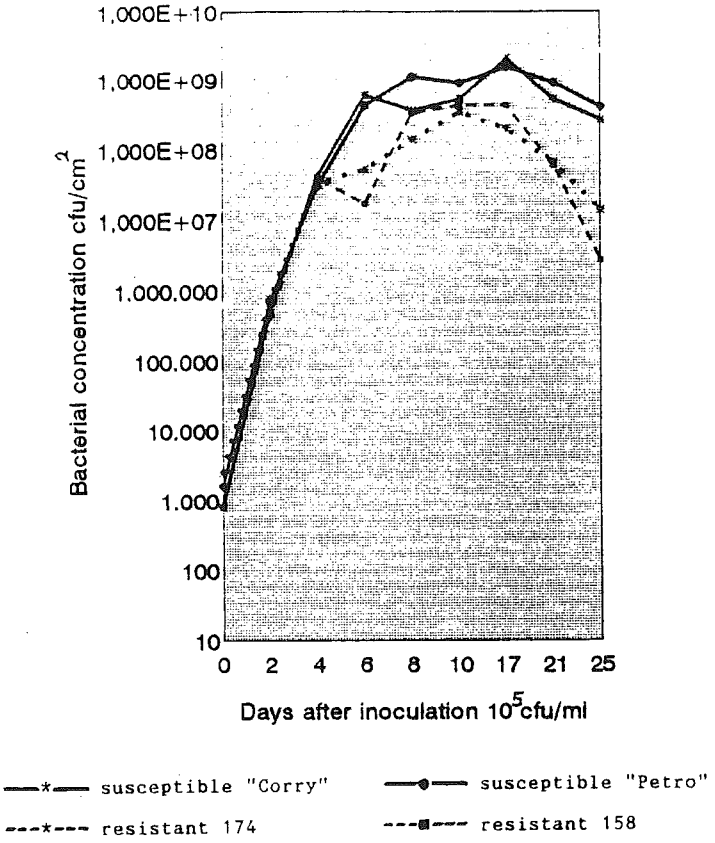

Figure 1: Bacterial multiplication in leaves of resistant and susceptible coriander lines.

From 267 lines tested in the glasshouse only 7 reacted highly resistant, 50 resistant, 44 weakly susceptible, 76 susceptible and 32 highly susceptible. The reaction of 58 lines could not be determined because the plants grew very fast without any rosette stage (Figure 2).

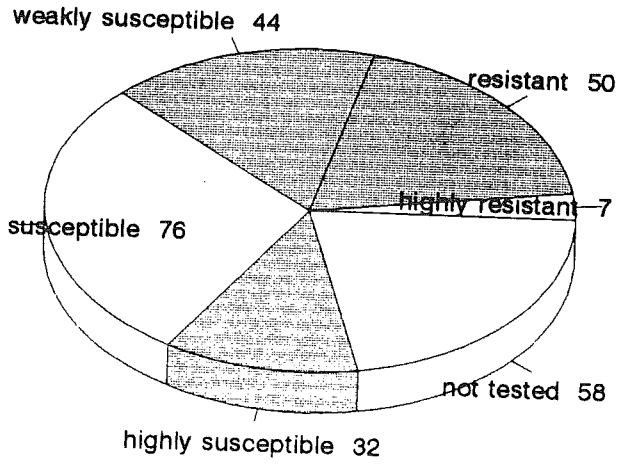

Figure 2: Reaction of coriander lines against *Pseudomonas syringae* pv. *coriandricola*.

Discussion

The heterogeneity of most of the lines presented a big problem in these studies. Therefore we selected and propagated single highly resistant plants. Another problem was presented by the fast growing plants which did not develop any rosette stage. These coriander lines will be screened for resistance in the umbel stage.
Unfortunately, we could not produce the disease on the field during the summer of 1994 because of unnormally high temperatures. These experiments are being repeated in 1995.

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THE POSSIBILITY OF APPLICATION ATB COMPUTER SYSTEM FOR IDENTIFICATION OF PHYTOPATHOGENIC GRAM NEGATIVE BACTERIA

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Introduction

Several methods for rapid identification of phytopathogenic bacteria have been developed in recent years. Many of them have been derived from methods which are applied in medical microbiology for human pathogens (1). One of contemporary methods, which has alredy been used for diagnosis of human diseases, is identification of pathogens by computer systems.
There are only a few data about usage of computer and similar systems for identification plant pathogenic bacteria in phytopathological literature. Reviews of available computer programs and various API systems were given (2).
The aim of this paper is to investigate the possibility of application ATB computer system (the ID 32 GN strips), for identification of phytopathogenic bacteria, which is firstly intended for diagnosis of human pathogens.

Material and Methods

STRAINS. - In these researches were included over 20 bacteria strains determinated by conventional methods.
The determinated strains originated from several phytopathological laboratories (Institute for Plant Protection, Novi Sad / Yugoslavia/ ; University of Horticulture and Food Industry, Department of Plant Pathology, Budapest / Hungary / ; Plant Health and Soil Conservation Station, Pecs /Hungary / ; Benaki Phytopathological Institute, Kiphissia-Athens / Greece / and Institut for Wheat and Sunflower "Dobrudja", G.Toshevo / Bulgaria/) (Table 1).
COMPUTER SYSTEM. - The investigations were carried out according to recommendations of producer of ATB system (BIO MERIEUH, France). The results were read after 24 and 48^{h} of incubation.
The ID 32 GN strips were used. These strips consist of 32 cupules, each containing the dehidrated carbohydrate substrates* . These substrates present specific biochemical line, adapted for identification gram negative bacteria.
List of taxa (ID 32 GN) include about 100 species / group , mainly of human pathogens. There are only several species of plant pathogens as *Pseudomonas cepacia* / Burkholder / Palleroni et Holmens, *Xanthomonas campestris* /Pammel/ Dowson, *Agrobacterium tumefaciens* / Smith et Townsend / Conn. ; genus *Erwinia* as *Erwinia* spp. 1 and *Erwinia* spp. 2 , and so-called "weakness" parasite as *Pseudomonas fluorescens* / Trevisan / Migula and *Pseudomonas aeruginosa* / Schroeter / Migula .
Pathovars of investigated bacteria don't exist in data base.

*Manitol, D-Glucose, Salicin, D-Melibiose, L-Fucose, D-Sorbitol, L-Arabinose, Propionate, Caprate, Valerate, Citrate, Histidine, 2-ketogluconate, 3-hydroxy-butyrate, 4-hydroxy-benzoate, L-proline, Ramnose, N-acetylglucosamine, D-Ribose, Inositol, D-Saccharose, Maltose, Itaconate, Suberate, Malonate, Acetate, DL-Lactate, L-Alanine, 5-ketogluconate, Glycogen, 3-hydroxy-benzoate i L-serine (biochemical line).

Results

The results of identification phytopathogenic bacteria by computer ATB system (the ID 32 GN strips) are shown in Table 1.

Table 1 Identification of phytopathogenic bacteria by computer ATB system (the ID 32 GN strips) after 24 and 48^{h} of incubation

Determinated bacteria and strains	Identificaion bacteria by computer** Estimates of identification	
	24^{h}	48^{h}
-P.cepacia /ICMP 5952/	P.cepacia -Excellent id.	P.cepacia -Excellent id.
-P.fluorescens /19/	P.fluorescens 2 -Excellent id.	P.fluorescens 2 -Doubtful profile
-P.aeruginosa /ATCC-27853/	P.aeruginosa-1 -Excellent id.	P.aeruginosa-1 -Excellent id.
-X.c.pv.vesicat. /X-12/	X.campestris -Very good id.	X.campestris -Very good id.
-X.c.pv.vesicat. /VES-10/	X.campestris -Good id.	X.campestris -Good id.
-X.c.pv.campestr. /Ku-1/	X.campestris -Very good id.	X.campestris -Good id.
-X.c.pv.phaseol. /SŠ/	X.campestris -Good id.	X.campestris -Doubtful profile.
-X.c.pv.phaseol./XB 90471/	X.campestris -Acceptable id.	X.campestris -Doubtfu profile
-E.c.ssp.carotovora /EC/	Erwinia spp. 1 -Very good id.	Erwinia spp. 1 -Very good id.
-E.carotovora /K-II 4/	Erwinia spp. 1 -Excellent id.	Erwinia spp. 1 -Excellent id.
-E.c.ssp.atros. /ATR-435/	Erwinia spp. 1 -Good id.	Erwinia spp. 1 -Good id.
-E.chrisanthemi /AGL-11/	Erwinia spp. 1 -Good id.	Erwinia spp. 1 -Very good id.
-E.amylovora /ERA 31/85 /	Erwinia spp. 2 -Low discrimination	Erwinia spp. 2 -Low discrimination
-E.amylovora /KR-1/	Erwinia spp. 2 -Low discrimination	Erwinia spp. 2 -Low discrimination
-E.amylovora /J-2/	Erwinia spp. 2 -Low discrimination	Erwinia spp. 2 -Low discrimination
-P.s.pv.syringae /KR-1-4/	P.fluorescens 2 -Acceptable id.	P.fluorescens 2 -Unacceptable profile
-P.s.pv.syringae /P-1 M/	P.fluorescens 2 -Doubtful profile	P.fluorescens 2 -Doubtful profile
-P.s.pv.syringae /K 1 2/	P.fluorescens 2 Unacceptable profile	P.fluorescens 2 -Doubtful profile
-P.s.pv.syringae /IM-21/	P.fluorescens 2 -Doubtful profile	P.fluorescens 2 -Unacceptable profile
-P.s.pv.phaseol. /Pph-8/	Different genera -Doubtful profile	Differenr genera -Uacceptable profile
-P.s.pv.glycinea /Sj-1/	Different genera -Doubtful profile	Different genera -Unacceptable profile

THE ESTIMATES OF IDENTIFICATION**

- Excellent identification % id ≥ 99,9 and T ≥ 0,75
- Very good identification % id ≥ 99,0 and T ≥ 0,5
- Good identification % id ≥ 90,0 and T ≥ 0,25
- Acceptable identification % id ≥ 80,0 and T ≥ 0

- % id - an estimate of how closely the profile corresponds to the taxon relative to all the other taxa in the data base.
- T index - an estimate of how closely the profile corresponds to the most typical set on reactions for each taxon. Its value between 0 and 1 and is inversely proportional to the number of atypical tests.

The results are shown in Table 1. Identification of phytopathogenic bacteria carried out by computer ATB system (the ID 32 GN strips), sufficiently corresponds to identification made by conventional methods, if investigated bacteria are included in data base, as *P.cepacia, X.campestris, P.fluorescens* and *P.aeruginosa.* These pathogens can be identified to the species level. Pathovars of *X.campestris* were identified as *X.campestris.* There aren't possibilities for distinguishing of *X.campestris* pathovars, because they don't exist in data base.
Erwinia spp. can be identified to the genus level, as *Erwinia* spp. 1 or *Erwinia* spp. 2, that could be coresponding to "cartovora" or "amylovora" groups. Identification of strains of group "carotovora" which are identified as *Erwinia* spp. 1, is more reliable ("good", "very good" or "excellent"), than identification of strains of bacterium *E.amylovora,* which are identified as *Erwinia* spp. 2 ("low discrimination"). Very complicated taxonomic structure (with different phenons and subphenons) of the genus *Erwinia* was shown (3) loc.cit.(2).
Identification of *Pseudomonas syringae* pathovars isn't possible by ID 32 GN strips, because the species *P.syringae* Van Hall isn't included in data base. The strains of *P.s.*pv.*syringae* are identified as *P.fluorescens* ("doubtful" or "unacceptable" profile) in most cases. These results indicate to reationship between *P.syringae* strains and *P.fluorescens.* Some pathovars of *P.syringae*, as *P.s.*pv.*phaseolicola* and *P.s.*pv.*glycinea* were classified in different genera ("doubtful" or "unacceptable" profile).
For complete application ATB computer system (with the ID 32 GN strips) in identification of phytopathogenic bacteria, it would be necessary to enlarge the data base with the important members of phytopathogenic bacteria (with pathovars) and to include still some specific bacteriological tests in biochemical line. Checking of pathogenicity can be used if the result of identification isn't enough satisfactory.

Conclusion

On the basis of given results it can be concluded that computer ATB system (the ID 32 GN strips), can be used sufficiently for identification of some phytopathogenic bacteria, if they are included in data base, as *P.cepacia, X.campestris* and some "weakness" pathogen as *P.fluorescens* and *P.aeruginosa.* They can be identified to the species level. Pathovars of *X.campestris* were identified as *X.campestris.* There aren't possibilities to disingushing pathovars of *X.campestris.*
Erwinia spp. can be identified to the genus level, as *Erwinia* spp. 1 or *Erwinia* spp. 2, that quite possibly could be corresponded to "carotovora" or "amylovora" groups. Identification of strains of group "carotovora" as

Erwinia spp. 1 is more reliable ("good" to "excellent"), than identification of strains which identified as *Erwinia* spp. 2 ("low discrimination").
The identification of *P.syringae* and his pathovars isn't possible, because the species *P.syringae* isn't include in data base. In most cases *P.s.*pv.*syringae* strains were identified as *P.fluorescens* 2 ("doubtful" or "unacceptable").

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A SIMPLE METHOD FOR EXTRACTION OF ELECTROPHORESED PROTEINS FROM SDS-POLYACRYLAMIDE GELS AND ITS USE FOR THE PRODUCTION OF MONOSPECIFIC POLYCLONAL ANTISERUM AGAINST THE SOFT ROT ERWINIAS.

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Introduction

The production of monospecific polyclonal antiserum (MPA) directed against plant pathogenic bacteria was reported earlier (1,5) and was based on the use of a unique polypeptide of the target organism as specific antigen for immunizing rabbits. For the production of MPA, the selected proteins were usually eluted by excising the corresponding protein band from an unstained, electroblotted nitrocellulose membrane (NC). After dissolving the NC stripe in dimethyl sulfoxide (DMSO), the protein-NC mixture was precipitated in buffer (PBS, pH 7.2) and the suspension was injected into rabbits (1). However, unique protein bands of the soft rot erwinias (*Erwinia carotovora* subsp. *atroseptica* (*Eca*), *E. c.* ssp. *carotovora* (*Ecc*) and *Erwinia chrysanthemi* (*Ech*)), after separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (3), were located close to each other. Even at a lower gel concentration, the selective elution of the specific protein from the unstained, electroblotted NC was difficult, without including neighbouring protein bands. This paper describes a simple method for extracting electrophoresed proteins from SDS-PAGE gels and its use for the production of MPA which can be used for the detection of soft rot erwinias. In addition, a method for elution of the specific proteins from unstained gels to immunize rabbits is also described.

Materials and methods

The bacterial strains, *Erwinia carotovora* subsp. *atroseptica* (strains SCRI 1039, 1092, 1054, 41), *Erwinia carotovora* subsp. *carotovora* (strains SCRI 133, 275, 320), *Erwinia chrysanthemi* (strains SCRI 4044, 4042, 4043, PD 483), *Pseudomonas fluorescens* (strains GSPB 1199, 1714) and *Pseudomonas marginalis* (strain GSPB 92) were provided by Dr. Perombelon (SCRI, Dundee, Scotland) and Dr. Mavridis (Institut für Pflanzenpathologie und Pflanzenschutz der Universität Göttingen, Germany). The bacteria were maintained on YDC agar slants at 4 and 18° C in screw cap test tubes. For the experiments, the bacteria were grown on YDC or NA agar plates (4) for 2 - 3 days at 25° C.

Protein extraction buffers (100 ml)

(A) Lysozyme buffer: 50 mM Glucose, 10 mM EDTA,and 25 mM Tris / HCl (pH 8.0). Lysozyme was added at a concentration of 2 mg / ml to the above solution just before use.
(B) Modified bromphenol blue buffer (BPB): 200 mM Tris / HCl (pH 8.8), 500 mM saccharose, 5 mM EDTA, and 3.0 % SDS.

Procedure for the extraction of eletrophoresed proteins from SDS-PAGE gels.

(1) Separation of the bacterial proteins by SDS-PAGE and staining of the gels with Coomassie blue, and subsequent destaining by acetic acid / ethanol treatment. The electrophoresis procedure was performed as described earlier (3);

(2) Electroblotting and immunostaining (Western blot) of proteins after separation by SDS-PAGE (2,5,6);

(3) Selection of a specific protein band of the target bacteria based on the results obtained in 1 and 2 above;

(4) Excision of the selected protein band from the Coomasie blue stained gel and maceration of the gel stripes in 100 µl of lysozyme buffer (A) in an Eppendorf tube;

(5) Add 150 - 200 µl of BPB buffer (B) and 7 µl of β-Mercaptoethanol to the macerate, mix properly and incubate in boiling water for 8 - 10 min. Cool 4 - 5 min.on ice;

(6) Extract the proteins from the macerate by centrifugation at 10,000 *g* for 10 min.. Carefully pipette the supernatant containing the proteins into sterile Eppendorf tubes;

(7) Repeat steps 5 and 6, 2 to 3 times using solution (B), until the gel pieces are colourless. Combine the supernatant with the previous extractions and store at -20° C.

Selection of specific proteins of *Eca* and *Ech*.

By comparing the molecular weight of the electrophoresed bacterial proteins with the reference protein bands (Merck standard 4, Merck, Darmstadt, Germany), proteins which were unique to *Eca* and *Ech*, respectively, and which bound the antibodies in immunoblots were selected as specific antigens and were extracted as described above. To include all possible antibody reactions to all occurring *Eca* and *Ech* strains, the protein bands of 4 representative *Eca* and *Ech* strains were chosen and used as antigen for immunization of rabbits.

Use of the extracted proteins of *Eca* and *Ech* to generate MPA in rabbits

The extracted proteins were again separated by SDS-PAGE. Using Coomassie blue stained borders as markers, the unstained middle part of the gel containing the proteins was excised, sealed in dialysis tube containing Tris / Glycine buffer (0.3 % Tris and 1.44 % Glycine (w/v)) and submerged in the same, ice cooled buffer in an agarose gel electrophoresis chamber. Elution of the proteins from the gel stripes was performed by passing constant current of 22 - 23 mA for three hours. The buffer in the dialysis tube was carefully pipetted into sterile Eppendorf tubes, centrifuged at 10,000 *g* for 5 min., and the supernatant was mixed with Freund's incomplete adjuvant (Difco, Detroit, USA) at a ratio of 1:1. The suspension was injected subcutaneously into a rabbit (repeated twice at 7 days interval). Extraction and purification of the antibodies directed against the proteins were performed as described earlier. (1,5).

Results

A comparison of protein bands of *Erwinia* spp. and other bacterial species separated by SDS-PAGE with reference protein bands (Merck standard 4) revealed a 43 kDa and a 40 kDa sized protein that was unique to *Eca* and *Ech*, respectively. The two protein bands were selectively extracted by using BPB buffer as described above. By using Western blot procedure, only one protein band was visible after the *Eca* extracted protein was electroblotted onto NC membrane filters and probed with *Eca* polyclonal antiserum (Fig. 1). Routine immunization of rabbits with the 43 kDa and 40 kDa proteins of *Eca* and *Ech*, respectively, eluted from unstained SDS-PAGE gels using Tris / Glycine buffer yielded MPA which showed in a Western blot, a strong antibody reaction only with the 43 kDa and 40 kDa protein band of *Eca* and *Ech*, respectively. (Fig. 2). The results of the detection of *Eca* in

potato extract using *Eca* MPA in the Western blot procedure is shown in Fig. 3. The result reveals a clear detection of *Eca* from the samples, because the antiserum reacted only with the 43 kDa protein of *Eca*. Similarly, using indirect ELISA procedure (4), *Eca* was also detected in an artificially inoculated potato tuber extract, however, only at higher bacterial concentrations (> 10^5 cells / ml).

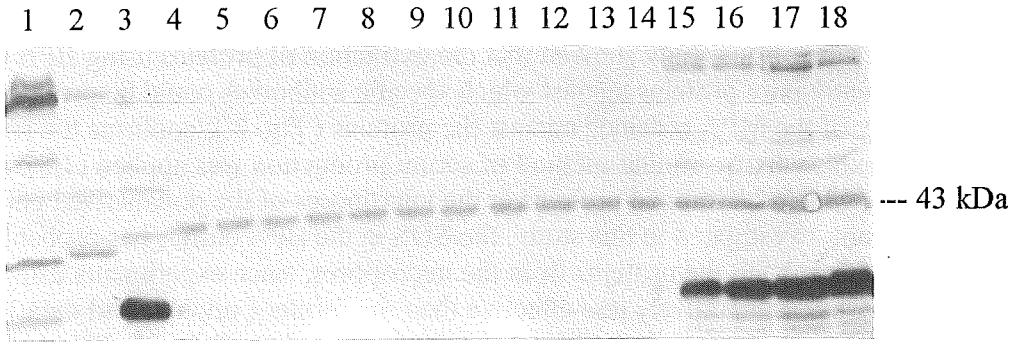

Fig. 1. Reaction of the extracted 43 kDa protein of *Eca* with *Eca* polyclonal antiserum (1: 300) in a Western blot. Lane 1: *Ech* 4044; lane 2: *Ecc* 275; lane 3: *Ecc* 133; lanes 4 - 14: 43 kDa protein of *Eca* extracted from Coomassie blue stained SDS-PAGE gels using BPB buffer; lanes 15 - 18: *Eca* strains 41, 1092, 1054, and 1039, respectively.

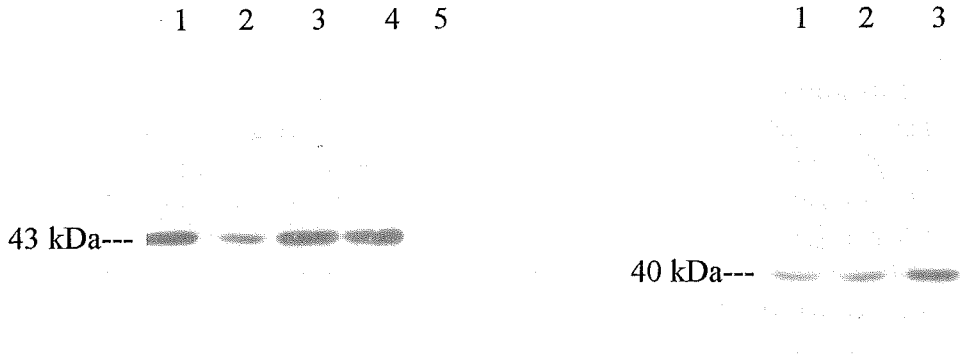

Fig. 2. (a) Reaction of blotted proteins of *Eca* strains with monospecific antibodies (MPA, 1: 10,000) raised against a 43 kDa protein of *Eca* strains (No. SCRI 1039, 1092, 1054, and 41). Lane 1: *Eca* 41; lane 2: *Eca* 1054; lane 3: *Eca* 1092; lane 4: *Eca* 1039 and lane 5: *P. fluorescens* (GSPB 1714). (b) Reaction of *Ech* strains with MPA (1: 10,000) raised against 40 kDa protein of *Ech* strains (No. SCRI 4044, 4042, 4043, PD 483) after Western blotting. Lane 1: *Ech* 4044; lane 2: *Ech* 4042; lane 3: *Ech* 4043, and lane 4: *P. marginalis* (GSPB 92).

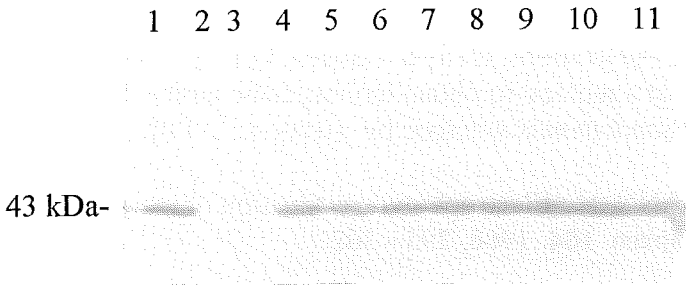

Fig. 3. Detection of *Eca* from artificially inoculated potato tuber extract using the Western blot procedure incubated with *Eca* MPA (1: 10,000) raised against the 43 kDa protein

fragment of *Eca* strains (SCRI 1039, 1092, 1054, and 41). The inoculated potato extract was incubated for 24 hrs at 20° C. Lane 1: *Eca* pure culture; lanes 2 and 3: Potato extract without *Eca*, and lanes 4 - 11: Potato extract with added *Eca* at concentrations of 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8, and 10^9 cells / ml, respectively.

Discussion

The results of this study showed that the extraction of proteins using BPB buffer, yielded apparently pure proteins against which specific antibodies could be produced in rabbits. The specificity of the MPA obtained against the proteins (Fig. 2), confirms the results of other publications where a similar method of serum production was applied (1,5). Although the mechanism by which the trapped proteins in the micropores of the polymerised gels were released was not investigated in this study, the SDS in the BPB buffer, has probably contributed in dissolving the proteins from the micropores, since experiments using the buffer without SDS was comparatively less effective than the former (unpublished data). In addition, incubation of the gel macerate in boiling water might have caused the expansion of the gel pores, facilitating the elution of the proteins during centrifugation. The physical maceration of the gel stripes might have, in addition contributed to the release of the proteins from the gels. The reaction of *Eca* MPA with the 43 kDa protein band using Western blot procedure and the detection of *Eca* in potato extract using the MPAs in an indirect ELISA showed that the method of protein extraction described above, probably does not alter the protein structure (antigenic sites) responsible for establishing a strong antigen-antibody reaction.

The monospecific polyclonal antiserum obtained after applying the protein elution procedure described earlier (1,5) revealed extensive cross reactions with the potato extract using the indirect ELISA procedure (Knapova, pers. comm.). In contrast, the MPA obtained, following immunization of rabbits with proteins eluted directly from unstained SDS-PAGE gels did not cross react with the potato extract. This showed that the method apparently yielded only pure proteins as compared to the former extraction procedure which contained dissolved nitrocellulose membrane against which non specific antibodies might have been produced. This fact might have resulted in the false positive reactions during the indirect ELISA test since all used plant extracts contained cellulose.

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USE OF DNA PROBES FROM PATHOGENIC BACTERIA FOR CHARACTERIZATION OF *X. CAMPESTRIS* PATHOGENIC TO SMALL GRAINS, IN VIEW OF IMPROVMENT OF SEED TESTING

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Introduction

Bacterial leaf streak (BLS) is a devastating bacterial disease of wheat or other small grains under warm and humid climates (10). It is considered to be caused by no less than five pathovars of *Xanthomonas campestris* (pv. : *cerealis, hordei, translucens, secalis* and *undulosa*), which are often grouped together under the denomination 'translucens group' or even considered as a single species or pathovar and therefore named *X. translucens* or *X. campestris* pv. *translucens* (8). The organism was showed to be seedborne but is also reported to occur on symptomless leaves of wheat where levels up to 10^7 cells have been recorded. Seed health policy based on *X. campestris* pv. *undulosa* (Xcu) detection on seeds is considered as the main control mean of the BLS (11), demanding therefore a sensitive and accurate detection method. Direct injection technique as well as other pathogenicity tests have been used for detection and identification of Xcu (5). Xcu is also routinely detected by dilution of seed extracts on semi-selective media, usually followed by pathogenicity testing of *Xanthomonas* strains (9, 15). Combination of such tests, which allows a sensitive and specific detection is nevertheless also very time-consuming and might be hampered by antibiotic resistance or antagonistic microorganisms occuring on some wheat seeds. Faster detection methods, like serological ones, have also been proposed for Xcu with polyclonal antisera (7, 12) or with monoclonal antibody (7, 9), using various techniques such as immunofluorescence, ELISA and DIA. Nevertheless, the specificity of these tests is, untill now, restricted to the 'translucens group', giving therefore no indications concerning pathogenic ability. Lazo *et al.* (14) proposed the use of RFLP for differentiating among pathovars of *X. campestris*. Recently, Verdier *et al.* (16) showed the usefulness of a rRNA probe for comparison between pathovars. Genes involved in the pathogenesis of xanthomonads have been described (1, 17). Considering the genetic and pathogenic diversity already demonstrated for the 'translucens group' by RFLP (3,5) and the already mentioned techniques for detection of Xcu (5, 9, 12, 15), the main focus of this study is to compare probes or primers issued from various phytopathogenic bacteria, to evaluate their ability to discriminate among xanthomonads strains pathogenic to small grains for a possible use as fast and precise molecular detection and identification tools

Materials and methods

Bacterial strains. The following reference strains were used : for *X. c.* pv. *cerealis* (NCPPB1944), *X. c.* pv. *hordei* (NCPPB2389 and UPB684 : UPB = unité de phytopathologie UCL, bacterial collection), *X. c.* pv. *phleipratensis* (NCPPB1837), *X. c.* pv. *poae* (NCPPB3230), *X. c.* pv. *translucens* (NCPPB973 and UPB787) and *X. c.* pv. *undulosa* (NCPPB2821 and UPB513).

Strains were stored lyophilised at 4°C. A pre-culture on LPGA medium was always made before use.

DNA extraction and preparation Genomic DNA has been extracted from overnight-grown cultures in medium O (peptone : 10 g, casamino acid : 1 g, yeast extract : 1 g, pH 7.2) following Boucher *et al.* (2).

Construction of a DNA library and probe preparation. Genomic DNA (10 µg) of strain *X. c.* pv. *undulosa* UPB513 was digested with EcoRI (Eurogentec) as recommended by the manufacturer. DNA fragments were ligated into the EcoRI site of pBleuescript M13 and used to transform competent cells of *Escherichia coli* DH5α. Recombinant plasmids were extracted by the alkaline lysis method from white colonies grown on Luria medium supplemented with ampicillin at 100 µg ml^{-1} and with Xgal at 30 µg ml^{-1} and analysed by agarose gel electrophoresis after EcoRI digestion. DNA clones were excissed from agarose gel and eluted with glassmilk (Bio101) before labelling. DNA probes were labelled with [^{32}P] deoxycitidine triphosphate by random priming (Multiprime, Amersham).

Southern blot and dot blot hybridization. For Southern blot experiments, DNA was digested with EcoRI, separated by electrophoresis through a 0.8 % (W/V) agarose gel in TAE and transferred to nylon membranes (Hybond N+, Amersham) according to the manufacturer's instructions. For dot blot experiments, DNA was directly spotted after denaturation in NaOH NaCl onto nylon membranes as recommended by the manufacturer. Prehybridization and hybridization were performed at 65°C in conditions described by the manufacturer (Amersham) for 3 hours. Blots were washed in 2 X SSC at room temperature for 20 min, then twice in 1 X SSC at 65°C for 10 min. Blots hybridized with probes from the *X. c.* pv. *undulosa* genomic library were washed twice with 0.1X SSC, for 10 min at 65°C. Autoradiography was done at -80°C with X-Ray film (Hyperfilm-MP, Amersham) with intensifying screens (Amersham). Dot-blot experiments were performed with DNA from 32 xanthomonads listed in table 1.

Probes. Probes related with virulence or pathogenicity in pathovars or species have been selected : two probes derived from plasmid DNA of *X. c.* pv. *manihotis* (pBSF2 and pF3eco)(16), a 2.3 kb EcoRI fragment of pVir2 from *Pseudomonas solanacearum* or the total hrp cluster pVir2 with pLAFR3 plasmid vector (3), hrp genes from *X. c.* pv. *campestris* pIJ3225 (1).

Additionally, 74 recombinant plasmids were obtained. This partial genomic library of strain UPB513 was investigated either arbitrarily or by analysis of selective hybridization with the 2.3 kb EcoRI pVIR2 fragment in either plaque colony or Southern hybridization of transformed *E. coli* cells or of digests of extracted plasmids, respectively. Inserts size varied from 0.5 to 20 kb, for tested plasmids. Plasmids PXCU1 and PXCU8 were choosen as probes.

Results

The ability of the probes to reveal polymorphism and to show differences between strains of various host range was determined on a set of 9 closely related reference strains representative of groups delineated previously (4). Selected recombinant clones, 32P-labelled, were hybridized with EcoRI digested genomic DNA of the set. All clones derived from strain UPB513 gave stronger signals with strains from 'translucens' group than well-characterized probes from *X. campestris* pvs. *campestris* and *manihotis*, or from *P. solanacearum*. The probe pXCU1 (fig. 1b), revealed for most of the strains more than 20 hybridizing bands generally well-distributed from 1 to 18 kb. Polymorphism was observed among strains, but complexity of the profiles did not allow numerical analysis. The probe pXCU8 was shown to be related to hrp cluster by colony hybridization and hybridization of the 2.3 kb EcoRI fragment of Pvir2 with the pXCU8 DNA fragment derived from UPB513 strain. It gave one or two hybridizing bands on the set of reference strains EcoRI digest (fig. 1c). The general polymorphism observed as well as the relative specificity (strains deviant for pathogenicity like UPB755 hybridized poorly) have led to an evaluation of the possible use of probes as diagnostic tool. Probes pXCU1 and pXCU8 were tested in dot-blot hybridizations with genomic DNA extracts of 46 strains of 32 xanthomonads species and/or pathovars. All strains from the 'translucens' group (NCPPB973, 1944, 2389, 2821 and UPB545, 882) reacted strongly (dark dots) at each concentration (1 and 0.5 µg) and whatever stringency used. The *X. c.* pv. *phleipratensis* strain NCPPB1837 reacted in the same manner. For all these strains, a 0.5 µg DNA spot was sufficient for production of a signal after 8 hours. Strains CFBP2038, 1948 ATCC10412, NCPPB2985, ATCC10601, NCPPB490, ATCC2623 of *X. ampelinus*, *X. c.* pvs. *oryzae, campestris, pelargonii, vesicatoria, mangifereindicae* respectively, were negatives. With the pXCU1 probe, only strains of the 'translucens group' plus strain of *X. c.* pv. *phleipratensis* reacted positively when the blot was washed with 0.1 X SSC. At a lower stringency (1 X SSC), strains of *X. albilineans* , *X. c.* pvs *cassavae citri, glycines, manihotis, phaseoli* and *poae* gave a strong signal while strains of *X. c.* pvs *aracearum, campestris, cassavae, diffenbachiae, mangifereindicae, manihotis, phaseoli-fuscans, pointsetticola, euphorbiae* and *ricini* hybridized poorly. The pXCU8 probe was less specific as it reacts with *X. albilineans, X. axonopodis* and 23 pathovars of *X. campestris*. The probe pF3eco from *X. c.* pv. *manihotis* reproduced (fig. 1, a) exactly the patterns already observed with pBSF2 probe (3). *X. c.* pv. *hordei* and *translucens* strains isolated from barley and pathogenic on this plant only, showed the same three-bands pattern. The strains of *X.c. pv. undulosa* shared 3 of their four bands pattern. Profiles of *X. c.* pv. *cerealis, poae* and *phleipratensis* were different with only one or 2 bands. Undetermined strain UPB755 did not or faintly hybridize, thus indicating a poor homology with the probe.

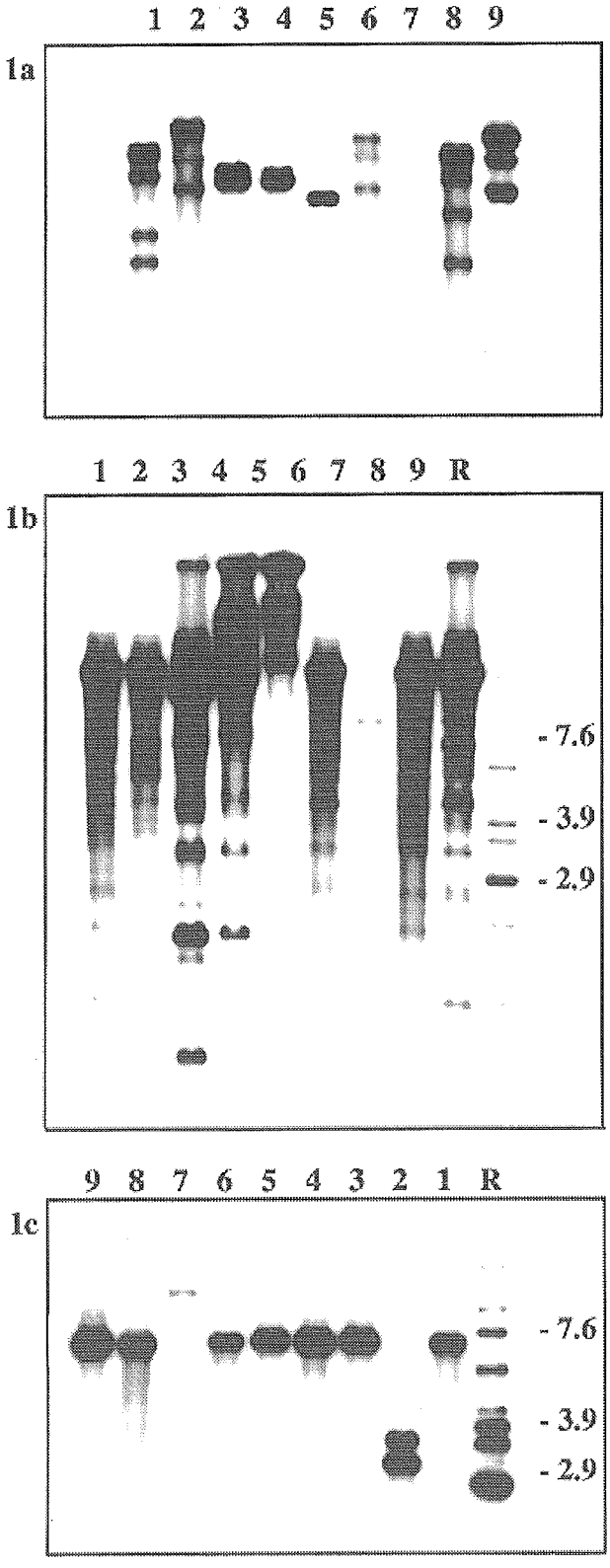

Figure 1, a, b, and c : Autoradiograph of southern blots of genomic DNA from reference strains of *X. c.* pv. *cerealis* (5-NCPPB1944), *hordei* (9-NCPPB2389), *phleipratensis* (4-NCPPB1837), *poae* (3-NCPPB3230), *translucens* (2-NCPPB973), *undulosa* (1-NCPPB2821, 8-UPB513) and undetermined (7-UPB755-Brazil) probed with pF3eco (a), pXCU1 (b) and pXCU8 (c). R = m.w. Raoul 1

Table 1 : : dot-blot hybridization of genomic DNA from xanthomonads with probes pXCU1 and PXCU8.

	PXCU1	PXCU8
X. axonopodis (NCPPB457)	0	+
X. albilineans (G7)	0	+
X. ampelinus (CFBP2038),	0	0
X. oryzae pv. *oryzae* (CFBP1948)	0	0
X. oryzae pv. *oryzicola* (NCPPB1585),	0	+
Pathovars of *X. campestris*	0	0
campestris (NCPPB528, ATCC10412)	0	+/-
aracearum (NCPPB2832)	0	+
begoniae (NCPPB3003)	0	+
cerealis (NCPPB1944)	+	+
hordei (2389)	+	+
incanae (NCPPB937)	0	+
diffenbachiae (NCPPB1833)	0	+
pelargonii (NCPPB2985)	0	0
citri (NCPPB409)	0	+
glycines (NCPPB554)	0	+
vesicatoria (ATCC10601)	0	0
malvacearum (NCPPB633)	0	+
translucens (NCPPB973, UPB545)	+	+
undulosa (NCPPB2821, UPB882)	+	+
vasculorum (NCPPB796)	0	+
phaseoli (XCP306)	0	+
phaseoli-fuscans (XCP072)	0	+
mangifereindicae (NCPPB490, 2623, 1717, 2926),	0	+/-
vignicola (UPB040)	0	+
poinsetticola (UPB073)	0	+
phleipratensis (NCPPB1837)	+	+
poae (NCPPB3230)	0	+
euphorbiae (LMG863, 7402)	0	+
ricini (UPB075, 076)	0	+
cassavae (NCPPB101, UPB899, 037)	0	+
manihotis (CFBP1851, LMG777, NCPPB1159, 2444, UPB079)	0	+

0 = negative reaction, + = positive reaction. A +/- indicates that some of the strains were positive

No radioactive signal was detected on blots probed with pIJ3225 at 65°C and autoradiographed for more than 3 days. Probing with pVIR2 and the 2.3 kb fragment of the same gene cluster gave only a low intensity signal. Those probes hybridized with a high number of fragments on the whole profile (data not shown).

Discussion

Seven probes from phytopathogenic bacteria were hybridized with reference neopathotype strains of *X. campestris* pathovars pathogenic on small grain cereals. In a general manner, the probes cloned from *X. c.* pv. *undulosa* strain UPB513 revealed higher intensity signal compared to more heterologous probes. The probe pXCU1, with patterns constituted by more than 20 bands of similar intensity, corresponds probably to a multicopy sequence. If analysis of such patterns by numerical analysis is difficult, the use of another restriction low-cutting endonuclease might give the opportunity of strains comparison. On the other hand, the probe pXCU8 gave one or two-bands signals, hence probably corresponding to a single-copy sequence. This last probe reflects also, as probe pBSF2, pF3eco and pIJ3225 (Alizadeh, personal communication), the polymorphism observed between the group of strains with a pathogenicity restricted to barley and the strains pathogenic equally on wheat and barley. To investigate such opportunity, 46 strains from 32 different species and pathovars have been analyzed by dot-blot. The probe pXCU1 might be specific to the 'translucens group' with adequate stringent conditions. The probe pXCU8 was, not surprisingly, less specific, but it was selected by hybridization with the pVIR2 probes and might therefore correspond to a fragment of hrp genes, highly conserved through phytopathogenic bacteria (1). The probes pVIR2, pBSF2, pF3eco gave multiple band signals and revealed polymorphism among the tested strains. Probes pBSF2 and pF3eco have been showed to be related to pathogenicity (16) and are highly homologous with avr genes already described for xanthomonads (Verdier, personal communication). They gave perfectly identical patterns in Southern blots with genomic DNA digested with EcoRI restriction endonuclease. These 2 probes, as well as the pVIR2 one, hybridize with the tested strains, except with deviant strain UPB755. This strain might be deficient in genes required for pathogenicity. The probe pIJ3225 gave no reaction with any of the tested strains, indicating a poor homology in our working conditions with strains of the 'translucens group'. Waney *et al.* (17) have already stated cross-hybridization between genomic DNA from *X. c.* pv. *translucens* strain Xct216 and hrp genes from *P. solanacearum.* Our results confirms such statment. Polymorphism detected among tested strains as well as previous results obtained pBSF2 (4) probe or pIJ3225 probe (Alizadeh, accepted for publication in Plant Disease) raises the feasibility of distinguishing clonal structure (13) between strains of the two pathogenicity groups, perhaps even strains more related to grasses like the *X. c.* pv. *cerealis* strain UPB453, by genotypic features at the opposite of the phenotypic traits currently used.

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SEROLOGICAL AND NUCLEIC ACID-BASED DETECTION OF *ERWINIA CAROTOVORA* SUBSP. *ATROSEPTICA* AND *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS*

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Introduction

Blackleg and ring rot are two important diseases of potato. The causal agents are the Gram-negative bacterium, *Erwinia carotovora* subsp. *atroseptica*, and the Gram-positive bacterium, *Clavibacter michiganensis* subsp. *sepedonicus*, respectively. The primary inoculum source for both diseases are the vegetative tubers used as "seed" for planting, but other sources of primary inoculum may also occur in agricultural areas (2, 5). Sensitive and specific procedures are, therefore, required to detect the pathogens in order to distinguish healthy from infected seed lots, and for research on the persistence of the pathogens in agricultural ecosystems.

Materials and Methods

A monoclonal antibody, 4F6, developed to the lipopolysaccharide antigen was used for detection of *E. c. atroseptica* by both immunofluorescence and ELISA. For *C. m. sepedonicus*, monoclonal antibody 9A1 directed toward a cell wall antigen was used for immunofluorescence, and monoclonal 1H3 (3) directed toward the extracellular polysaccharide was used in ELISA. Both the immunofluorescence procedure and ELISA were carried out on bacterial cells from pure cultures and on extracts of potato tissue. The standard indirect immunofluorescence staining procedure was used with an anti-mouse fluorescein conjugate. Polyclonal rabbit antibody was used to capture antigen in an indirect double antibody sandwich ELISA and bound monoclonal antibody was detected with anti-mouse alkaline phosphatase conjugate.

Specificity and sensitivity of the serological procedures were compared to detection based on amplification of a DNA fragment by the polymerase chain reaction (PCR). To extract DNA, 1 ml of cell suspension or plant extraction fluid was pelleted by centrifugation. After resuspending the pellets in 100 µl of Tris-EDTA buffer (pH 8.0) containing 1% sodium dodecyl sulfate, they were heated to 50° C for 3 hr (proteinase K at 10 µg/ml was added to plant samples prior to heating). Bacterial and plant debris were precipitated by addition of one half volume of 7.5 M ammonium acetate and removed by centrifugation. DNA was precipitated by addition of one volume of isopropanol and, after centrifugation, the pelleted DNA was washed with 70% ethanol and dried.

The PCR test for *E. c. atroseptica* utilized primer sequences selected from a specific hybridization probe. The PCR primers for *C. m. sepedonicus* were selected from the intergenic

spacer between the 16S and 23S rRNA genes. PCR reactions (20 µl) contained 1 µl of DNA template, 0.5 µM of each primer, 100 µM of each dNTP, 2.0 mM Mg^{++}, and 0.5 U of Taq DNA polymerase in the buffer provided by the manufacturer. After an initial denaturation step at 95° C for 5 min, PCR conditions for *E. c. atroseptica* were 62° C for 45 sec, 72° C for 45 sec, and 94° C for 30 sec. For *C. m. sepedonicus*, annealing was at 62° C for 45 sec, extension at 72° C for 90 sec and denaturation at 94° C for 90 sec. A final elongation was performed at 72° C for 5 min following 40 cylces of amplification.

Results

All strains of *E. c. atroseptica* tested positive in PCR, yielding a 690 base pair (bp) product, while only strains in serogroups I and XXII were positive in ELISA and immunofluorescence with the monoclonal antibody (Table 1). Other subspecies of *E. carotovora* and saprophytic bacteria, except for two bacteria isolated in the Netherlands, were negative by ELISA and immunofluorescence; all these were negative in PCR.

Most of the potato samples that were positive in ELISA were also positive in PCR (Table 1). Some ELISA-negative samples were positive in PCR , and some samples were positive in ELISA but negative in PCR. The negative PCR result of ELISA-positive samples was not due to the presence of inhibitors because when 10 ng/µl of *E. c. atroseptica* DNA was added to the samples amplification of DNA proceeded normally.

Table 1. Pure cultures and potato samples tested for reaction in ELISA with monoclonal 4F6 and in PCR using primers to *E. c. atroseptica*

Bacteria/Sample	No. of samples	Number of positive samples ELISA	PCR
Pure cultures			
E. c. atroseptica	50	19	50
E. c. carotovora	44	0	0
E. c. betavasculorum	2	0	0
E. c. odorifera	4	0	0
E. c. wasabiae	4	0	0
E. carotovora (subsp. unknown)	8	0	0
E. chrysanthemi	1	0	0
Unidentified saprophytes	13	2	0
Field samples			
Potato stems	85	33	42
Seed tubers	20	6	4
Progeny tubers	65	40	40

The primers selected for PCR of *C. m. sepedonicus* resulted in specific amplification of a 215 bp fragment. All strains of C. m. sepedonicus that were tested yielded the fragment, but other subspecies of *C. michiganensis*, other coryneform bacteria, and unidentified bacteria isolated from potato were negative (Table 2). All PCR positive strains were positive in ELISA with monoclonal 1H3 and in immunofluorescence with monoclonal 9A1.

All the tuber samples that gave a positive reaction in ELISA, also contained immunofluorescing cells, and yielded the 215 bp PCR product (Table 2). The ELISA-negative tubers that yielded PCR products also contained immunofluorescing cells but generally at less than 10 cells/microscope field. Two samples had more than 30 cells/field. Fifteen samples in which no immunofluorescing cells were observed were also negative in PCR.

Table 2. Pure cultures and potato samples tested by PCR with primer sequences selected from the rRNA intergenic spacer region.

Bacteria/Sample	No. of samples	No. positive in PCR
Pure Cultures		
C. m. sepedonicus	24	24
C. m. michiganensis	5	0
C. m. insidiosus	7	0
C. m. nebraskensis	2	0
C. m. tessellarius	2	0
Rathayibacter rathayi	2	0
Rathayibacter iranicus	2	0
Rathayibacter tritici	2	0
Erwinia carotovora atroseptica	1	0
Unidentified isolates from potato	50	0
Potato Samples		
ELISA positive tubers	17	17
ELISA negative tubers	24	9

Discussion

In contrast to the serological tests, all serogroups of *E. c. atroseptica* could be detected by PCR. Monoclonal 4F6 reacted only with serogroup I strains, and, more weakly, with serogroup XXII strains. Although the monoclonal antibody showed a high degree of specificity, two saprophytic bacteria were isolated in the Netherlands (van der Wolf, personal communication) which cross-reacted with it. DNA from non-*E. c. atroseptica* strains were not amplified by PCR using the selected primers.

In tests with pure cultures, PCR was more sensitive than ELISA, and some ELISA-negative potato samples were positive with PCR. However, some potato stem and tuber preparations that were positive in ELISA for *E. c. atroseptica* did not yield amplification

products in PCR. The possibility that the negative PCR test was due to inhibitory products in the extract was ruled out by retesting the preparations with added *E. c. atroseptica* DNA from pure cultures. All but a few samples were PCR positive in the retest. Perhaps in potato samples, intact DNA is less persistent than the lipopolysaccharide antigen detected by ELISA.

Serological testing of seed potatoes for the bacterial ring rot pathogen has been relatively successful. However, a few strains of saprophytic bacteria that cross-react with monoclonal antibodies 9A1 and 1H3 have been isolated previously. These bacteria were negative in PCR with primers selected from the rRNA intergenic spacer region. Thus far only authentic *C. m. sepedonicus* strains have yielded PCR products when these primers were used. Since these primers are based on genomic nucleotide sequences, the presence of a plasmid is not required for a positive test. This may be important because at least one pathogenic, plasmidless strain has been identified (4).

Detection of *C. m. sepedonicus* by PCR using these primers was more sensitive than ELISA and immunofluorescence tests based on monoclonal antibodies 1H3 and 9A1, respectively. The 215 base pair amplification product was obtained by PCR for all potato tuber samples that were positive by the serological tests. In addition, the presence of the ring rot pathogen could be confirmed by PCR in samples in which the density of fluorescing cells were below the positive/negative threshold. Tuber samples in which no fluorescing cells were found were also negative in PCR.

For both the blackleg and ring rot pathogens, PCR provides an alternative test to confirm serological assay results. However, since serological tests detect specific antigens and PCR detects specific DNA sequences, discrepancies can occur if the persistence of the respective target molecules differ. On the other hand, application of both serology and DNA-based tests may provide stronger evidence for the presence of bacterial pathogens in field samples than either approach alone. The use of complementary tests to identify bacteria in field samples is particularly important when isolation of the bacterium is not possible.

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A FOREIGN LYSOZYME AS A NEW TOOL FOR ANTIBACTERIAL RESISTANCE BREEDING IN TRANSGENIC PLANTS

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Introduction

Antibacterial resistance is extremely difficult to achieve by conventional breeding methods as no suitable resistance traits are available in current breeding material. Only in some wild species of the genus *Solanum* valuable resistance traits can be found. On the other hand, sexual transmission of these genes to breeding material is difficult because of the wide genetic distance. Genetechnology might be a new means for an efficient approach to reduce susceptibility to bacterial pathogens as *Erwinia carotovora*.

Lysozymes are a widespread family of bacteriolytic enzymes. In many plant species endogenous mostly bifunctional enzymes with a strong chitinase and a weak lysozyme activity have been detected. These basic proteins are located in the vacuole or the cell wall and may represent a natural antibacterial defence reaction (1). In potato tubers only very weak bacteriolytic activities could be detected (2).

The introduction of bacteriophage T4 lysozyme into the intercellular spaces of transgenic potato plants might enable an early interaction of the enzyme with the invading bacteria. T4 lysozyme has been shown to possess bacteriolytic activity against several gram-positive and gram-negative bacteria including *E. carotovora* and *Pseudomonas solanacearum*. (3)

A chimeric fusion gene containing the barley α-amylase signal peptide and the bacteriophage T4 lysozyme coding sequence under the control of the 'constitutive' CaMV 35S promoter has been cloned into two different vectors. They were used for *Agrobacterium tumefaciens*-mediated transformation of the tetraploid potato genotype Z2 (in cooperation with M. Fladung, MPI for Plant Breeding, Cologne) (4) and the cultivar Désirée.

Material and Methods

Experimental procedures were as described in (4).

Results

All the tested Z2 transformants contain one copy of the T-DNA. Northern blotting reveals different levels of expression of the foreign gene in the different Z2 and Désirée transformants. As detected by Western blotting, the foreign protein is synthesized in a glycosylated form to about 0.001% of total soluble protein in greenhouse tissue. It is found in intercellular washing fluids and detected by electron microscopic immunogold labelling in intercellular spaces and cell walls.

All the resistance experiments show a general tendency towards some regenerants for highest reduction in maceration. These plants provide the highest levels of expression.

Infection of potato tuber discs with different numbers of bacteria exhibit a clear dependence of percentage of maceration on the infection pressure. Significant results were obtained in the range of 1,000 to 150,000 bacteria. Maceration of control tissue ranges between 60 and 100% for increasing numbers of bacteria. In contrast, the mean value of all transformants analyzed ranges between 20 and 65%. The regenerant T424 shows even better effects. When inoculated with 1,000-3,000 bacteria, tissue maceration only reached 5% of the disc surface. As naturally only around hundred bacteria are sufficient for a successful infection we assume that conditions used in these experiments are harsh compared to those which may occur in the field.

In a greenhouse biotest with each 78 explants the sprouting capability of infected tubers was assayed. After inoculation with *E. carotovora atroseptica* T424 tuber pieces efficiently produced shoots and developped healthy plants without any symptoms of blackleg during a complete life cycle, whereas the Z2 tuber pieces were more or less completely unable to generate sprouts.

A modified binary vector with a minimal T-DNA (5) has been used for generation of transgenic Désirée potato plants bearing the same foreign lysozyme gene. About 40 transformants have been examined at molecular level (selection scheme: protein → DNA) and 17 transformants further at DNA, RNA and protein level. Protein expression levels reach up to 0.02% of total soluble protein in sterile culture tissue. 5 lines have been selected to be tested in a field resistance assay. This material is being further characterized and prepared for the field release experiment.

Discussion

Two different potato lines (Z2 and Désirée) had been transformed with the bacteriophage T4 lysozyme gene. Northern and Western analysis revealed varying levels of expression. Infection tests of potato tubers with *Erwinia carotovora* showed higher resistance to tissue maceration in comparison to untransformed plants. T4 lysozyme therefore seems to be an effective means against phytopathogenic bacteria.

The conditions tested can only serve to evaluate a possible antibacterial resistance of transgenic potato plants producing T4 lysozyme in the field under natural conditions. Deduced from the data presented here we expect that it should be possible to observe a resistance to *Erwinia* also in the field especially as the initially infecting number of bacteria seems to be very low. Multiplication of these natural inocula in intercellular spaces could be prevented by the T4 lysozyme.

As the T4 lysozyme approach is not specific and this lysozyme provides activity against gram-positive and gram-negative bacteria as well there might be a wide application potential to other host-pathogen systems.

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ELECTRON MICROSCOPICAL INVESTIGATIONS FOR RESISTANCE INDUCTION AGAINST BACTERIAL CANKER OF TOMATO

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Introduction

Bacterial canker is one of the diseases most feared in the cultivation of tomato plants. It is caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). Cmm, a vascular wilt pathogen which penetrates the vascular system, quickly spreads along the xylem vessels of their host plant (1; 5). NCPPB 3123 is an apathogenic isolate of Cmm that induces resistance in tomato plants against subsequent inoculations with aggressive Cmm-isolates. Electron microscopical studies were made to compare the different Cmm-isolates with regard to their morpholgy, serological reactions, multiplication and spread in plants as well as their effect on tissue and cells of the host plants.

Material and methods

Plant and bacterial material. Tomato plants (*Lycopersicon esculentum* MILL.) cultivar "Harzfeuer", grown in a greenhouse, were inoculated at the three-leaf stage by injection into the axil of stem near the leaf with bacterial suspension of the apathogenic Cmm-isolate NCPPB 3123 and virulent Cmm-isolates (Cmm 3.1.4.-S, Cm 63 and Cm 71). Inoculation with distilled water constituted the control.
Electron microscopy. Bacterial suspensions were negatively stained with uranyl acetate or prepared for immuno labeling of the Cmm-cells with gold conjugates EM goat anti-rabbit IgG, 5 nm. For cutting ultrathin sections bacterial suspensions were embedded in agar. Samples of these as well as of stem and leaf of tomato plants, healthy and inoculated with apathogenic and different virulent Cmm-isolates were fixed in glutaraldehyde postfixed in osmium tetroxid (OsO_4), dehydrated in ethanol and embedded in Epon. The ultrathin sections cut on a LKB Ultrotome III with a glass knife were contrasted with uranyl acetate and lead citrate.
The preparations were viewed with a transmission electron microscope JEOL 100 B or EM 902/ZEISS. For scanning electron microscopy cross-sections were cut with a razor from stem at various points of healthy and inoculated tomato plants. The samples were fixed in glutaraldehyde and OsO_4, dehydrated in acetone and by critical point drying, sputtered with gold-platinum and viewed with ISI-40.
Light microscopy. For light microscopy transverse sections from tomato stem were also cut and coloured with toluidin blue.

Results

The Cmm-cells in electron microscope showed no distinctions between cells of the apathogenic and the different isolates with regard to their shape and size (fig. 1). Also immuno gold labeling indicated no distinction between both apathogenic as well as virulent Cmm-cells (fig. 2).
In ultrathin sections the Cmm-cells of avirulent and also virulent isolates embedded in agar accumulated mainly in intercellular spaces of plant tissue covered with thick slime, often with clear limit lines (fig. 3d). Similar multiplication and rate of spread of bacterial cells of these Cmm-isolates were observed within the vascular system of the host plants (fig. 3a-c).
Macroscopic observations showed that in tomato infected with virulent Cmm-isolate the vascular system at several sites of stem were stained dark brown and accompanied with tissue

maceration. Few days after inoculation both apathogenic and virulent Cmm-cells colonized the vascular system from the site of inoculation to the apex of tomato plants before wilt symptoms appeared.
Specially electron dense particles were seen mainly at the tonoplast of parenchyma cells especially in tomato plants inoculated with the apathogenic Cmm-isolate 3123(fig. 4). When the tonoplast was destroyed, we also found such particles at the cover membranes of chloroplasts and mitochondria.

Discussion

The bacterial cells of different Cmm-isolates are, in general a little flexible, club-shaped short rods and variable in form and size (most 0.6 ... 0.7 µm x 0.7 ... 1.2 µm) (4), without distinctions in morphology and survey. In ultrathin section, where the multiform of the Cmm-cells is very clearly seen (fig. 3d), there is also no distinctions between apathogenic and virulent isolates.
The serological reaction was similar and all the cells were immuno gold labeled (fig. 2). Presently, with the available poly- and monoclonal antibodies no different immuno gold labeling between the apathogenic and aggressive Cmm-cells was achieved.
After inoculation of the different isolates of Cmm-cells into different tomato plants the spread within the tissues was about similar. The bacteria seen at first only in the tracheids, invaded some days later mainly adjacent intercellular spaces and at times the vascular parenchyma cells. Similar but extensive ultrastructural investigations of tomato plants after infected with Cmm were made by BENHAMOU (1).
The effect of preimmunity is probably not caused by antagonism or rivalry for place or mechanism for active trapping the virulent Cmm-cells.
Very interesting are the electron dense particles which we found in mesophyll cells of mainly tomato plants inoculated with the apathogenic Cmm-isolate 3123. Probably these particles were formed at tonoplast. At the beginning they were elliptical and later when nearly spherical they moved from tonoplast into the centre of plant cell in the large vacuole (fig. 4). Electron dense particles in the system French bean *Phaseolus vulgaris/Pseudomonas phaseolicola* seen by EBRAHIMNESBAT et SLUSARENKO (3) predominated in a resistant cultivar of bean, although small amounts of similar material were observed in a susceptible cultivar. Also, BENHAMOU et al.(2) have observed electron dense droplets deposited in the vascular parenchyma cells of tomato plants after seeds treatment with chitosan, which is believed to induce systemic resistance against fungal pathogens. From their histochemical studies the induction of these droplets is believed to be the beginning of the synthesis of phenolic compounds.
Regarding the electron dense particles which we have observed, it is probable that the origin of these particles is a specific defensive reaction of the host plant in our resistance inducing system, tomato/Cmm.

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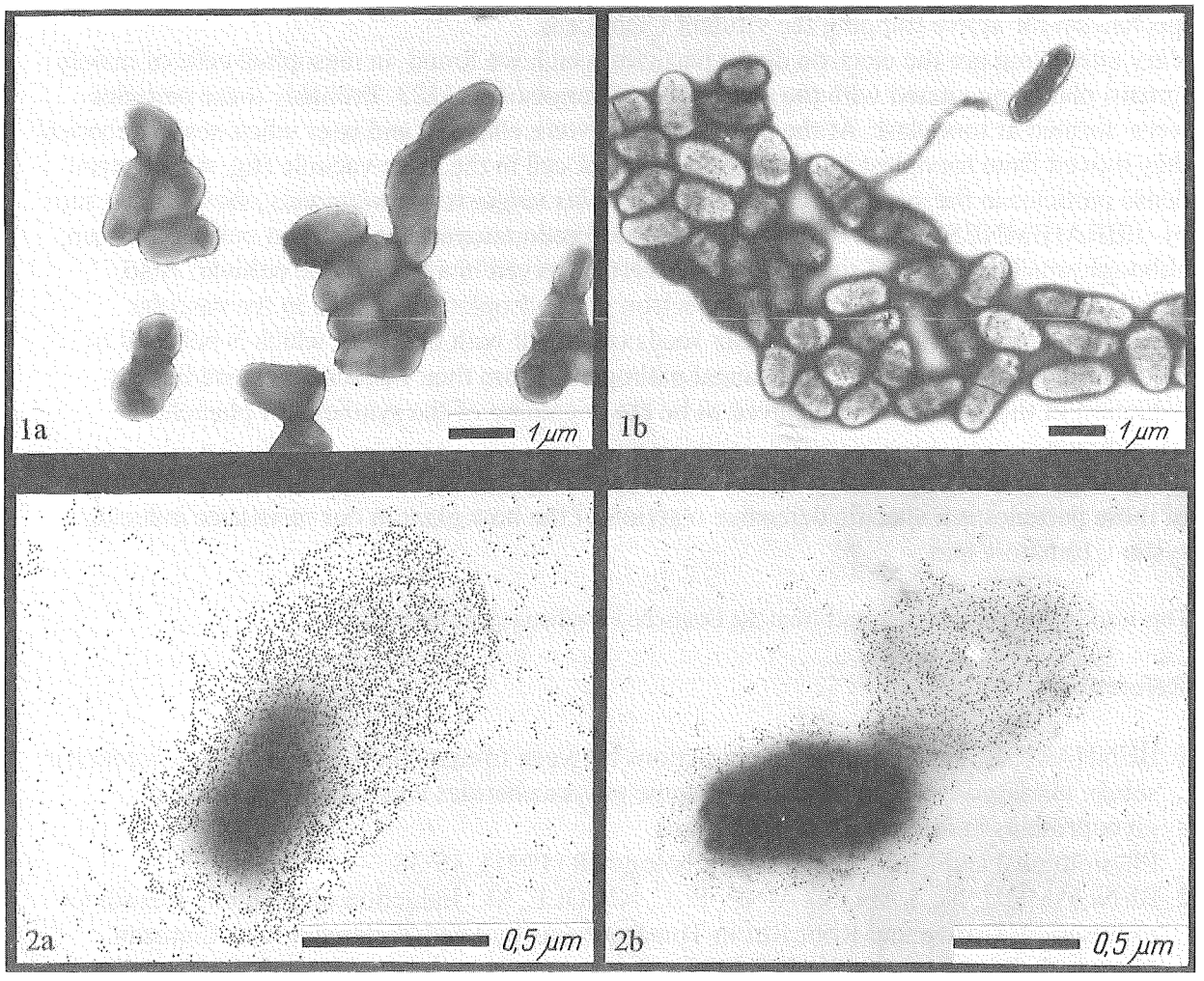
Legendes to the photopraphs

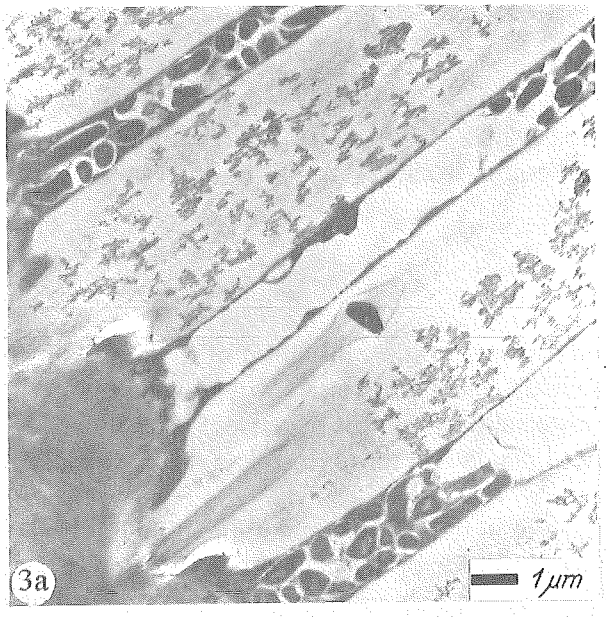
Fig. 1: Morpholgical comparison between Cmm-cells of apathogenic (a) and virulent (b) isolates in suspension.

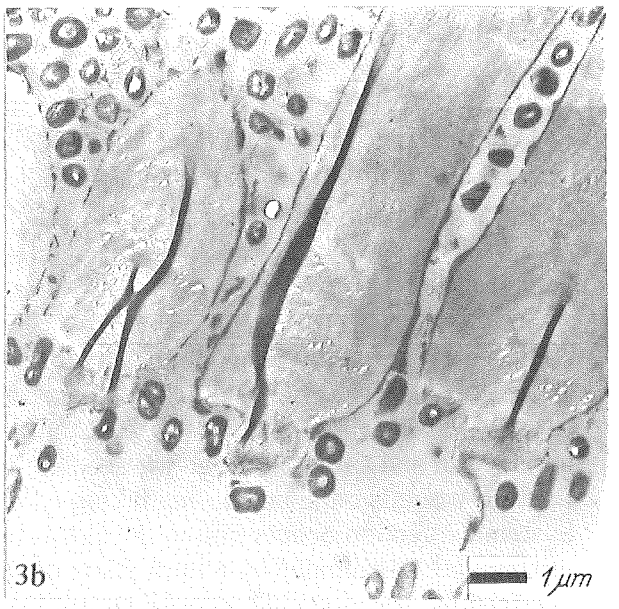
Fig. 2: Immuno gold labeling with goat anti-rabbit gold colloides (5 nm) - (a) apathogenic Cmm 3123-cells, (b) virulent Cm 3.1.4.-cells.

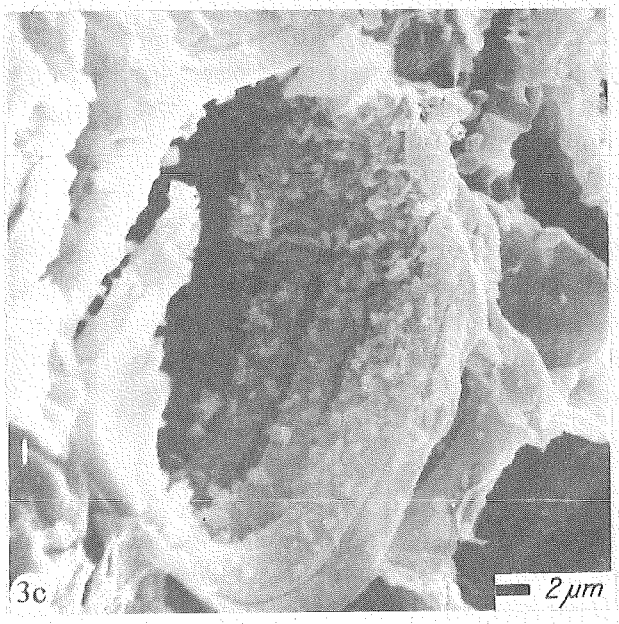
Fig. 3: Multiplication and spread of Cmm-cells within the xylem of tomato plants. - Ultrathin sections from stem with bacterial cells between the screw of tracheids: (a) Cmm 3123, 2 d p.i., and (b) Cm 3.1.4., 47 d p.i. - (c) Scanning electron microscopical photograph: Tracheid with Cm 63-cells, 5 d p.i. - (d) Ultrathin section with apathogenic cells within intercellular space, 6 d p.i.

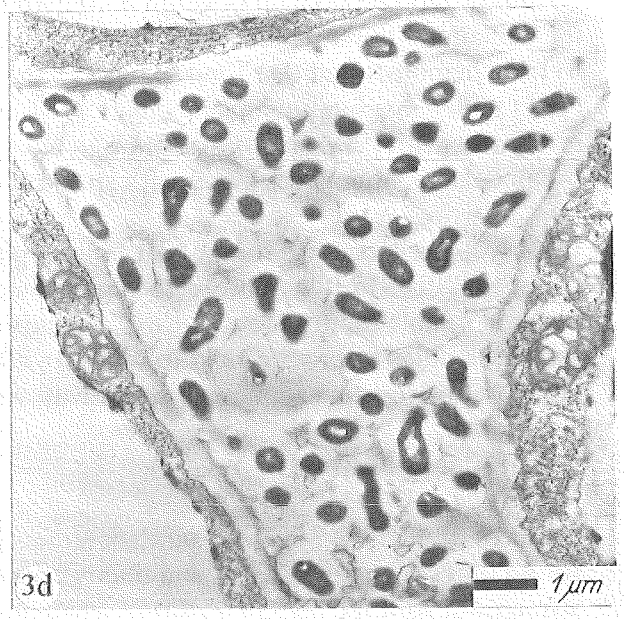
Fig. 4: Electron dense particles within xylem parenchyma cells from top of the stem of the host plant. Particles (a) at tonoplast and (b) moving from tonoplast.

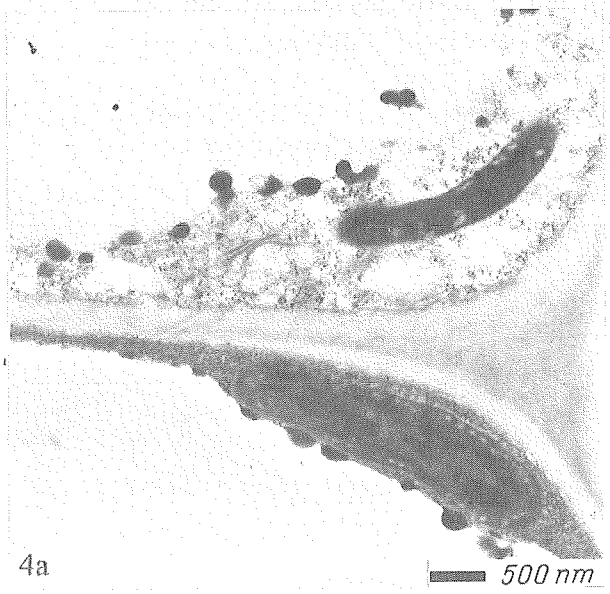


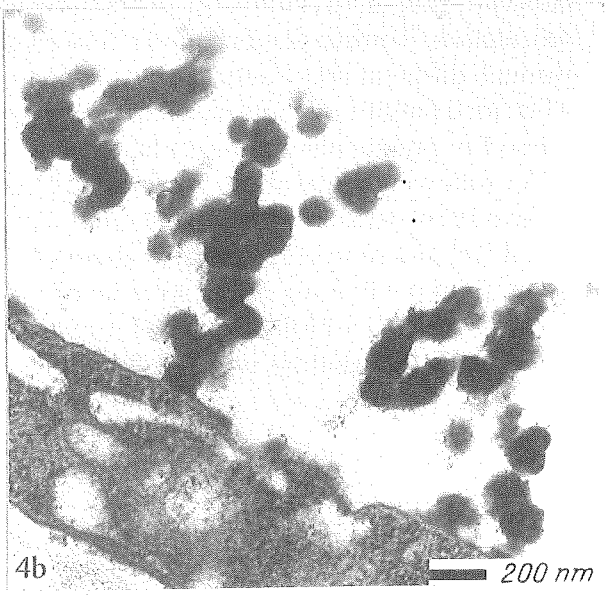

3a
1 μm


3b
1 μm


3c
2 μm


3d
1 μm


4a
500 nm


4b
200 nm

INDUCTION OF RESISTANCE TO BACTERIA CANKER OF TOMATO BY PREIMMUNIZATION

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Introduction

Bacterial canker and wilt is one of the most feared diseases in the cultivation of tomato plants. Often, this disease is the cause of heavy losses in yields. The pathogen - *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) - is especially dangerous because it is easily transmitted and spread through the seeds of infected plants and through soil as well as mainly through wounds on plants during taking care.
Systemic infection occurs first in the xylem tissue, later in the phloem and in the pith (1). Cells of Cmm are surrounded by a thick slimy cover consisted of exopolysaccharides (EPS). This extracellular glycopeptide induced an unspecific wilting not only on tomato plants and cuttings but also on cuttings from other plants (2).
Until now a direct control of Cmm in tomato cultures is not possible. One possibility to reduce the damage of tomato plants is by preimmunization of tomato plants. Different authors (3, 4, 5) have demonstrated that tomato plants were protected against local (3) or systemic infection (4, 5) with Cmm by previous inoculation with different members of the *Pseudomonas syringae* group (3), with *Ps. aeruginosa* (4) or an auxotrophic mutant of Cmm (5), when the preinoculated Cmm were killed with streptomycin immediately after superinfection.

In early experiments we found, that tomato plants preinoculated with the avirulent strain of Cmm NCPPB 3123 were protected against superinfection by virulent strains of Cmm (6). This paper is a short report about our experiments using different methods and technics to measure the possibilities to produce and to optimize effects of preimmunization and in order to compare the resistance inducing strain with virulent Cmm.

Material and Methods

Cultures of NCPPB 3123 and virulent strains of Cmm (3.1.4.-S, Cm 63, Cm 71) were grown for 48 or 72 h on YDCa agar and than suspended in water. We counted the cells within suspension with the help of THOMA-chamber. Depending on the different inquiry the applied inoculum concentration differed between 10^2 to 10^9 cfu/ml. But mostly we used a concentration of 10^8 cfu/ml for the preinoculation and 10^5 or 10^6 cfu/ml at the following inoculation. Tomato plants cultivar "Harzfeuer" were inoculated at different stages of age through different sites roots, sprouts and wounds.

- For inoculations of roots seedlings were removed from the soil and the tips of roots were cut. For preinoculation these plants were put in a suspension of NCPPB 3123 for 24 h. For following inoculations we removed the seedlings, cut 1 mm of the end of roots and put them in suspension with virulent Cmm for 2 h. After this the seedlings were planted in the soil and grown in greenhouse at about 25 °C.
- The sprouts of young plants were inoculated by injection of the avirulent and virulent strains each in the axil of fully expanded topmost leaf. The period of time between pre- and following inoculations was 7, 10 or 14 days.

- The buds were inoculated through fresh wounds created after removing them by covering with the suspension of NCPPB 3123 and 5-10 min later with virulent isolates. In some cases we used for the preinoculation heat-killed cells of NCPPB 3123 (10, 20 or 30 min at 100 °C in a water bath).

Symptom expression (SE) was classified following: 1 = no symptoms; 2 = little wilting of some leaflets; 3 = < 30 % of leaves wilted; 5 = 30-50 %, 7 = 51-75 % and 9 = > 75 % of leaves wilted.

Spreading of different Cmm strains in tomatoes was examined by microbiological, serological and microscopical methods.

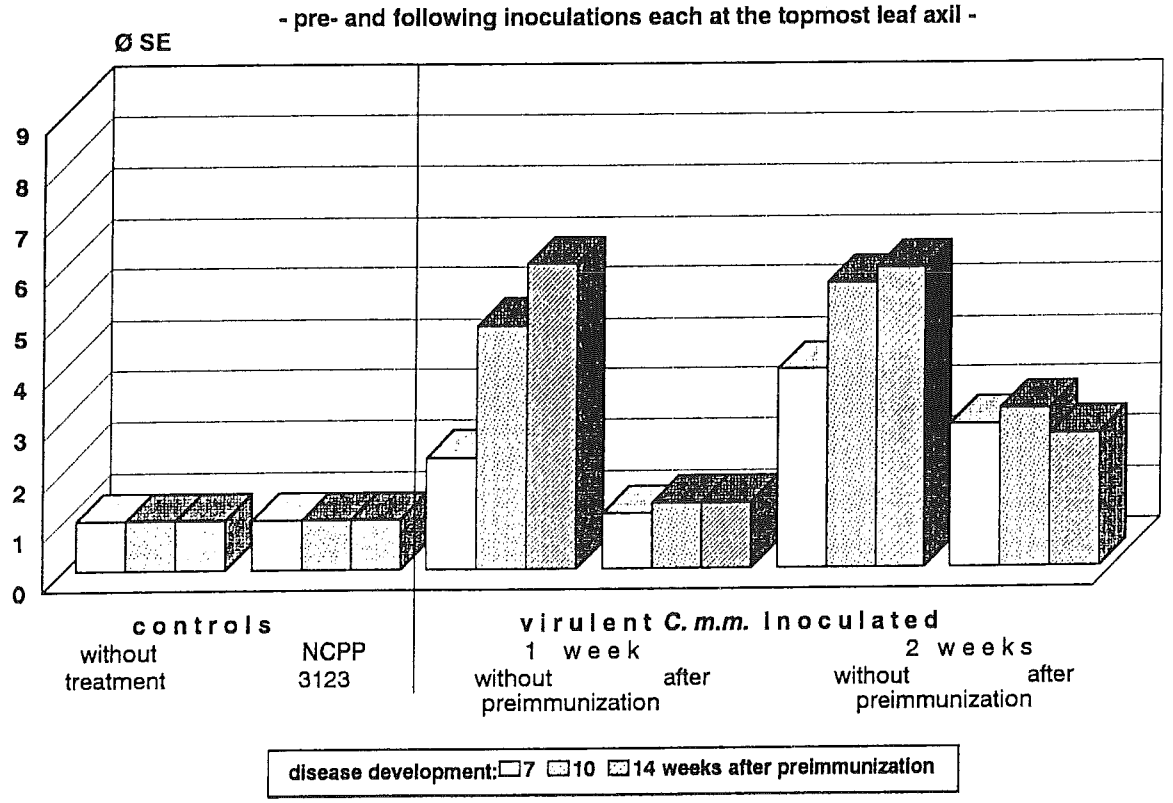
Randomly amplified polymorphic DNA PCR (RAPD-PCR) was used to generate DNA profiles for the characterization and differentiation of Cmm strains.

Results

The investigations on the optimization of the preimmunization showed that preinoculation with the avirulent NCPPB 3123 induced a protective effect in tomato plants in all tested cases. But this differs depending on the age of tomatoes, inoculation sites, time intervals and the inoculum concentrations for pre- and challenge inoculation.

On young plants (4 or 5 leaf stages) the optimum effect of preimmunization was observed when pre- and following inoculations were carried at time interval of 8 to 10 days each in the axil of fully expanded topmost leaf. The figure below demonstrates, that the induced protective effect was preserved for more than 3 months.

Development of wilting symptoms on tomato after preimmunization of seedlings and following inoculation with *C.m.m.* 63 after 1 and 2 weeks



In the case of root infections the results of repeated trials were inconsistent. We also observed that covering of fresh branch wounds with suspension of NCPPB 3123 was a good protection against challenge infection with virulent Cmm-strains at debuding. The results of using

different concentrations of NCPPB 3123 for preinoculation before challenge inoculation with virulent strains of Cmm (10^5 or 10^6 cfu/ml) were nearly insignificant for development of wilting on tomato plants. But the best effect of preimmunization was induced at 10^8 cfu/ml.

The level of protection after preinoculation of tomato plants with heat-killed cells was not so high when compared with the application of living cells of NCPPB 3123. But also in this case the differences between tested variants were not significant.

Numerous researches carried out to examine the ability of NCPPB 3123 to induce preimmunization effects on other host/pathogen-systems showed that this strain was unable to induce resistance against other bacterial pathogens of tomato (*Pseudomonas solanacearum*, *Ps. syringae* pv. *tomato*, *Xanthomonas campestris* pv. *vesicatoria*), potato (*C. michiganensis* subsp. *sepedonicus*) or *Phaseolus*-bean (*Ps. syringae* pv. *phaseolicola*). In this connection we found that the on tomato avirulent NCPPB 3123 was virulent on pepper.

The results of investigations with regard to the characterization of the resistance inducing NCPPB 3123 are compiled in the table shown below. There were striking similarities between

Characterization of resistance inducing NCPP 3123 and comparison with virulent *C.m.m.* - strains

Characteristics	Resistance inducing *Cmm*		Virulent *Cmm*
Resistance induction through:			
intact cells	yes		-
heat-killed cells	yes		?
Wilt-induction on tomato	no		visible 10. days p.i.
Spreading in tomato		in vegetative parts about equal	
virulent for pepper	yes!		yes
Cellmorphology and size		similar	
Colony consistance	variable/dry		slimy
Antagonism		no	
Protein bands		similar	
DNA composition		differences detectable	
EPS - developments *	rel. low		very plentiful
EPS components *	very low	Fucose synthesis	plentiful
Reaction with *Cmm* - antisera		similar	

* for this results we are grateful to Dr. M. Ramm / University of Jena

virulent strains and resistance inducing strain in view of the spread in tomato plants, their cell morpholgy and size, protein bands, reaction with monoclonal antibodies and polyclonal antisera to Cmm and virulence on pepper. However, there were differences with respect to DNA composition and EPS components.

Discussion

The results indicate that on tomato avirulent Cmm strain NCPPB 3123 is able to spread in tomato plant about the same way as virulent isolates of Cmm. The fact that the protective effect is not only at the sites of inoculations but also in other parts of tomato plants showed that the effect of preimmunization is systemic. The protective mechanism is considerably

influenced by the inoculation sites and cell concentrations used for the pre- and challange infection and also by the time interval between the both inoculations.The protection occurs very quickly if pre- and subsequent inoculation were carried at the same site. It needs about one week for the induction of resistance if the inoculation sites were at different parts or organs of the plant.

The fact that NCPPB 3123 induced resistance only on the host/pathogen-system tomato/Cmm and that heat-killed cells induced protection with about the same intensity like living cells indicate that resistance induction in this system is a very specific biochemic effect.

NCPPB 3123 is virulent only on pepper whereas the other Cmm-strains are virulent on both tomato and pepper. However, the differences observed in the DNA and EPS of these strains but especially the differences in host specificy could be an indication that the NCPPB 3123 is an other pathovar or a race of Cmm.

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STUDIES ON BACTERIAL LIPOPOLYSACCHARIDES AS A FACTOR OF VIRULENCE AND PATHOGENICITY

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Introduction

Plant-pathogenic bacteria are able to produce different symptoms appearing on leafs, fruits, stems and roots. The causative agents of leaf-spot-diseases belong predominantly to the genera *Pseudomonas* and *Xanthomonas*. They include species and pathovars, which are highly specific concerning their host-plants.

Mostly, the bacteria invade the intercellular space of the leaf-tissue through stomata. In the intercellular space the bacteria can either multiply (compatible interaction) or not (incompatible interaction). In many cases the incompatible interaction is characterized by a hypersensitive reaction (=HR) consisting in a fast tissue collapse. As a consequence of the HR the plant tissue desiccates, so that the bacteria cannot multiply anymore. Compatible bacteria are capable to damage the leaf tissue without causing a hypersensitive reaction, so that the typical leaf-spots develop.

The mechanisms for the narrow host-specifity of pathovars of *P.syringae* and *X.campestris* are unknown. The pathovars are not distinguishable by established microbiological methods.

For example the chemical composition of the host-plants is not the decisive factor, because the nutrient requirements of the bacteria are not specific. Apparently the host cell membrane is damaged during the compatible as well as the incompatible reaction, so that nutrients are released into the intercellular space. The main factors responsible for the compatible interaction between host and parasite are the mechanisms which include water-soaked spots together with membrane damaging. Possibly, pathogenic bacteria are able to slow down the process of desiccation of the host tissue by excreting exopolysaccharides (=EPS) and producing a gel containing water, which allows continuous bacterial multiplication. In this way water-soaked spots and exudates, consisting of bacteria and bacterial slime, appear in diseased leaves.

The components of the bacterial slime of *P. syringae* pvs are alginate, levan and lipopolysaccharides (=LPS). Xanthomonads produce xanthan instead of alginate and levan.

Alginate, levan and xanthan are important virulence factors However, these components are unspecific, and thus cannot explain host-specifity. The LPS may be one of the factors responsible for the highly specific host-pathogen-interaction.

Results of other laboratories indicate that the O-specific chains of the LPS-molecules may be specific for the different pathovars of *Pseudomonas syringae (1)*. As determinants of the serological specifity of humanpathogenic Gram-negative bacteria the O-specific chains are already well-known.

Material and Methods

LPS of different origins were purified by hot phenol-water extraction and dialysis. Plant-polysaccharides (pectins) were obtained by extraction in a buffer containing EDTA followed by ammonium sulphate precipitation and dialysis.

The interactions between bacterial LPS and pectins extracted from plant-tissue were examined by rheological methods. Both stock-solutions, LPS and pectins, were examined separately and in mixtures of different proportions. We measured the shear stress of the solutions depending on different shear rates in a cone-plate-viscosimeter (Brookfield DV-3). With these data the viscosity (here: consistency index) and the yield stress (trend of gel formation) of the mixtures were calculated.

In this way we examined the interactions in several 'compatible' and 'incompatible' mixtures. In cases with no measurable interactions between the two molecule types, we expected a direct correlation between LPS/pectin concentration and viscosity and yield stress respectively. Occurrence of interactions was indicated when a mixture of LPS and plant extract reacted differently from a mathematical calculation.

Results and Discussion

By rheological examinations of the 'compatible' mixtures (fig.1, fig.2, fig.5) we obtained data showing that the molecules interacted in a way that increased viscosity and yield stress. In these cases mixtures of the two components were more viscuous and more gelly than in the separate condition. Figs.1 and 2 show rheological interactions between LPS of *P.syringae* pv *tomato* (race 0, and race 1, respectively) with pectins of a tomato-cultivar susceptible to bacterial races 0 and 1.

Experiments with ' incompatible' solutions resulted in oppositive effects (figs. 3, 4) Fig. 3 shows the rheological interactions between LPS of *P.s.* pv. *tomato* (race 1) with pectins of a

non-host-plant (bean, cv. Red Kidney). In the mixture viscosity and yield stress of the solutions decreased. Even by mixing LPS of *P.s. pv. tomato* (race 0) with pectins of a resistant host plant (tomato cultivar Ontario, susceptible to race 1, but resistant to race 0) we obtained these data (fig.4). Mixing these Ontario-pectins with LPS *of P.s. pv. tomato* (race 1) viscosity and yield stress increased again (fig. 5).

Thus, the LPS of a pathogenic bacterium interacting with polysaccharides of a susceptible host-plant caused a viscous gelly solution.

Similar results were obtained by studying different systems of bean-plants and LPS of *P. s.* pv *phaseolicola* (data not shown).

These results indicate that also *in planta* bacterial LPS and plant-polysaccharides may be involved in specific host-pathogen-interactions.

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Figs. 1-5:

Rheological interactions between LPS-molecules and plant-pectins. The abscissa shows the relation between LPS and pectin stock solution in the sample. The ordinate shows the consistency index (CI) respectively the yield stress (YS) of the samples.

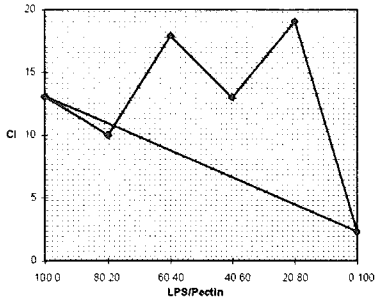
Fig. 1: Rheological interactions between LPS of *P.syringae pv. tomato* (race 0) and pectins of tomato cv. 47 susceptible to races 0 and 1.

Fig. 2: Rheological interactions between LPS of *P.syringae pv. tomato* (race 1) and pectins of tomato cv. 47 susceptible to races 0 and 1.

Fig. 3: Rheological interactions between LPS of *P.syringae pv. tomato* (race 1) and pectins of a bean cultivar.

Fig. 4: Rheological interactions between LPS of *P.syringae pv. tomato* (race 0) and pectins of tomato cv. ONTARIO resistant to race 0 and susceptible to race 1.

Fig. 5: Rheological interactions between LPS of *P.syringae pv. tomato* (race 1) and pectins of tomato cv. ONTARIO resistant to race 0 and susceptible to race 1.



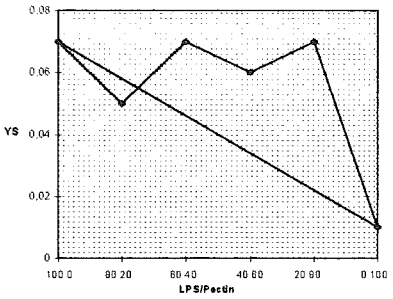
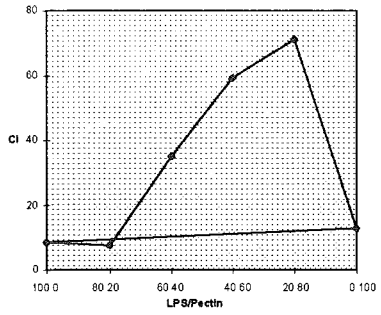


Fig.1



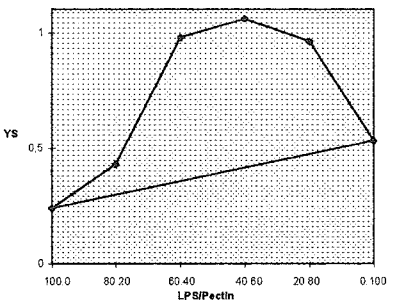
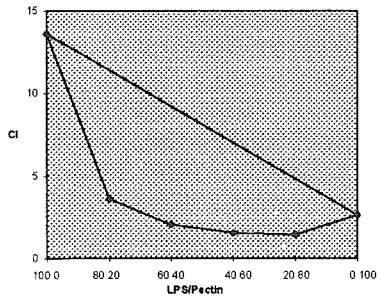


Fig.2



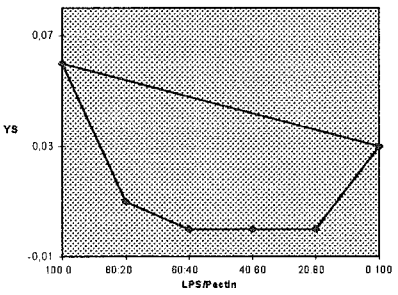
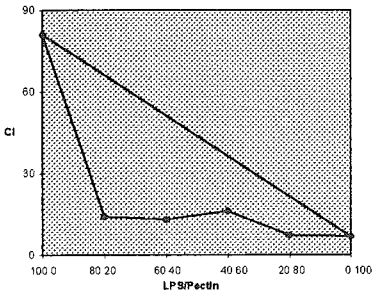


Fig.3



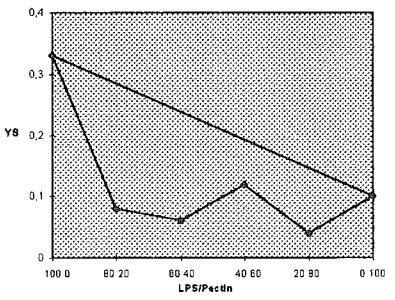
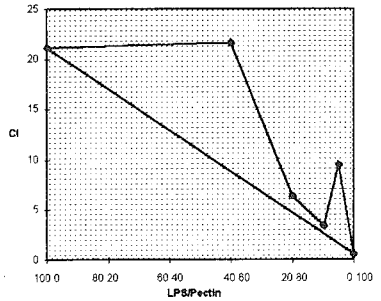


Fig.4



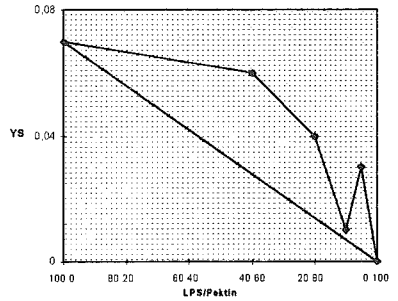


Fig.5

EVALUATION OF SEROLOGICAL METHODS AND SEMISELECTIVE MEDIA FOR DETECTING *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS*, THE CAUSAL AGENT OF BACTERIAL RING ROT OF POTATO

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Introduction

Clavibacter michiganensis subsp. *sepedonicus* (*Cms*), the causal agent of bacterial ring rot of potato, is a quarantine organism. Inspections of seed lots have to be carried out to prevent introduction of this disease. The currently prescribed procedure for detection of *Clavibacter sepedonicus* consists of the removal and homogenization of conical tissue cores from the heel ends of 200 tubers/lot and subsequent filtration and centrifugation. The resuspended pellet is used for the immunofluorescence (IF) test. A positive IF result has to be confirmed in a pathogenicity test on eggplants. If typical symptoms occur, the pathogen has to be isolated to test several physiological activities for identification. These methods are extensive and time consuming.

To facilitate detection of the ring rot pathogen, different serological methods and semiselective media were tested for their suitability in detecting *Cms* . 3 serological methods were presented: the isolation by magnetic immunotrapping, the immunosorption of bacterial cells on a solid phase with subsequently adding of an agar medium and growth of colonies, and the immunostaining of tiny colonies grown in an agar medium. Furthermore, a new semiselective medium helps to detect and to isolate *Cms*.

Material and Methods

Trapping of *Cms* - cells in a liquid sample with antibody-coated magnetic particles

The "magnobeads" are 0.5 - 1.5 mm in diameter, the material is iron oxide. Goat anti - rabbit antibodies are bound to the surface of the particles. Prior to use, the magnobeads were washed with sterile 0,01 M PBS. The first step of the procedure is to link the target cells on an anti-*Cms*-antibody from rabbit. The incubation time was 1h at room temperature. Than a suspension containing the magnetic beads was added, reaching a final concentration of 10%. After incubation for 1h at room temperature, a magnet was placed beneath the suspension, so

the beads were put to the bottom of a well of a culture plate. In this position the supernatant can be removed by a pipette tip connected to a vacuum pump. 1 ml of sterile PBS was added and the content resuspended. To evaluate the effectiveness of isolation, the resuspended particle-bound cells were streaked out onto agar medium or poured into agar medium.

Immunoadsorption of *Cms*-cells on a solid phase and adding of an agar medium.

Bacterial cells were immunoadsorbed onto antibody-coated solid phases, for instance petri dishes, and an agar medium added at 45 °C. Adsorbed bacteria could then form colonies at the bottom of the plates. Removing of liquids took place with an pipette tip connected to a vacuum pump.

Coating of antisera was done at 27 °C for 1h or at 4 °C over night on test areas, that were delineated on a petri dish. The plates were washed 2 times with Ringer´s Tween, PBS or PBS-Tween. After washing the plates were desinfected with 70% ethanol. Coated plates were stored dry at 4 °C for up to 5 days. To detect the target bacteria, 5 ml of the sample was added to petri dish and incubated at 27 °C for 1h. The petri dish was coated with different antisera dilutions on 6 circles. In a later stage of investigations, 24-well culture plates were used. Plates were washed 2 times for 1 min and then 10 ml of 0.8% YGM-Agar was added at 45 °C. After incubation at 23 °C for 4-6 days the grown colonies could be observed.

Immunofluorescence colony staining

The samples or test suspensions were mixed with an agar medium and poured into petri dishes. The bacteria in the sample formed colonies within the medium. When the colonies were grown on the surface of the agar medium, they were covered with water agar. On a heating plate or with a warm air dryer the agar medium was dried to a transferable, thin material. After placing it in a well of a culture plate, the dried agar was incubated with anti-*Cms*-FITC-conjugate. Using the indirect mode, incubation was first with an anti-*Cms*-antiserum and second with a FITC.conjugate against the first antibody. Incubation was at 27°C and the washing steps were done with 1mM PBS + 0,01% Tween 20. When using this method with plant material, a *Cms*-suspension as a positive check was added to healthy tubers. As a negative check we used a heterologous organism.

Semiselective medium for *Clavibacter michiganensis* subsp. *sepedonicus*

To selectively enrich *Cms*, a semi selective agar medium was developed, containing a special carbon source and some antibiotics. The medium is based on the YGM-medium (anonym 1993) and contains : 2.0 g yeast extract, 2.5 g mannitol, 0.25 g K_2HPO_4, 0.25 g KH_2PO_4, 0.1 g $MgSO_4$ x 7 H_2O, 0.015 g $MnSO_4$ x H_2O, 0.05 g NaCl, 0.005 g $FeSO_4$ x 7 H_2O, 16.0 g Agar (Oxoid N°·1), ad 1000 ml aqua dest, pH 7.2. After autoclaving and cooling

to 50 °C, 2.0 mg nalidixic acid, 100 mg trimethoprim and 10 mg amphotericin B was added. When working with plant material, the concentration of trimethoprim was reduced to 70 mg.

Depending on the kind of the test, colonies were also observed after 2-3 days of growth with a binocular.

Results

Isolation of bacterial cells with antibody-conjugated magnetic particles from liquid samples and plating the trapped cells onto agar medium was successfully performed. The growth of unwanted organisms was reduced to a satisfactory level. However, the detection limit for *Cms* was in the range from 20.7 to 49.5 %, due to the several washing steps. Washing with Ringer´s Tween resulted in a higher level of *Cms*-losses than washing with PBS. These results were not satisfactory, so other techniques were needed to obtain the necessary sensitivity.

Immunoadsorption of *Cms* cells on a solid phase and subsequent adding of an agar medium was performed successfully: *Cms*-cells bound to the bottom of the test areas were able to form visible colonies. When the bacterial suspensions were diluted in PBS + 2% PVP, the amount of grown colonies was reduced compared to PBS without PVP. Blocking of the plates after coating with antisera resulted in an increased number of *Cms* - cells within the test circles. However, also outside of the test circles more target bacterial cells were observed. Washing the plates with 10 mM PBS-Tween gave resulted in more trapped cells than washing with 1 mM PBS-Tween. Because of the washing steps, losses of the target bacteria were inevitable, resulting in a decrease of sensitivity. This is similar to the results in magnetic immunoisolation.

The staining of *Cms*-colonies was specific and with brillant intensity. *Cms*-cells from stained colonies could be removed by a sterile syringe or pasteur pipette and plated onto appropiate agar medium. Single cells were able to form colonies, resulting in a pure culture of *Cms*. Dried preparations could be stained, by a modification of the procedure above, after storage for 18 months. Stained preparations could be observed 7 months after staining without loss of quality. The agar concentration, the drying of the preparations and the washing steps influenced the quality of staining.

In table 1 the result of a test with natural infected tubers is shown. An input stage of the medium was used, containing trimethoprim and Amphotericin B as inhibitory substances. On these media non-*Cms* bacteria were inhibited up to 100%, compared to the YGM and YMM media. Therefore, detection of *Cms* from tubers was much easier. Regarding strain HJ4/91, the tendency of slightly reduced growth of *Cms* was observed on media containing antibiotics. Growth of *Cms*-starin HJ4/91 was better on YMM than on YGM. Probably the inhibitory effect can be more than equalized using mannitol instead of glucose.

Table 1: colonies of bacteria (*Cms* and non-*Cms*) from a natural infected tuber lot and from a suspension of the strain HJ4/91 on 4 agar media. YGM = Yeast extract glucose mineral salts medium; YMM = Yeast extract mannitol mineral salts medium; *Cms*-colonies were counted after 6 days, other bacteria after 4 days of growth at 23 °C
AB = antibiotics Trimethoprim (100 ppm) and Amphotericin B (10 ppm).

sample/ dilution	medium							
	YGM		YGM + AB		YMM		YMM + AB	
	Cms	non *Cms*	*Cms*	non *Cms*	*Cms*	non *Cms*	*Cms*	non *Cms*
tubers 10^{-3}	13[1)]	229	37	0	3[1)]	208	34	2
10^{-4}	6[1)]	28	5	0	6[1)]	27	7	0
10^{-5}	1[1)]	4	1	0	0[1)]	6	1	0
10^{-5}	0[1)]	2	2	0	0[1)]	2	1	0
HJ 4/91 10^{-5}	294		268		340		299	
10^{-5}	276		277		316		334	
HJ 4/91 in %, YGM = 100	100		95,6		115,1		111,1	

1): Minimal amount of present colonies, because of saprophytes not countable exactly.

Discussion

Both the trapping of *Cms*-cells in a liquid sample with antibody-coated magnetic particles and the immunosorbent plating were working. Probably the methods could be further optimized, for instance by coating anti-*Cms*-antisera directely to the "magnobeads" or by adsorbing bacteria to another solid phase than petri dishes. Also optimization of buffers and solutions could be investigated. However, these attempts can only reduce the losses of target cells. By immunofluorescence staining of small colonies, grown in a suitable agar medium for 2-3 days, the disadvantage of losses by washing steps could be avoided. The immunofluorescence staining of colonies combines specificity (depending on the quality of antisera used), sensitivity (depending on the agar medium used) and fast isolation of the pathogen. The combination of immunofluorescence colony staining and semiselective medium for *Cms* could be a helpful tool for the detection and isolation of *Cms* from plant material, e.g. potato tubers or inoculated eggplants.

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COMPARISON OF PCR-TECHNIQUES AND CONVENTIONAL METHODS TO DETECT *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* IN POTATO TUBERS

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Introduction

Clavibacter michiganensis subsp. *sepedonicus* (*Cms*) is the cause of bacterial ring rot of potato. In accordance with the status of a quarantine organism within the EC, seed lots have to be tested on *Cms* by an established EU procedure. Because these methods are often time consuming and extensive, different PCR approaches have been developed by the EU experts group. The methods were tested for their suitability to detect *Cms*-cells in potato tubers and eggplants, and to identifcate *Cms*-isolates.

The polymerase chain reaction (PCR) is an effective technique, amplifying DNA to detectable quantities occuring otherwise in undetectable amounts.To increase the sensitivity of the PCR detection, a so-called nested primer PCR - i.e., a second, smaller DNA fragment is made from the first amplified DNA fragment - was performed.

In comparative test performed in different EC-countries, 2 x 7 potato tubers were distributed, from which 5 out of 7 were artificially inoculated with *Cms*.. In order to obtain the most efficient method, two procedures of sample preparation (the homogenisation- and the shaking-procedure) and two procedures of DNA extraction (phenol/chloroform extraction and spin column purification) were applied. To determine the level of infection and subsequentely the detection sensitivity, immunofluorescence (IF) and eggplant test was compared with polymerase chain reaction (PCR). New approaches were used to increase the specificity of PCR detection. The results of the extractions and the detection techniques are discussed.

Material and Methods

Each of 2 x 7 tubers was mixed with 199 tubers that were known to be free of *Cms*. Two controls were included, representing a bacteria-free sample of 200 tubers and a sample with 1 ml of a known concentration of *Cms* (~ 10^8 cells) added to 200 heel end cores. Therefore, 9 samples were tested on the homogenisation method and 9 samples on the shaking

method, and each of these sample was tested by IF-test, eggplant test, phenol/chloroform extraction, spin column purification and PCR (2 protocols).

Homogenisation method

According to the council directive 93/85/EEC (1), annex 1, heel end cores were removed from tubers and crushed with a hammer in a small heavy duty bag, containing 25 ml of 10 mM PBS-Tween. After soaking of the macerate for 30 min at 4°C, macerate was filtered (Whatman N° 1), enhanced by using vacuum. The filtrate was centrifuged at 10 000 x g for 20 min and the resulting pellet suspended in 2 ml of 0,01 M PBS. (= samples "A")

Shaking procedure

0,5 cm of the heel ends from the tubers were removed and cut in pieces. Potato tissue was placed in bottles and tapwater added until the material was covered. Bottles were shaken on a rotary shaker for 18 hours at 100 rpm. Samples "C" of 100 ml were taken and centrifuged at 6 000 x g for 20 min. The pellet was suspended in 2 ml of water. Samples "B" were taken without centrifugation (results not shown).

Immunofluorescence (IF) test

An aliquot of the suspended pellet was diluted and 25 µl of each sample transferred to a well of a multitest slide. Slides were air dried and fixed in acetone for 10 min. Slides were covered with polyclonal antisera against *Cms* and with the monoclonal antibody 9A1. After incubation for 30 min, slides were washed and incubated with the appropriate FITC-conjugates. After incubation slides were washed and covered with a mounting fluid. Observation was done at 1000 x magnification using a microscope with a mercury lamp and a suited filter system. Morphologically typical fluorescent cells were counted in 50 microscope fields and the mean number per ml of the pellet calculated (1).

Eggplant test

According to (1) 25 plant of *solanum melongena* (cult. Black Beauty) were inoculated with an aliquot of the pellet by syringe inoculation. The number of plants showing typical symptoms was observed.

Lysozyme-SDS lysis and Phenol/Chloroform extraction

An aliquot of the resuspended pellet was used for lysis of the cells by treatment with lysozyme and SDS. DNA was separated by phenol/chloroform extraction and precipitated with ethanol (2).

Alkaline lysis and spin column purification

In comparison to phenol/chloroform extraction, the *Cms*-Dna was purified by lysis of the cells with 50 mM NaOH and by using a spin column (Diagen), according to the companies protocol.

Polymerase chain reaction (PCR)

PCR was performed by 2 pairs of primers running 15 circles with the first and 30 circles with the second oligonucleotides. In the "one tube" nested primer PCR, both primer pairs were added at the beginning of the amplification. Some samples were also tested by running 15 circles with the first primers, adding the second primer pair to the tubes, and afterwards running 30 further circles ("two tube").

Results and Discussion

Results are listed in tables 1 and 2. In the homogenisation method all positive samples were detected by IF-test, eggplant test and PCR after Qiagen column purification. The phenol/chloroform extraction gave negative results in 3 out of 6 positive samples. Sample 7 showed a very light false positive band in the agarose gel after "one tube"-PCR, probably due to contamination. After shaking tuber pieces, positive result were observed in 4 out of 6 positive samples with IF-test, PCR (Qiagen column purification) and eggplant test. Results after phenol/chloroform extraction differed in 3 samples from the other techniques (table 2).

Table 1: Results of samples "A" (homogenisation method)

sample	IF-test	PCR one-tube		PCR two-tube		eggplant - test	inocu-lated
		Qiagen column	phenol/ chlorof.	Qiagen column	phenol/ chlorof.	(number of plants with symptoms)	
A 1	+	+	+	+	n.p.*	+ (12)	+
A 2	+	+	-	+	n.p.	+ (2)	+
A 3	-	-	-	-	n.p.	-	-
A 4	+	+	-	+	n.p.	+(18)	+
A 5	+	+	+	+	n.p.	+ (21)	+
A 6	+	+	-	+	n.p.	+ (22)	+
A 7	-	(+)	-	-	n.p.	-	-
A 8	-	-	-	-	n.p.	-	-
A 9	+	+	+	+	n.p.	+ (21)	+

*= not performed

Table 2: Results of samples "C" (shaking method)

sample	IF-test	PCR one-tube		PCR two-tube		eggplant - test	inocu-lated
		Qiagen column	phenol/ chlorof.	Qiagen column	phenol/ chlorof.	(number of plants with symptoms)	
C 1	-	-	-	-	n.p.*	-	+
C 2	+	+	-	+	n.p.	+ (8)	+
C 3	-	-	-	-	n.p.	-	-
C 4	+	+	+	+	n.p.	+ (15)	+
C 5	+	+	-	+	n.p.	+ (12)	+
C 6	-	-	(+)	-	n.p.	-	+
C 7	-	-	-	-	n.p.	-	-
C 8	-	-	-	-	n.p.	-	-
C 9	+	+	-	+	n.p.	+ (1)	+

*= not performed

Regarding the performed protocol, the shaking method seems to be less sensitive than homogenisation method. However, some modifications of the protocol (e.g., reducing the size of the tuber pieces to be soaked) could enhance sensitivity. In principle, this method is more convenient than the homogenisation procedure, because preparations are almost devoid of plant debris.

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EFFECTIVENESS OF ERWINIA HERBICOLA STRAINS AND CHEMICALS TO BLOSSOM FIRE BLIGHT CONTROL

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Introduction

Fire blight, caused by Erwinia amylovora, is widespread and destructive disease of pear, apple and other rosaceous plants, f. i. quince, hawthorn, mountain ash and cotoneaster.

Fire blight may be controlled in pome-fruit orchards by the application of chemicals including copper compounds and antibiotics, i.e. streptomycin. Copper compounds are often phytotoxic and less effective than streptomycin, that is not approved for use in many countries due to problems with patogen resistance. Biological control, using microbial agents, represents an alternative measurement.

Material and Methods

Saprophytic bacteria were isolated from host plants of fire blight (hawthorn, apple tree, pear tree, mountain ash) in various localities of Bohemia. Isolated bacteria were mostly classified with Erwinia herbicola group (LÖHNIS) Dye, 1964. Obtained bacterial isolates were screened for inhibitory activity against the fire blight agent using test of agar plates seeded by *Erwinia amylovora* (2) and using the immature pear fruit assay (1). Selected antagonistic strains were than tested for ability to control blossom-blight of various host plants (*Crataegus* sp., *Pyrus* sp., *Malus* sp., *Sorbus* sp. *Cotoneaster* sp.). Blossoming branches, maintained in growth chamber, were sprayed first with potential biological control agents (10^8cfu/ml) or chemicals and than 24 h later with patogen (10^6cfu/ml). Chemical preparations were used in these concentrations: Streptomycin - 0,02% (streptomycin sulfate), Aliette - 0,3% (fosetyl Al), S-0208 - 0,3% (oxanilic acid analogue), Trimiltox forte - 0,3% (mancozeb, copper), Champion 50 WP - 0,2% (hydroxide copper) and Kuprikol 50 - 0,3% (oxychloride copper). Effectivity of tested antagonistic strains and chemicals were compared.

Results

Streptomycin was the most effective preparate with 82% effectivity, followed by the preparate Aliette 80 WP (78 %). Antagonistic strains Eh 198/1/1 and Eh 97/5/0 with preparations S-0208 and Trimiltox forte had approximately 62% effectivity. The lowest effectivity had antagonistic strains Eh 91/4/0, Eh 147/1/1 and the preparate Champion 50 WP (55 %) and the absolutely lowest effectivity was found at Kuprikol 50 (47 %). With the exception of streptomycin and Aliette 80 WP, the effectivity of antagonistic strains was comparable with tested chemicals. In comparison with Kuprikol 50, their effectivity was higher. When compared chemicals and potential biological

control agents, significant differences ($P \leq 0.05$) were found in effectivity of several of them (Table 1). There were no significant differences ($P \leq 0.05$) between host species (Fig. 1).

Table 1: **Effectivity of chemicals and antagonists against** *Erwinia amylovora* **on detached blossoming branches of host plants** (average)

Treatment	Infected blossoms (%)	*Effectivity (%)	Differences
Water check	19,3	-	a
Streptomycin	29,9	82,0	a b
Aliette 80 WP	32,7	78,0	a b c
S-0208	39,5	66,4	b c d
Trimiltox forte	41,4	63,5	b c d
Eh 198/1/1	43,6	58,9	b c d
Eh 97/5/0	44,3	57,8	b c d
Eh 91/4/0	45,4	56,8	c d
Eh 147/1/1	46,2	54,0	c d
Champion 50 WP	47,0	53,9	c d
Kuprikol 50	50,0	47,2	d
Ea 531	79,3	-	e

Legend: Ea - *Erwinia amylovora*, Eh - *Erwinia herbicola*
* water check = 0 and *E. amylovora* = 100 %
$P \leq 0.05$ - significant differences between particular treatments (a,b,c,d,e)

Discussion

In fire blight control, they are prospective such chemicals and biological control agents that are comparable with effectivity of streptomycin. Comparison of the selected strains of *Erwinia herbicola* with streptomycin and other chemical preparations showed that the biological control agents can provide an effective control of blossom blight on various host plants. Streptomycin and Aliette 80 WP were effective than copper compounds. However, no antagonistic strain was as effective as streptomycin.

High effectivity of preparation Aliette 80 WP in our experiments is in accordance with results of many reserchers that recommend this preparation to be used mainly against blossom blight, because it is not phytotoxic to blossoms (6). Preparation S-0208 was not as effective in our experiments as we would suppose. Some reserchers obtained in tests with preparation S-0208 the same or higher effects than with streptomycin, however in field trials (3,7). Unfortunately this preparation failed in respect of environmental criteria and was excluded from further evaluation.

The most effective preparation of copper compounds was Trimiltox forte that was verified with good results in Holland, too (4). Other copper preparations (Champion 50 WP and Kuprikol 50) had lower effectivity against blossom blight than selected antagonistic strains. Similary in experiments on

cotoneaster, the weak effect of copper compounds for blossom blight was performed, but preparation S-0208, streptomycin and biological control agents ensured sufficient control (8).

Effectivity of all four verified antagonists was almost similar. In glasshouse conditions, there were not any statistically significant differences between host species. We supposed that infection of pear and hawhthorn clusters would be more severe than infection of apple and mountain ash clusters in accordance with different sensitivity of these host species to fire blight (5).

Results confirmed that antagonistic strains could substitute copper compounds in fire blight control.

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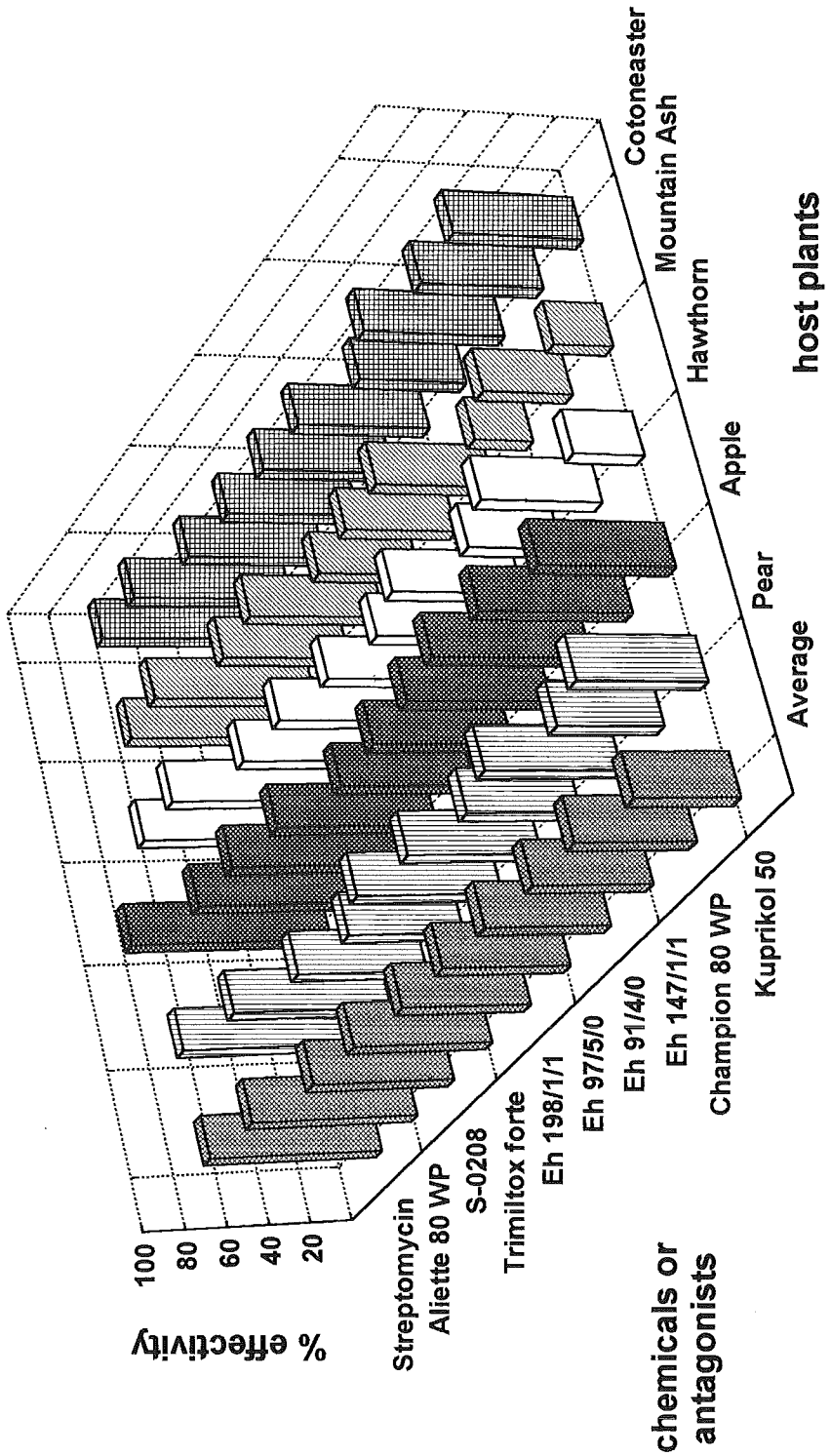
Fig. 1 Effectivity of chemicals and antagonists against Erwinia amylovora on detached blossoming clusters of host plants of fire blight



POTATO BROWN ROT - ISSUES IN THE EUROPEAN UNION AND RESULTS OF SEROLOGICAL TESTS

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Introduction

Potato brown rot is caused by *Pseudomonas solanacearum* (Smith) Smith. This bacterium is especially for tropical and subtropical areas of economical importance. It is classified into 5 biotypes because of its acid production from various disaccharides and sugar alcohols and into 5 races according to its pathogenicity. Race 3, which is adapted to moderate climate and specific to potatoes and other Solanaceae, is of particular importance for the European Union.
YABUUCHI *et al.* (1992) proposed to transfer seven species of the genus *Pseudomonas* Homology Group II to *Burkholderia* New-Genus, mainly because of the 16S rRNA sequences, but also of other features (1). This concerned also *Pseudomonas solanacearum* for which the name *Burkholderia solanacearum* was proposed.

In the beginning of the 1970's, this potato disease was reported in Sweden and then from 1989 to 1994 in the United Kingdom, the Netherlands and Belgium, in total in 8 locations at 15 fields. Due to strict inspection of ware potatoes, an increasing number of infected lots imported from third countries have been found and rejected.
In view of this, the phytosanitary regulations of the Plant Health Directive 77/93/EEC aimed at protecting Member States were extended (2). They now describe more detailed requirements for potato production in Member States and third countries, in order to determine, for instance, whether a potato-growing site is free from infection or not. Other plants, such as pepper, tomato, banana, tobacco and eggplants intended for planting, are covered, too.

As far as *Pseudomonas solanacearum* is concerned, there are no fixed EU-wide test methods such as those for *Clavibacter michiganensis* ssp. *sepedonicus* (bacterial ring rot). The application of the indirect immunofluorescence antibody staining (IFAS) combined with the examination of potato tubers for the presence of *Clavibacter michiganensis* ssp. *sepedonicus* could be a possible method as a first detection step in routine testing. Various polyclonal antisera produced at the Institute for Pathogen Diagnostic in Aschersleben have been tested for their suitability.

Material and methods

Antisera, conjugates and isolates used

Four antisera were prepared in rabbits with two dates of bleeding and one in goat. Various strains of *Pseudomonas solanacearum* were used as antigens. The goat serum was a serum against several strains (Table 1).
To test the reaction to *Pseudomonas solanacearum* (Ps) by IFAS five different isolates were used, among them four of race 3, biotype 2 from potato. Cross-reaction was tested with two isolates of *Clavibacter michiganensis* ssp. *sepedonicus* (Cms), three of *Erwinia carotovora* ssp. *atroseptica* (Eca), two of *Erwinia carotovora* ssp. *carotovora* (Ecc) and three of *Erwinia chrysanthemi* pv. *chrysanthemi* (Ech).

Table 1: Antisera tested for their suitabiliy to detect *Pseudomonas solanacearum* and possible cross-reactions by IFAS

Code-number used	Animal	Bleeding	Source of antigen
A 1	rabbit	08.04.1991	Solanum tuberosum
A 5	N° 129	29.04.1991	- „ -
A 4	rabbit	29.04.1991	Solanum tuberosum
A 8	N° 128	06/1991	- „ -
A 2	rabbit	08.04.1991	Solanum dulcamara
A 7	N° 139	29.04.1991	- „ -
A 3	rabbit	23.04.1991	Solanum dulcamara
A 6	N° 148	29.04.1991	- „ -
A 9	goat	04.09.1990	Solanum tuberosum, Phaseolus vulgaris; Musa

FITC goat/anti-rabbit IgG, whole molecule and FITC rabbit/anti-goat IgG, whole molecule, both from SIGMA, were used as conjugates in 100-fold dilution in 0.01 M PBS.
In principle the method for IFAS, described by JANSE, 1990 (3), was used with some changes.

Sample preparing:
Pure cultures were used as a 10^6 cells/ml suspension in 0.01 M PBS (2.7g $Na_2HPO_4x12H_2O$, 0.4g $Na_2H_2PO_4x2H_2O$, 8.0g NaCl ad 1l, pH 7.2). Potato sap with bacteria, added in defined density, was filtered on a Whatman paper filter n°1 which was placed in a sintered glass filter n°2 (90-150µm) using vacuum. The filtrate was collected in a centrifuge tube, centrifuged for 20 minutes at 10,000 g and the pellet resuspended in 1 ml 0.01 M PBS and diluted 10- and 100-fold.

Procedure for IFAS:
20 µl of suspensions were applied to two succeeding wells of a 10-well multitest slide and heat-fixed on a hot plate for 30 min at 60 °C. This was followed by a refixation. The slides were covered with a mixture of ethanol:chloroform:formaldehyde (60:30:10 vv) for 30 min in a humid chamber, then gently rinsed with 0.01 M PBS and airdried. 20 µl of antisera/window in 4 dilutions (1000, 3000, 6000, 12000) were applied, incubated for 30 min at 37 °C in a humid chamber, washed three times in 0.01 M PBS and dried on a hot plate at 60 °C (without direct contact, in a distance of about 30 mm!). 20 µl conjugate were then added and incubated, washed and dried as described above. The slides were covered with 0.1 M phosphate-buffered glycerin and a coverglass and then examined with a microscope with an epifluorescent light source and blue-filter (450-490nm) with 1000-fold magnification. 10 to 50 microscopic fields/window were scanned for the presence of fluorescing cells.

Results and discussion

None of the antisera have shown a positive result at a dilution of 1:12000. The rabbit-antisera with code numbers A 4 and A 8 have crossreacted with all isolates and were aspecific.
The reaction of the other antisera with isolates of *Pseudomonas solanacearum* and cross-reactions with isolates of other species by IFAS are summarized in Table 2.
The intensity of fluorescence was determined with a scale. No fluorescence is expressed by „-" and excellent fluorescence by „**4+**".
Refixation with the mixture of ethanol:chloroform:formaldehyde produces a more uniform fluorescence of the whole cell wall of the bacteria. Without this step an accumulation of fluorescence at the cell ends was observed.

Table 2: IFAS reaction of various antisera with different isolates of *Pseudomonas solanacearum* and cross-reactions with isolates of other species

	antisera																	
	A 1			A 5			A 2			A 7			A 3			A 6			A 9		
isolates	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c	a	b	c
Ps 1	(+)	(+)	(+)	(+)	(+)	(+)	2+	2+	+	3+	2+	+	(+)	(+)	(+)	+	+	+	3+	3+	2+
Ps 2	4+	3+	3+	3+	2+	2+	3+	2+	2+	4+	3+	2+	2+	2+	2+	3+	3+	2+	4+	3+	2+
Ps 3	4+	3+	+	2+	(+)	(+)	3+	(+)	(+)	4+	3+	2+	3+	2+	2+	(+)	2+	2+	3+	3+	2+
Ps 4	3+	2+	2+	2+	2+	+	3+	+	(+)	3+	2+	2+	2+	(+)	(+)	+	+	+	4+	4+	2+
Ps 5	3+	2+	+	(+)	(+)	-	2+	(+)	-	3+	2+	(+)	(+)	(+)	(+)	(+)	-	-	4+	4+	3+
Cms 1	+	(+)	(+)	-	(+)	(+)	(+)	-	-	-	-	-	2+	2+	2+	2+	2+	+	+	(+)	(+)
Cms 2	+	(+)	(+)	(+)	(+)	(+)	(+)	-	-	-	-	-	-	-	-	(+)	(+)	-	(+)	(+)	-
Eca 2	(+)	(+)	-	(+)	-	-	(+)	-	-	-	-	-	(+)	-	-	-	-	-	(+)	(+)	(+)
Eca 4	(+)	-	-	(+)	-	-	(+)	-	-	(+)	-	-	-	-	-	-	-	-	+	(+)	(+)
Eca 6	-	-	-	-	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-	+	+	(+)
Ecc 5	(+)	(+)	-	(+)	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ecc 1	(+)	+	+	(+)	+	+	+	(+)	-	+	-	-	(+)	(+)	(+)	(+)	-	(+)	(+)	(+)	-
Ech 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	(+)	-	-
Ech 7	-	-	-	-	-	-	+	-	-	-	-	-		-	-	-	-	-	-	-	-
Ech 8	(+)	-	-	-	-	-	(+)	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-

antiserum dilution: a = 1:1000; b = 1:3000; c = 1:6000

intensity of fluorescence: – none; (+) very weak; + weak; 2+ medium; 3+ good; 4+ excellent

The antisera A7 (rabbits) and A9 (goat) showed at dilutions of 1:1000 and 1:3000 excellent and good reactions by IFAS to all used isolates of Ps with regard to the intensity of fluorescence. The rabbit serum A1 exhibited sufficient fluorescence only to Ps 2 and Ps 3, the other sera had no satisfying reaction to the investigated dilutions. Therefore, lower titres should be tested for their suitability. The sera showed different cross-reactions according to titre and species mostly with very small to weak fluorescences. Only the rabbit serum A7 did not show by IFAS any cross-reaction at dilution 1:3000. Because of the better reactions to all used Ps isolates and the higher titre, the goat serum A9 was used in further experiments to detect certain bacteria densities in potato sap (Table 3).

Table 3: Detection of *Pseudomonas solanacearum* in potato sap by IFAS using defined densities of bacteria

	series 1		series 2	
sample	a	b	a	b
1	0.7×10^8	3.4×10^7	1.0×10^8	4.1×10^7
2	0.7×10^7	4.5×10^6	1.0×10^7	3.0×10^6
3	0.7×10^6	6.4×10^5	1.0×10^6	3.0×10^5
4	0.7×10^5	4.8×10^4	1.0×10^5	0
5	0.7×10^4	0	1.0×10^4	0

a = number of bacteria/ml, added
b = number of bacteria/ml, detected

Pseudomonas solanacearum: **Ps 2**
Antiserum: **A9**, **1:3000** diluted

After sample preparing it was not possible to detect the added bacterial densities by IFAS. The losses of bacterial cells led near the detection limit for IFAS (10^4-10^5 cells/ml) to the fact that no fluorescing cells were found under microscope. In any case the preparation of the samples is of decisive importance and should be improved in future to optimize IFAS results.

The goat serum A9 prepared in Aschersleben proved in the tests to be suitable for routine testing for *Pseudomonas solanacearum* using potato samples which were prepared for testing for latent infection with *Clavibacter michiganensis* ssp. *sepedonicus*.

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DETECTION OF BACTERIAL DISEASES OF TOMATOES BY THE POLYMERASE CHAIN REACTION (PCR) AND SEROLOGY

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INTRODUCTION

Tomatoes contribute a large portion of vegetables grown in Turkey. The production is still increasing. Concomitantly bacterial diseases are causing higher yield losses (1). Because effective bactericides are not available and tomatoes are mainly grown by small farms with limited budgets disease control is insufficient (2,3). The present strategy to reduce yield losses by bacterial diseases is to use healthy seeds and resistant cultivars in combination with chemical, biological or cultural measures (4,5,6). Therefore highly sensitve methods have to be developed to determine bacterial infestation of seeds, so that these methods can be used by the plant protection service in Turkey in the future.

MATERIALS AND METHODS

Bacterial strains.

Bacterial strains originated from the collection "Göttinger Sammlung phytopathogener Bakterien" and were grown overnight in Rhodes´ medium (7) at 28 °C under gentle agitation.

DNA extraction, PCR assay and restriction analysis

The oligonucleotides used for PCR based diagnosis were derived from a region of the *cfl* gene of *Pseudomonas syringae* pvs. as described by Bereswill et al. 1994 (8). Total DNA was extracted according to Ausubel et al. 1988 (9).
For PCR amplifications 10 ng of total DNA were used. Two oligonucleotide primers (25 pmol each) and a 50 µl PCR mixture containing 0.8 U Taq-polymerase (Fermentas), 0,2 mM dNTPs, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08% Nonidet P40, 5% DMSO, 10 mM 2-mercaptoethanol, and 100 µg bovine serum albumin were used. The PCR mixture was overlayed by 50 µl paraffin oil. First denaturation was done at 94 °C, further denaturations at 92 °C for 2 min. After the first denaturation the polymerase was added (hot start). The primers were annealed by a touch-down protocol (2 cycles each beginning at 52 ° C for 2 min, decreasing the annealing temperature for 0.5 °C at each second cycle down to 48 °C followed by 11 further cycles), the primers were extended at 72 °C for 2 min. In these experiments a thermocyler of Hybaid-OmniGene was used. An aliquot of 8 µl was analysed on a 1.5 % agarose gel (10). Restriction analyses were performed with approximately 500 ng of the amplification product. The digestions were carried out according to the manufacturer (Fermentas) with 10-fold overdigestion.

DNA cloning, plasmid DNA extraction and DNA-sequencing.

The amplified DNA fragment was cloned into pBluescript SK- (Stratagene) after creating blunt ends with DNA polymerase I. Ligation reaction was performed in the presence of 2.5 % polyethyleneglycol 6000 (Serva). Plasmid DNA was extracted according to Birnboim 1983 (11) and sequenced with the T7-sequencing kit derived from Pharmacia using α-dATP-S35 according to the manufacturer´s recommendations.

Serological methods

Serological methods were used as described Baharuddin et al. 1994 (12).

The solubilized proteins were separated by PAGE in SDS. After Western-blotting the samples were incubated with polyclonal antibodies against *Xanthomonas campestris* pv.*vesicatoria* obtained from Dr. van der Wolf, Wageningen, and visualized by reaction with secondary antibodies and Fast Red.

RESULTS

The primers derived from Prof. Geider, Heidelberg, used for the amplification of a region from an enzyme involved in the coronatine biosynthesis (*cfl*) led to a successful amplification of a 650 bp fragment out of *Pseudomonas syringae* pv. *tomato* GSPB-No. 487. The identity of the amplification product was checked by both restriction analyses and sequencing. Fig. 1 shows the expected fragment sizes according to the published data from Bereswill et al. 1994 (8). The cloning and sequenzing of the amplification product confirmed the identity of the product (98% identity). Further *in vitro* tests showed successful amplification with other coronatine producing plant pathogenic bacteria. Non-producing bacteria showed no signal (see Tab. 1).

Tab. 1: Amplification of a region of the *cfl* gene out of different plant pathogenic bacteria

Isolate	GSPB-No.	Amplification
Pseudomonas syringae pv. *tomato*	487	+
Pseudomonas syringae pv. *glycinea*	2011	+
Pseudomonas syringae pv. *morsprunorum*	1013	+
Pseudomonas syringae pv. *phaseolicola*	1715	-
Pseudomonas corrugata	1424	-
Pseudomonas marginalis pv. *marginalis*	92	-
Burkholderia (Pseudomonas) solanacearum	1958	-
Erwinia carotovora subsp. *carotovora*	1405	-
Xanthomonas campestris pv. *vesicatoria*	2050	-

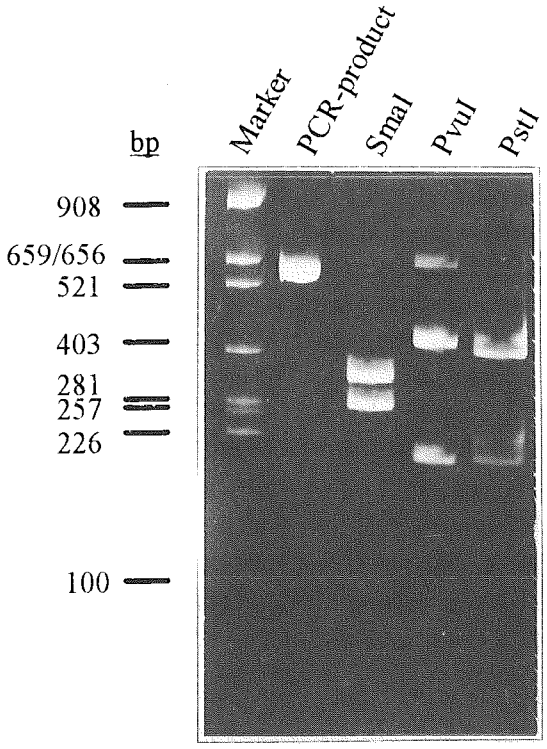

Fig. 1. Restriction analysis of the *cfl* PCR-product out of *P. s.* pv. *tomato*

An antiserum against *Xanthomonas campestris* pv. *vesicatoria* was tested with three strains (GSPB-Nos. 2050, 2045, 224). Cross reactions were tested with *Pseudomonas corrugata, Pseudomonas syringae* pv. *tomato, Burkholderia (Pseudomonas) solanacearum, Clavibacter michiganensis* ssp. *michiganensis, Pseudomonas fluorescens.* The three *Xanthomonas campestris* pv. *vesicatoria* strains showed an identical protein band of 28 kDa which did not occur in any other bacterial species or pathovar tested (Fig. 2).

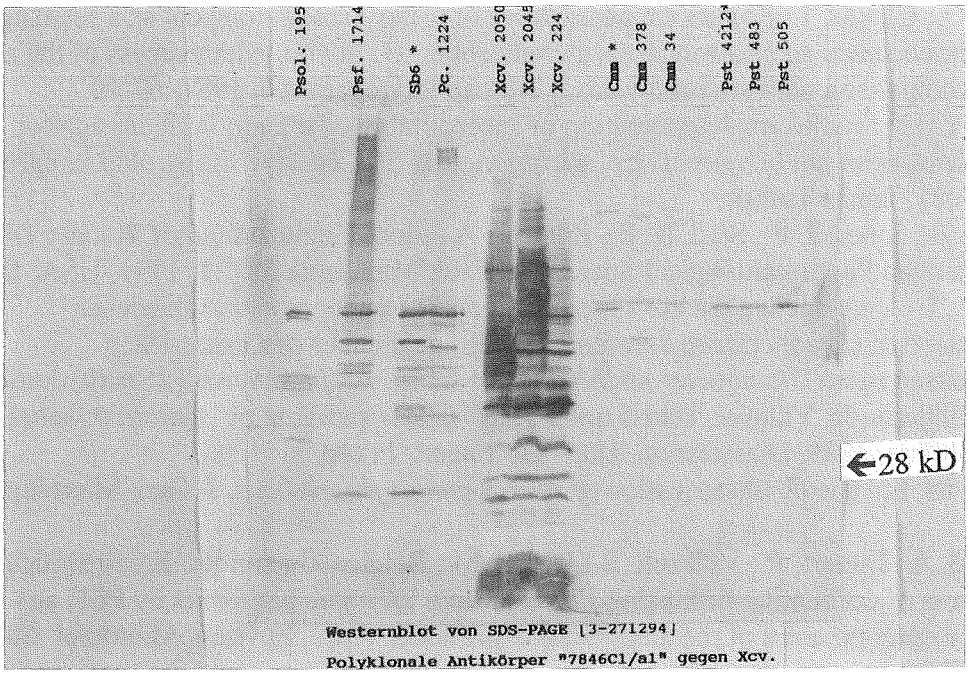

Fig. 2. Western blot developed with polyclonal antiserum against proteins of whole bacterial cells of *X. c.* pv. *vesicatoria.*

DISCUSSION

The objective of our work is to develop diagnostic methods for sensitive detection of bacterial pathogens on tomato seeds. The intention was to use both serological and DNA-techniques. The PCR technique was successfully applied to amplificate a DNA sequence (*cfl*) coding for coronafacate ligase (8). This enzyme catalyses the coupling of coronafacic acid and coronamic acid. Other results revealed that non-coronatine-producers did not result in a positive PCR-signal. Therefore, this technique might be useful for a sensitive detection of *P. syringae* pv. *tomato* in tomato-plants, especially since all virulent *P.s.* pv. *tomato* seem to produce coronatine (13).

The incitant of bacterial spot, *Xanthomonas campestris* pv. *vesicatoria*, could be characterized by a specific protein band of 28 kD. Further experiments have to reveal whether this protein band can be used to prepare a highly specific antiserum.

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RESISTANCE EVALUATION OF APPLE BREEDING MATERIAL AGAINST FIRE BLIGHT (*ERWINIA AMYLOVORA)*

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Introduction

Fire blight, caused by *Erwinia amylovora* (Burrill) Winslow et al., is an important bacterial disease of pome fruit. Choosing resistant fruit varieties must be the first priority in preventing and controlling fire blight (1).
The first programme for apple breeding (to improve scab resistance) in Germany was started in 1928. At present the aim of resistance breeding consists in a multiple resistance against fungal and bacterial diseases and pests. In 1974 a special programme for fire blight resistance was initiated at Dresden-Pillnitz in cooperation with the Institute of Phytopathology, Aschersleben.

Material and Methods

Resistance in apple breeding material was evaluated on seedlings and graftings on "MM 106" - rootstocks at Aschersleben. For the inoculation of succulent shoot tips we used a suspension composed of three virulent isolates (10^9 cells/ml) of *Erwinia amylovora* (Burr.) Winslow *et al.*

Virulence analysis. Every year *Erwinia amylovora*- isolates from orchards and nurseries with fire blight outbreaks were collected and tested on the variety 'Prima', 'Malus robusta No. 5' and a resistant hybrid in the glasshouse. Reference strains from the USA and isolates from France, Switzerland and the Tschech Republic were involved. In January after shoot inoculation with each isolate (10^9 cells/ml) the severity (= length of necrosis / length of shoot x 100%) was calculated and the three virulentest strains selected.

Since 1993 we inoculate shoot tips by cutting through two unfolding leaves with contaminated scissors. Blossoms were inoculated with a painting brush or by spraying. The tests we carried out in March and April under glasshouse and later under field conditions. Shoot length and necrosis were measured 4 weeks p.i. For the evaluation of blossom susceptibility the progress of the disease into branches and stem were registered .

Results and Discussion

Virulence analysis. In 1995 for examble we selected the isolates 197, 222 and 237 as the virulentest on the three varieties (Table 1) and used them for the resistance evaluation. Whereas we had no infections in the resistance evaluation on the hybrid ZS 4352 in 1994 shoot infections with the strain 222 from the Tschech Republic could be observed in the virulence test. It seems nessesary to collect and select virulent *Erwinia* amylovora- isolates every year for the resistance evaluation ecording to the virulence development.

Resistance evaluation. In many cases blossom and shoot susceptibility respectively resistance corresponded (2). In some varieties we found no correlation between blossom and shoot resistance (Table 2). Therefore, it is nessesary to test both. Under the climatical conditions of central Germany blossom infections are very dangerous, seemingly more than shoot infections.

Table 1 Virulence analysis of *Erwinia amylovora*- strains from different hosts in 1995: Shoot infections (% attacted shoot lenghts) on different apple varieties

strain	'Prima'	M. robusta no. 5	ZS 4352	isolation	hostplant
77	56,7	72,1	0	USA, 266	*Malus*
91	13,9	0	0	D, 1985	*Pyrus*
143	17,9	0	0,7	D, 1993	*Cotoneaster*
180	23,2	0	0	CH, 1993	*Cotoneaster*
197	19	**13,3**	9,2	D, 1994	*Malus*
222	32,5	0	**8,8**	CS, 1992	*Cotoneaster*
223	61,3	0	0	CS, 1993	*Pyrus*
237	**73,4**	0	0,8	D, 1994	*Malus*
243	44,8	0	5,2	D, 1994	*Malus*

In the last years we found apple hybrids with a high level of fire blight resistance combined with scab and mildew resistance. Varieties with multiple resistance are: 'Remo', 'Rewena', 'Reanda' and 'Reglindis'. In the apple breeding without resistance genes two varieties were obtained with a low level of susceptibility - 'Pilot' and 'Pinova'.

Table 2. Resistance of apple varieties and hybrids against Fire Blight.
(1) artficial shoot infections in the glasshouse from 1980 to 1988,
(2) artificial shoot and (3) blossom infections in the field 1986 and 1988,
(4) natural infections in 1985 (Blight score 9 - no symptoms)

Variety/ Hybrid	(1) score	assess-ment	(2) shoots infected (%)	(3) blossoms infected (%)	(4) score	assess-ment
Alkmene	5,9	m	1	53	7,0	l
Jonagold	2,7	h	6	37	5,0	m
Red McIntosh	1,3	vh	8	30	5,0	m
Prima	4,2	m	-	-	9,0	ns
James Grieve	4,0	h	77	100	1,0	vh
Breuhahn	5,3	m	29	44	5,0	m
Jonathan	5,1	m	32	57	5,0	m
Gloster	3,4	h	33	31	5,0	m
Pi-A-9	8,6	r	0	1	9,0	ns
Pi-A-173	8,0	r	1	3	8,5	ns
Rewena	**8,5**	**r**	**0**	**2**	**9,0**	**ns**
Reanda	8,5	r	0	2	9,0	ns
Remo	**9,0**	**r**	**0**	**19**	**9,0**	**ns**
Reglindis	6,0	m-l	9	2	9,0	ns
Pinova	**7,0**	**l**	**2**	**21**	**7,0**	**l**
Pilot	**6,0**	**m-l**	**9**	**33**	**8,5**	**l-r**

susceptibility: **l** - low **m** - middle **h** - high **vh** - very high
resistance: **r** - resistant **ns** - no symptoms

'Pilot' and 'Pinova' result from the breeding without resistance genes. They are dessert apple varieties with very high fruit quality and low susceptible to fire blight and scab.
The "Re"-varieties are from the resistance breeding. 'Remo' and 'Rewena' have 5 resistances (Table 3).

Table 3. Resistances of new apple varieties from the Institute of Fruit Breeding Dresden-Pillnitz (until 1994)

variety	scab	mildew	Fire blight	bacterial canker	red spider mite	winterfrost	blossom frost
Remo	x	x	x	o	o	x	x
Reglindis	x	(x)	(x)	o	x	x	n.i.
Retina	x	(x)	o	o	(x)	-	x
Reka	x	(x)	o	x	-	(x)	-
Remura	x	x	o	o	n.i.	x	o
Reanda	x	x	x	o	-	o	x
Renora	x	(x)	o	o	o	(x)	x
Rewena	x	x	x	x	o	o	x
Rene	x	o	x	(x)	-	o	x
Relinda	x	(x)	o	x	-	n.i.	n.i.

x resistant, frost resistance high (x) low resistant o middle susceptible
- high susceptible n.i. no informations

'Remo', a cider apple variety, is a crossing of 'James Grieve' and a scab resistant hybrid of *Malus floribunda*. 'Rewena' is a crossing of the hybrid 'Cox Orange'x 'Oldenburg' and a F_3 - hybrid of *Malus floribunda*. Varieties with multiple resistances should be used to replant orchards in heavy attected regions. They are very interesting for ecological production methods to reduce chemical spraying.

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ON THE ROLE OF BACTERIAL EXOPOLYSACCHARIDES IN PATHOGENESIS OF LEAF SPOT CAUSING PSEUDOMONADS

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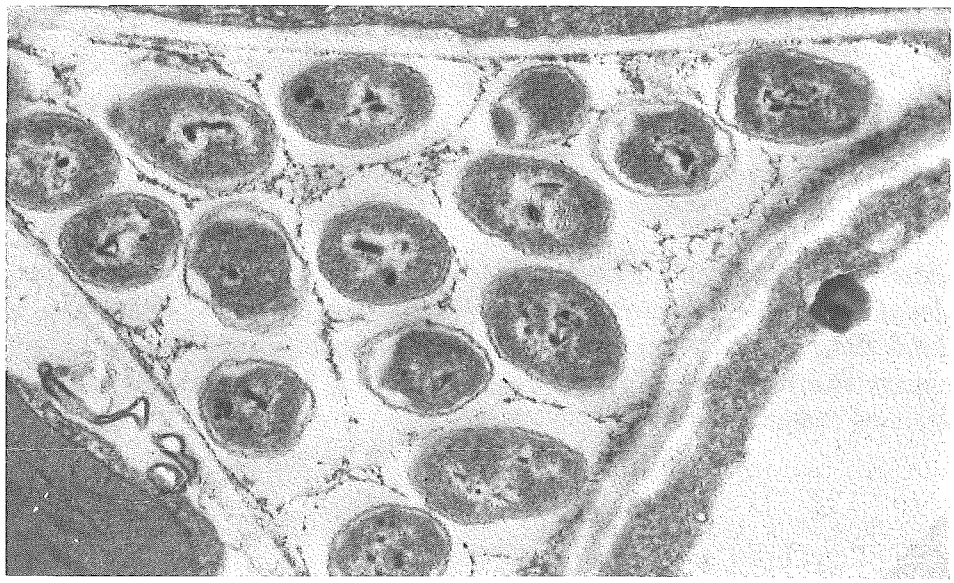
Leaf spot causing pseudomonads colonize the intercellular spaces of the leaf mesophyll. Under these conditions, the main hindrance to bacterial multiplication is the non-availability of water (1).

Several lines of evidence indicate that only the production of large masses of slime or extracellular polysaccharides (EPS) allow the bacteria to fill the intercellular space of the leaf mesophyll (2,3). Thus, electron microscopic studies revealed that the bacterial cells in the intercellular spaces are embedded in masses of slime (Fig. 1)

Fig. 1: Cells of *P.s.* pv. *phaseolicola* in the intercellular space of bean leaf are embedded in a network of fibrillar material visualized by staining with Ruthenium-Red



Similar results were obtained for several pseudomonads as well as xanthomonads (4,5). The main components of the slime matrix are bacterial exopolysaccharides, such as levan, alginate, and lipopolysaccharides (LPS), and possibly aggregates of bacterial and plant polymers.

Levan (ß-2,6-D-fructan) is synthesized by the exoenzyme levansucrase from many phytopathogenic pseudomonads (6) as well as by several non-phytopathogenic bacteria. The features of the enzyme demonstrate a remarkably high resistance against unfavourable environmental conditions, so that the enzyme seems to be well adapted to the adverse milieu in a living host organism. Levansucrase was produced constitutively by all *P. syringae* pvs. tested, but was inducible in *P. fluorescens*. Since sucrose is the predominant soluble sugar in

the intercellular space of leaves, a highly active, constitutively synthesized levansucrase may have the function to create a protective shield around the bacterial cells by synthesizing levan, which retains moisture and thus can protect micro-colonies from sudden desiccation. Simultaneously, glucose is provided as carbon source for bacterial growth. This role of levansucrase may be critical during the early phase of pathogenesis.

Semiquantitative data of Albers (7) and El-Shouny et al. (8) exemplified the wide-spread capacity of *P. syringae* pathovars and of *P. andropogonis* to produce **alginate**. On the other hand, the opportunistic pathogens *P. marginalis* and *P. viridiflava*, which do not induce the water-soaking symptom, produced no or very low amounts of alginate *in vitro*. Similar results were obtained when the *algD* gene encoding for GDP-mannose dehydrogenase was cloned and used as a probe in hybridization experiments (Koopmann and Nöllenburg, unpublished). Obviously, the capability to synthesize alginate is essential for all leaf spot causing pseudomonads.

Low concentrations of alginate are characterized by extraordinary rheological properties. Due to cross-linking by Ca^{2+} ions alginate forms an insoluble gel.

When we compared the EPS-production *in vitro* of several *Pseudomonas* species and *P. syringae* pathovars we found that alginate was the decisive EPS-component responsible for the viscosity of the culture filtrate. Several experiments led to the conclusion that alginate is predominantly responsible for the **water-soaking effect** exerted by bacterial slime in leaves (3). Thus, alginate-rich EPS caused the longest persisting water-soaked spots, whereas all our experi-ments to induce persistent water-soaking in leaves by purified levan or levansucrase failed.

In the *P. syringae* pathovars *phaseolicola, tomato* and *aptata* the alginate content of the EPS correlated with the water-soaking capacity and virulence of the bacterial strains (7) (table 1). This correlation was not so clear for different strains of pathovars *lachrymans* and *pisi*.

Recent studies of Sonnenberg (9) showed that in bean leaves inoculated by *P.s.* pv. *phaseolicola* alginate was produced in higher amounts than levan throughout the infection period and that in susceptible leaves the proportion of alginate to levan was higher than in resistant leaves during the later infection period. We calculated from our studies (3) that the total EPS concentration of the bacterial slime in susceptible bean leaves amounts to 5%. This concentration of alginate plus 1/10 levan results in a highly viscous solution which can by no means infiltrated manually into leaves. Therefore, the most crucial experiment, to infiltrate naturally occurring concentrations of bacterial slime aseptically into leaves in order to induce persistent water-soaking, cannot be carried out.
The induction of persistent water-soaking in leaves by highly viscous alginate solutions or alginate-gels may not only be due to the pure alginate but may also involve **interactions** with macromolecules of the host.

Table 1: Relation between EPS composition *in vitro* and induction of water soaking in three pathovars of *Pseudomonas syringae*

Pseudomonas syringae pathovars	strain	Alginate %	Levan %	Degree of water-soaking
phaseolicola	N7	76	28	7
	FV	7	48	3
	Ro	1	88	1
tomato	2 schr	81	25	7
	2 sl	19	75	5
	487	21	45	3
aptata	1087	31	64	3
	1287	30	51	5
	1092	19	70	5
	62	4	78	2
	61	3	70	3
	1289	0.4	80	1

Interactions with host polymers seem to be even more important for the third EPS-component, the bacterial **lipopolysaccharides** (LPS). From the three components of LPS, the core and the lipid A represent highly conserved structures which also occur in non-pathogenic pseudomonads (10). The O-specific side chains, on the other hand, appear to be specific for certain pathovars of *P. syringae* (11) and may play a role in host-specificity.

Bacterial mutants of *P.s.* pv. *phaseolicola* which were deficient in the O-specific side chain proved to be non-pathogenic. Electron micrographs revealed these bacteria to be very densely packed *in planta* without any space of EPS between them (2). These bacteria were obviously not able to grow into the intercellular space. It is assumed that the rough LPS more easily induces a resistance reaction in the host plant which can be modified by interaction with plant polymers (12). Thus, the intact LPS may play a role in pathogenesis by induction of water-soaking and release of nutrients from the plant cytoplasm due to membrane damage (13).

Recent experiments of Grolms (see her contribution in these Proceedings) revealed specific synergistic interactions between purified LPS-preparations and polymers of susceptible leaves, but not with polymers of resistant or non-host plants.

In **conclusion**, it is assumed that EPS act like a protective shield around the bacterial cells *in planta*, so that water is attracted and micro-colonies in the normally air-filled intercellular spaces do not desiccate and can take up nutrients more easily. In addition, the bacterial slime prevents close contact of bacterial cells with plant cell walls and thus blocks recognition and triggering of the hypersensitive reaction. EPS can also protect bacterial cells from agglutination by plant polymers, or from bacteriostatic compounds (phytoalexins) and stress molecules (oxygen radicals).

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IDENTIFICATION AND UTILIZATION OF SOFT ROT RESISTANCE IN DIHAPLOID POTATOES

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Introduction

Bacterial soft rot of potatoes is one of the most destructive diseases in storage and transit. The contaminated tubers under favourable environmental conditions may be responsible for rotting in storage and disease development in the field (2). The most efficient measure for soft rot control is able to bred resistant cultivars. Potato cultivars with a high level in soft rot resistance do not exist in this time. However, potato cultivars do vary in their relative susceptibility to soft rot.
The tetraploid nature (2n=4x=48) of Solanum tuberosum L. complicates potato breeding. Therefore, the production of dihaploides (2n=2x=24) has more and more importance for the potato research. Also in our institute a collection of dihaploids was produced with following characteristics: good yield, attractive tuber appearence, product quality, resistance to viruses and other pathogens. Dihaploids were produced by pollination of tetraploid cultivars and breeding clones with the pollen of Solanum phureja. The primary dihaploids and interdihaploids were also crossed with the species S. phureja, S.vernei, S.kurtzianum, S.goureayi and S. famatinae (3, 4). A negative correlation was found between the characters total rot and pendulum index (5).
The objectives of this study are to identify resistent soft rot genotypes on the dihaploid level as sources for potato breeding.

Material and Methods

64 dihaploid genotypes were tested under laboratory conditions for three years. These genotypes possess different descents and they belong to middle early and middle late maturity groups. All tubers were stored at 8 °C after harvest and investigated after five month storage period.
The isolates of Erwinia carotovora subsp. atroseptica and subsp. carotovora were used for artifical inoculation (10^8 CFU/ml).The soft rot pathogen Erwinia carotovora could always be proved in our potato tubers by the Enzyme-Linked Immunosorbent Assay (Elisa) (7, 8). The bacteria in the lenticels represent the most dangerous infective potential. Based on this fact, we applied two methods of resistant screening:
1. Tubers were mechanically damaged and following inoculated with Erwinia carotovora. A hole of 7 mm deep and 7 mm wide was made with a sterilized screwdriver.
2. Damage of lenticels with sterile needles according to Karwasra et al. (1). In this case 40 lenticels over the tuber were damaged in the deep of 2 mm and the tubers were incubated in a humidified atmosphere at 26 °C for 72 h.

Additional 42 dihaploid genotypes were screened by the method of inoculating stems. Stems of potted plans at 10 to 15 cm high were inoculated 5 cm above soil level by picking the stem with a needle (10^7 CFU/ml) (6).

Results and Discussion

Figure 1 indicates the high variability of the 64 genotypes in the level of soft rot resistence. All genotypes with an infestation index <1.25 have the highest soft rot resistance. There is 14 % of all tested genotypes. Of all tested genotypes it was selected ten genotypes with highest and ten with lowest degree of resistance. Figure 2 shows the year-to-year influence of these genotypes. The genotypes with the highest resistance level have little differences within the years, but the year-to-year influence was significantly different at the 10 genotypes with the lowest resistance. We classified the reaction of the genotypes to soft rot resistance after mechanical damage in the following manner: comparatively resistant = 14.0 %, medium resistent = 17.2 %, slightly resistant = 39.1 %, not resistant = 29.7 %. Fully immune geno-

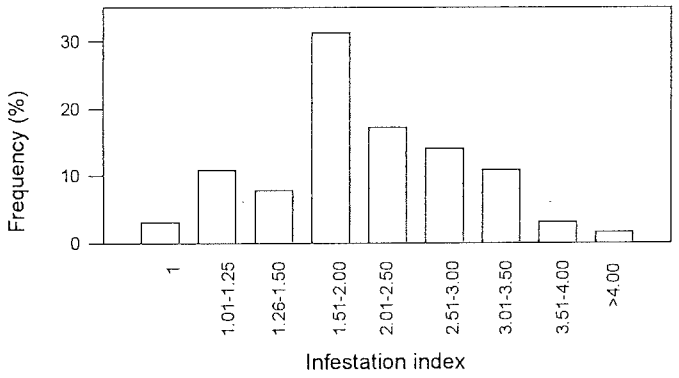


Fig. 1. Soft rot resistance with inoculation method in 64 genotypes of different dihaploid combinations (mean for period 1992-1994)

types were not found. The result of damage of lenticels in 42 of the 64 genotypes is demonstrated in Fig. 3. Our proposal is that all genotypes in which only very small rotten tuber surface occurs up to 1 % named resistant. In this case it was found 23.8 % resistant genotypes, but here there are are genotypes with a high of suceptibility, although no Erwinia suspension was used. The figure 4 demonstrates the number of rotten lenticels in 42 genotypes of different dihaploid combinations. Only three genotypes have not rotten lenticels.The variation of the susceptibility between the genotypes is high. Figure 5 shows the results of blackleg resistance tested inoculation of stems in 40 genotypes of different dihaploid combinations. A lot of genotypes show no reaction to the treatment (outside 62.2 %, internal 45 %). The investigation of infestation indicated that there was only small differences between the outside and internal part of the stems.

The results of our investigations are the basis for studies about the possibility to increase the soft rot resistance via sexual and asexual hybridization and to develop resistant cultivars.

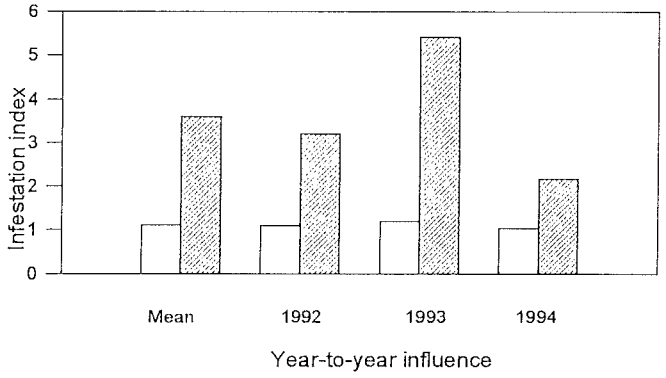


Fig. 2. Soft rot infestation in dihaploids with highest (n=10) and lowest (n=10) resistance

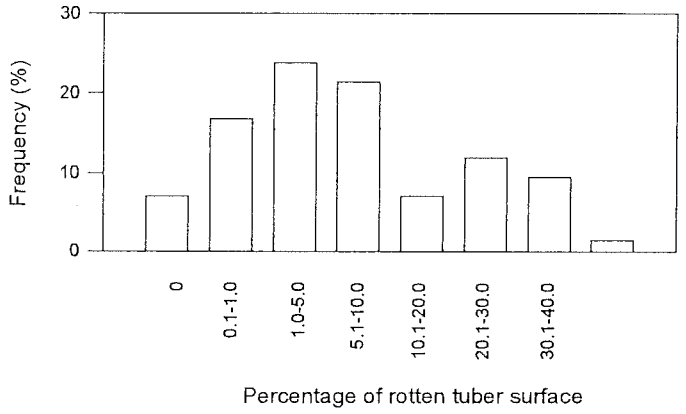


Fig. 3. Soft rot resistance tested with damage of lenticels in 42 genotypes of different dihaploid combinations (1994)

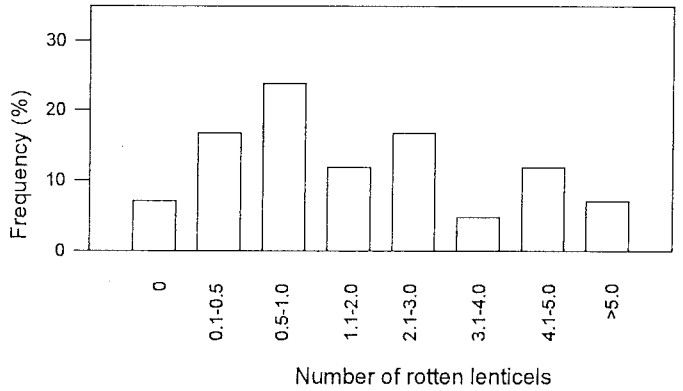
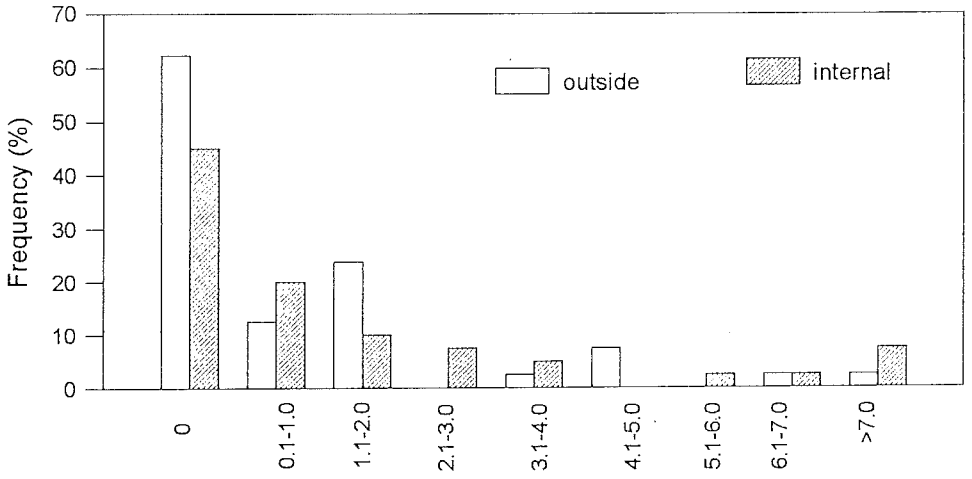


Fig. 4. Soft rot resistance with damage of lenticels in 42 genotypes of different dihaploid combinations (1994)

Fig. 5. Blackleg resistance tested with inoculation of stems in 40 genotypes of different dihaploid combinations (1994)



Infestation of outside and internal part of stems (cm)

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DETECTION AND IDENTIFICATION OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *SEPEDONICUS* WITH SPECIAL ATTENTION TO FLUORESCENT *IN-SITU* HYBRIDIZATION (FISH) USING A 16S rRNA TARGETED OLIGONUCLEOTIDE PROBE

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Introduction

Ring rot of potato, a bacterial disease with a quarantaine status in the EU, is caused by *Clavibacter michiganensis* subsp. *sepedonicus* (*C.m.s.*). This bacterium was first described from Germany by Spieckermann and Kotthoff in 1914 (17). *C.m.s.* proved to be a slow growing, Gram-positive, small, rod-shaped bacterium with a low temperature optimum (c. 21°C) and to cause a vascular disease. Bacterial ring rot is mainly seed transmitted and cutting seed potatoes has been an efficient way of spreading the disease (18). Also non-cutted seed may present a risk because *C.m.s.* may occur in a latent form (without symptoms) and may be spread in this way from infected to non-infected areas.

Classical diagnosis of (visual) infected plants and tubers is the following: a) visual inspection for symptoms (intercostal yellowing, rolling of leaf margins, necrosis and wilting of leaves, yellowish ring of macerated vascular tissue in tubers); b) Gram stain directly on smears from leaf, stem or tuber; c) fluorescence of vascular ring of tubers under UV-light (proved to be non-specific, 11); d) serology, using (latex)agglutination, immunofluorecence (IF) or ELISA with poly- or monoclonal antisera, isolation on (selective) media or through a host plant (2,7,8,16). Identification of the organism took place by Gram stain of a pure culture, biochemical tests (*C.m.s.* being very inert in many tests, 2,3), serology and a pathogenicity test, followed by reisolation of the pathogen (2,16).

These tests, however, do not allow detection of latent infections by *C.m.s.* For this purpose an official EU-method has been developed by the EU working group 'Bacterial disease of plants' (2). This method uses IF as screening method. Since serolgy was shown not to be completely reliable due to disturbing cross-reactions of mono- and polyclonal antisera with saprophytic bacteria (6), the method had also to comprise a laborious and time-consuming host test on eggplant. This also in order to obtain the pathogen for identification and fulfilment of Koch's postulates. For identification the method uses biochemical tests. More recently biochemical tests can be replaced by the more rapid fatty acid analysis or whole cell protein electrophoresis, both methods using computerized databases.

Developments in molecular biology offer possibilities for a more reliable and more rapid detection and identification of *C.m.s.* In an EU-funded project where 10 laboratories cooperate, DNA dot-blot hybridization using a plasmid-based probe, the polymerase chain reaction (PCR) and *in-situ* hybridization using a 16S rRNA targeted oligonucleotide probe have been developed and (partly) evaluated over the last four years. For identification restriction fragment length polymorphism (RFLP) analysis has been investigated. It was found that DNA dot-blot hybridization for detection is more reliable than IF, but less sensitive. PCR is promising, but plant inhibiting substances offer problems and PCR products have to be hybridized with a probe or analyzed with restriction enzymes to be more sure about identity of the PCR product. Koch's postulates are difficult to fulfil with PCR. RFLP enables identification up to strain level, but a certain identification at subspecies level needs the creation of RFLP libraries (9,14,15).

A new strategy for detection and identification is the application of oligonucleotide probes complementary to rRNA sequences. Its sequence information is readily accessible and it is present in high copy numbers (10^4-10^6 per cell) and there are highly specific sequences. *In-situ* hybridization has already been successfully applied to study complex bacteriological communities using both 16S rRNA and 23S rRNA sequences (1,4,5,10,12,19).

In this paper we report about a 16S rRNA targeted oligoprobe that showed dot blot-specificity for *C.m.s.* in a previous study (13). It is shown that this probe can be used to visualize *C.m.s.* cells in smears of pure cultures and of potato heel end macerates on a microscope slide using a fluorescein-labeled probe and epifluorescence microscopy. A preliminary protocol for this fluorescent *in-situ* hybridization (FISH) is presented as well as results on the influence of some parameters in the FISH test.

Material and methods

Bacterial strains

The following microorganisms and extracts were used in this study: *Clavibacter michiganensis* subsp. *sepedonicus* (PD (PD= culture collection, Plant Protection Service, Wageningen, the Netherlands) 37, 221, 323, 406, 1975); *C. m.* subsp. *insidiosus* (PD151, 1837, 1839, 1840, 1841); *C. m.* subsp. *nebraskensis* PD522); *C.m.* subsp. *michiganensis* (PD223, 1386, 1692); *C. iranicus* (PD164); *C .rathayi* (PD227); *C. tritici* (PD222); *Erwinia chrysanthemi* (PD551, 483); *E. carotovora* subsp. *atroseptica* (PD230); *E.c.* subsp. *carotovora* (PD578); *Pseudomonas solanacearum* (PD441); *P. fluorescens* (PD1702); *Rhodococcus fascians* (PD521); saprophytic bacteria from potato (PD1685, 1687, 1691); serologically cross-reacting lactic acid bacteria (PD71, 1192); Naturally infected potato macerate (from an EU-ring experiment, tubers received through J. van Vaerenbergh, Merelbeke, Belgium, who inoculated plants); *C.m.s.* negative potato macerate; potato macerate artificially contaminated with 10^7 cells.ml^{-1} of *C.m.s.* PD323. Macerates were obtained by following the EU method (1). For a full identification of strains and origin, see reference 15.

Oligonucleotide probes used

a) Eub 338, a probe complementary to to the 16S rRNA of all eubacteria (1) 5'-GCTGCCTCCCGTAG-GAGT-3', *Escherichia coli* positions 338-355.
b) 16S CMS1, complementary to 16S rRNA of *C.m.s.* strain PD323 (13) 5'-TTGCGGGGCGCA-CATCTCTGCACG-3', *E.coli* positions 1011-1034 in the variable V6 region.

Protocol used for in-situ hybridization of C.m.s.

The protocol for FISH of *C.m.s.* developed in our laboratory, closely following the one published by Zarda & al., 1991 (19) is as follows. Pure cultures were grown in liquid or on solid yeast-peptone-glucose (YPG) medium (containing in g.l^{-1}: Difco Bacto yeast extract, 5, Difco Bacto peptone, 5, D(+)glucose, 10, agar if solid, 15 at pH 7.0) to the mid-logphase, harvested and suspended in sterile PBS (0.13M NaCl, 7mM Na_2PO_4, 13mM NaH_2PO_4, pH 7.2). Potato macerate suspended in PBS or diluted 1:10 in PBS was used directly.

To 50 μl (or more) of suspended cells or macerate an equal volume of ethanol 96% was added and aliquots fixed for 30 min. at RT (Note: bacterial cells and macerates can be stored in PBS/ethanol for at least two months without apparant influence on the hybridization results). 10 μl of the fixed cell suspension or macerate was spotted on a 10 well multitest slide (Flow Laboratories, UK) and air dried. Thereafter heat fixation by gently moving the slide three times through the top of a Bunzen burner flame. Subsequently cells were dehydrated by placing the slides in 50, 80 and 100% ethanol (2 min. for each concentration). After dehydration cells were air dried.

It was found that for better penetration of the probe through the Gram-positive cell wall of *C.m.s.* and other Gram-positive bacteria a mild lysozyme treatment was necessary. Each well containing fixed Gram-positive bacteria was incubated with 10 μl freshly prepared lysozyme solution (lysozyme Sigma L-6876, 5 mg.ml^{-1}; buffer 100 mM Tris/HCl, 50 mM EDTA, pH 8.0) for 30 min. at RT. Alternatively each well could also be incubated with 40-400 μg.ml^{-1} proteinase K in buffer (containing 20 mM Tris-HCl, 2 mM $CaCl_2$, pH 7.4) for 30 min. at 37°C. After incubation cells were washed for 10 min. in lysozyme or proteinase K buffer and thereafter slides were rinsed with ultrapure water. Another dehydration in the above mentioned ethanol range was performed.

Hybridization was performed by applying 9 μl hybridization solution (hybridization solution contains 0.9M NaCl, 0.01% SDS, 10 mM Tris/HCl, 5mM EDTA at pH 7.2) containing 50 ng labeled oligonucleotide probe (5' fluorescein labeled by ISOGEN, Leiden, the Netherlands) and checked for labeling efficiency by photospectrometric scanning). Hybridization was performed in an air tight chamber containing a piece of filterpaper saturated with hybridization wash solution for 1 h at 46°C in a hybridization oven. After hybridization slides were washed in pre-warmed wash solution (600 ml containing 200 ml 3x hybmix, 6 ml 1% SDS and 394 ml sterile ultrapure water) for 30 min. at 48°C (refreshment of washing solution after 15 min). After washing slides were rinsed briefly with ultrapure water and quickly air dried under the stream of a hair blower.

Slides were mounted in Citifluor AF1 (R1320, Citifluor Ltd, London, UK), to avoid fading of fluorescence. After a coverglass and a drop of immersion oil was added, slides were examined with a Zeiss Axioplan microscope fitted for epifluorescence microscopy, with FITC filtersets 09 and 15 and a 50W mercury high pressure lamp. For photography Kodak Ektachrome 400 ASA was used.

Results and Discussion

We tested several parameters of FISH in order to obtain a maximum penetration of the probe and a maximum fluorescence. Initially fixation in 4% paraformaldhyde on gelatin coated slides, as described by Hahn & al. in 1993 (12) was used. Later it was found that simple fixation in ethanol and use of non-coated slides gave similar results. There are indications that even only the flame fixation will be enough for good results.

The first fluorescein-labeled batch of the *C.m.s.* specific probe received. proved to be only 16.6% upon scanning with a spectrophotometer. At that time we only got positive results using the eubacterial probe. The second labeling was about 58%, giving much better results (Fig.1). Even though the fluorescing of bacteria, when one label per oligonucleotide is present, is still not really strong. This makes until now the use of a very good fluorescent microscope with as few optics as possible and bandpass filter sets necessery as well as an antifading reagent. It will be tested if more than one fluorescent label per sequence will give improvement, but it may be suspected that binding of the probe will be impaired. Preliminary experiments using the DIG-labeling system gave unsatisfactory results.

To obtain optimal penetration of the probe through the Gram-positive cell wall of *C.m.s.* mild lysis was found to be necessary. Lysozyme was tested in concentrations of 0, 2, 4, 6, 8 and 10 $mg.ml^{-1}$. Above 4 $mg.ml^{-1}$ there was no increase in signal. For lysozyme Sigma L7651 with special activity against bacterial cell walls 500 $\mu g.ml^{-1}$ was found to be sufficient, at higher concentrations bad results were obtained. When no lysozyme was applied, results were very poor. A good signal was also obtained by using 40-400 $\mu g.ml^{-1}$ proteinase K.

Hybridization temperature of 46°C gave most specific and strongest signal, 48 and 50°C gave no better results. Specificity could possibly be increased by making the (second) washing step more stringent (temperature used was 48°C, SDS concentration 0.01%), because when bacteria mentioned under *'bacterial strains'* were tested a positive reaction was still obtained with strains of *C.m.* subsp. *insidiosus*, *C.m.* subsp. *nebraskensis* and *C.m.* subsp. *tesselarius*. All the other bacteria were tested negative. In dot-blot hybridizations using this *C.m.s.* probe it was found that by changing the washing conditions the probe could be made specific for *C.m.s.* alone or for the subspecies of *C. michiganensis* (13). The method was also applicable to potato extracts. We obtained positive results even with naturally infected materal from an EU-ring test that had been stored for more than one year at -20°C. Background (auto)fluorescence of tissue was not high and the use of crystalviolet (1 $mg.ml^{-1}$) to suppress the background even had a negative influence on the signal. If FISH appears to be robust and reliable in future studies it could be used in a verification/identification procedure after initial screening with immuno-fluorescence. In this case also the eggplant test could be omitted in routine screening. For critical cases and the complete fulfilment of Koch's postulates the classical methods will still be necessary.

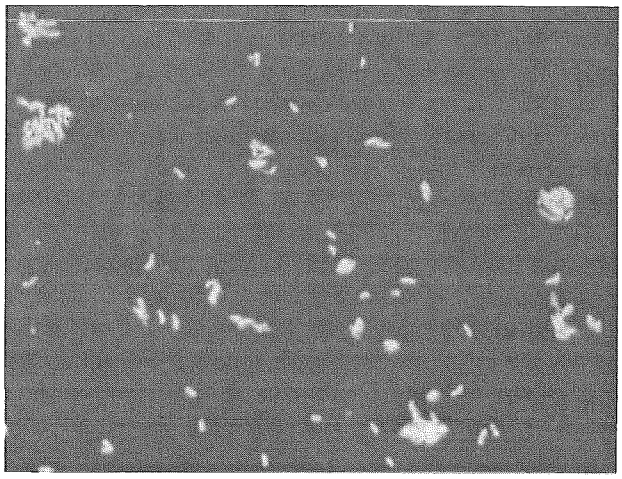

Fig.1 Light microscopic photograph of *Clavibacter michiganensis* subsp. *sepedonicus* cells treated with fluorescein-labeled 16S rRNA targeted oligo probe CMS1. Pure culture of strain PD323.

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STUDY ON THE BACTERIAL DISEASES OF FABA BEAN (*VICIA FABA* L.) AND SOME ATTEMPTS FOR CONTROL

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Introduction

Government observations in our country elite seeds of Faba bean found out 14 and 21% bacterial infestation, respectively, in the period 1985-1986. At present, only bacterial disease of *Vicia faba* with symptomology like a „potato blackleg" have been noticed in Bulgaria (2). No special investigation on the causal agent were done. The authors proved that most of the known bacterial diseases of *V. faba* were seed-borne. In relation to causal agents there were contradictory positions (1, 5, 10).
The aim of this study was to determine: 1) Bacterial species involved in pathogenesis of *Vicia faba*; 2) Symptoms on the different organs of *V. faba* and some characters into life cycle of the pathogens; 3) Some attempts at control against the most widespread bacterial pathogen of Faba bean elite seeds in our country.

Materials and Methods

Seed and plant samples were collected from the 1985 through 1994 yields and from different parts of naturally infected plants in the vegetative period. Plants were grown in the laboratory, glasshouse and in the field following the IPGR technologies. Tests were done (7) for crude estimation of some agronomic characters (tabl. 2). Pure cultures were isolated (8, 11). Their pathogenicity were tested on the tobacco leaf (6) and in the petiols of *V. faba* (12). Bacterial virulence were studied by infectivity titration (3). Pathogenic isolates were identified following the methods of Fahy, Persley (4) and Schaad (9). Symptoms of bacterial diseases of Faba bean, as well as characters into life cycle of the pathogens were studied on naturally and artificially infected seeds and plants. The final decision for bacterial involvement in the pathogenesis was done only for this event when Koch postulates were fulfilled (11).
Trials for eradication of seed-borne bacterial pathogen were carried out by:
1) dry heating 50 °C; 2) water heating 60 °C; 3) chemicals and antibiotics (Tiram; Vitavax 201, Vitavax 300 and Vitavax 200 FF; Kasumin, Streptomycin).
Trials for field control of seed-borne bacterial pathogen were carried out for:
1) preventive effect: plant spraying before symptoms appearance; 2) curative effect: plant spraying after symptoms appearance. We used wide spectre of bactericides, (copper compounds, Kasumin, Streptomycin) insecticides (Agria 1050, Omit 77EC) and fungicides (copper compounds, Rovral FF, Folicur +, Chlortosip) in four sprayings at seedling stage, initial forming of the buttons, flowering and initial forming of the pods. Pest control were applied only if insects presented. Controls were no treated at all or usually dressing of the seeds before sowing were done .

Results

Five hundred pure culture from seed samples were isolated. The main part (87%) of the pathogenic isolates were white fluorescent pigmented bacteria (tabl. 1). Twelve

selected isolates from the most widespread bacterial pathogen with higher virulence were used for the final identification.

Table 1. Tests for identification of the isolated fluorescent pigmented pathogenic bacteria of *Pseudomonas*

Test/species	*P. syringae*	*P. marginalis*	*Vicia faba* isolates
Oxidase	-	+	+
Arginine dihydrolase	-	+	+
Levan	+	+	+
β- glucosidase	-	+	+
Nitrate to N_2	-	+	+
Growth at 41 °C	-	-	-
PHB accumulation	-	-	-
Potato rot	-	+	+
HR on tobacco leaf	+	-	-
Pathogenicity on *Vicia faba*	+	+	+
Used for growth:			
β - аланин	-	+	+
Trehalose	-	+	+
Sucrose	+	+	+
D - tartrate	-	+	+
M - tartrate	-	+	+
Sorbitol	+	+	+

+ up to 90% from the isolates were positive; - up to 90% from the isolates were negative; PHB - poly-β-hydroxybutirate

Their growth on the King B (KB) medium were abundant and became pink-orange. Some of them produced brown diffusible pigment on KB medium, besides the fluorescent one. All of them were with a tuft of polar flagella; not accumulated poly-β-hydroxybutirate (PHB). Levan, oxidase and potato rot were positive, arginine dihydrolase and tobacco hypersensitivity were negative. The bacteria studied utilized for their growth β - alanine, trehalose, sucrose and sorbitol. They were nitrate reductase positive and β- glucosidase producing . No growth at 41 °C were observed. The first symptoms we observed on the roots and seedlings. Diseased roots were observed when no visible symptoms on the seedlings were appeared. Symptoms were more frequently reproduced in the paper blotter test (7) than in glasshouse and field experiments. In the case of glasshouse and field conditions, symptoms were escaped of our notice because of their position on the root and the base of the stem. The similar symptoms were reproduced after artificial inoculation with the isolates. Later on, at the stage of blossom, symptoms on the leaves, petiols and florets could be confused with some lesions caused by some fungi from *Alternaria, Botritis, Fusarium* species, etc. When factors for bacterial but not for plant

growth were favourable all the organs of green plant (roots, seedlings, stems, leaves, florets and pods) were covered with numerous small brownish spots or chlorotic stripes coalesced in big black or reddish necrotic lesions. Sometimes, they caused cracking of the petiols and stems. Leaf spots were usually a marginal necrosis. Necrotic areas often withered and leaves died. Pods and seeds were spotted by subconcaved, small, numerous or single wide lesions spread all over the grain. Dense black lesions covered the roots and seedlings grown from these seeds. Similar symptoms were observed from the apparently healthy seeds too. Usually the heavy infested bacterial seeds have died.
Glasshouse method showed more reliable estimation closer to the field experiments (tabl. 2) than blotter test (not shown). Controlling the disease by disinfecting of seeds for eradication of the pathogen as well as disinfecting by chemicals and antibiotics were not promising. Results not presented here. When conditions for plant growth were better the disease were reduced, without visible symptoms, but still seed-borne at lower percentages.

Table 2. Healthy and diseased plants (%) from lines and cultivars at glasshouse and field conditions

Name	Glasshouse		Field	
	healthy	diseased	healthy	diseased
Maris Beaver	80	20	80	20
Bourdon	75	25	70	30
Banner	85	15	90	10
Bulldog	80	20	80	20
Daffa	70	30	70	30
Maris Beagle	85	15	90	10
Webo	95	5	90	10
Polar	85	15	90	10
Throws M.S.	95	5	95	5

Discussion

On the basis of morphological, biochemical and pathogenic properties from twelve selected isolates, the most widespread bacterial pathogen in our country elite seeds of Faba bean was identified as *Pseudomonas marginalis* pv. *marginalis* (Brown) Stevens. In addition, several isolates belonged to *Bacillus, Erwinia* and *Xanthomonas* species. The results were similar of German authors Griesbach et al. (5). More recently (1991-1994), we have isolated *Pseudomonas syringae*. Symptoms on the different organs of *V. faba* and some characters into life cycle of the pathogens suggested that we had an opportunistic pathogen damaging of crop in the years with bad condition for plant growth and bad agronomical measures applied, as suggested previously for Pseudomonas fabae (10), the bacterial name omitted at present. Further investigation on the bacteria involved in the pathogenesis could be carried out, because of severe damages on the broad bean crops in recent years.

Blotter test gave us possibility to express more reliable the bacterial attack, but glasshouse method showed more precisely estimation closer to the field experiments.
Unfortunately, our attempts at controlling the disease by disinfecting of seeds for eradication of the pathogen, as well as disinfecting by chemicals and antibiotics alone or combined with vegetative spraying were not promising. When conditions for plant growth were better than for pathogen development, rating of the disease and percentages of the seed-borne infection were reduced. Hence, every agronomical measures applied in time supported the better plant growth could restrict bacterial growth and give a good yield of *Vicia faba* grain with lower seed-borne infection.

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Erwinia carotovora - the role of its pectate lyases in soft rot pathogenesis

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Introduction

The phytopathogenic bacterium *Erwinia carotovora (Ec)* causes soft rot on a wide spectrum of host plants. Tuber soft rot and blackleg of potatoes are of greatest agricultural importance in temperate regions (9). The *Erwinia* pathogenicity is based on the maceration of plant tissue by extracellular cell wall degrading enzymes like pectate lyase (PL) polygalacturonase (PG), pectin methylesterase (PME), endo-1,4-ß-glucanase (CEL) and protease (PRT). The PL (EC 4.2.2.2) including several isoforms is the principal enzyme secreted by Ec bacteria and is to be seen as a virulence determining factor (1,3). PL depolymerizes pectic polymers of plant cell walls. The enzyme cleaves the α-1,4-galacturonide linkages in pectic polysaccharides by ß-elimination releasing series of unsaturated oligogalacturonids (10). The optimal substrate for PL enzyme action is assembled in cell walls of potato tubers, one of the main Ec host plant. Thus 55 % of the total cell wall material consists of pectic substances and the degree of pectin esterification ranges from 41 % to 45 % (12). We found recently that PL isoenzymes acted not only on unmethylated pectate but also on 31 % and even on 68 % esterified pectin (2). Production of enzymes by Ec bacteria spreading in the intercellular space of host tissue and the immediate action of enzymes secreted on plant cell walls are close connected in pathogenesis because products of enzyme action are not only carbon sources for bacterial propagation but also inducers for enzyme biosynthesis (4,5) and even elicitors for plant defence reactions (8, 13, 16). We focused the investigations on the enzyme production of natural occuring Ec bacteria as well as on the action of enzymes secreted and the plant tissue responces.

Material and Methods

Bacteria and culture conditions: The wild-type Ec bacteria were isolated from soft rotted tissue of different plant species by means of Mac Concey-Agar. For detection of enzyme activities cells were grown on Luria Bertani (LB)-agar plates containing 0,4 % of the enzyme substrate at pH 6,8-7,0 and 25 °C for 20 h. Enzyme producing bacteria were subcultivated on the same medium. Shaken cultures were performed in 25 ml LB-broth containing 0,2 % citrus pectin in 100 ml Erlenmeyer flasks at 25 °C for 20 h at 150 cpm. For PL induction cells were grown in LB-medium supplemented with 0,2 % pectin and in 25 g of sliced plant tissue. Eca C18 cells were grown in soya/ molasses-medium (15) in a laboratory fermentor. Genes encoding PL isoenzymes were derived from the strain Eca C18 using standard methods (10). For overexpression of PL isoenzymes pT7-7 derived plasmids were transformed into *E. coli* BL21 (DE3) pLysS cells (Novagen). Bacteria were grown in M9 medium with the appropriate antibiotic (11) in a laboratory fermentor.
Enzyme assays: Pectinases- citrus pectin was used as substrate and 0,05 % ruthenium red for staining the plates; *Cellulases-* carboxymethylcellulose as substrate and 0,1 % congo red for staining; *Proteases-* casein as substrate and 0,2 M citric acid for flooding the plates to visualize enzyme activity. Activity of enzymes secreted in liquid media was analysed in the culture filtrate. PL activity was determined spectrophoto-metrically at 235 nm by monitoring the formation of unsaturated products from 0,1 % polygalacturonic acid in 0,1 M Tris buffer at pH 8,0 supplemented with 0,1 mM $CaCl_2$. One PL unit is the enzyme activity liberating 1µM of unsaturated oligogalacturonides at 25 °C per 1 min.
Enzyme preparation: Eca C18 extract- enzyme proteins of the culture concentrate were precipitated with ethanol and lyophilized. PL isoenzymes were chromatographed on an S-Sepharose (Pharmacia) cation exchange column and separated by gelfiltration chromatography (Superdex D-200).
Maceration tests: Enzyms were infiltrated into potato tuber disks (diam. 10 mm, 2 mm thick) using a vacuum of 200 mbar. After incubation the disks were vital stained with neutral red. The absorbed dye was extracted and OD_{535} was measured. OD_{535} obtained from samples incubated with the inactive enzyme corresponds to 100 % vital staining (14).
Microscopy: Enzymes were infiltrated into potato tuber disks. For scanning electron microscopy (Rem-Cam Scan 44) separated cells were either dried by air or using a Critical Point Dryer.

PL3 preincubation: Enzymes were infiltrated into potato tuber disks. Following 2 h of preincubation tuber disks were inoculated with serial dilutions of Ec bacteria. Maceration of tuber tissue was measured after 20 h of bacterial propagation at 25 °C. Controls were pretreated with the inactive PL 3 enzyme.

Results and Discussion

Enzyme production of Ec bacteria: 20 000 Ec isolates derived from soft rotted tissue of different plant species were screened for their enzyme production. 30 % of them secreted enzymes after growing on LB-agar plates containing the specific enzyme substrate. But only 1000 isolates remained stable in their enzyme production over ten subcultivations. Bacteria of the enzyme active phenotype produced the complete set of cell wall degrading enzymes. This indicates an adaptation of the Ec enzyme spectrum to the plant cell wall with the different polysaccharide component. The observation that pectinolytic, CEL and PRT activities of individual isolates disappeared simultaniously may be an additional sign for a concerted action of enzymes in cell wall degradation and for a joined mechanism of gene regulation. PL was the main enzyme secreted. In pectin containing media 61 % of the isolates revealed PL activities which were lower than 1 Uml^{-1}, activities of 38 % ranged from 1 to 2 Uml^{-1} and only 0,8 % produced activities which were higher than 4 Uml^{-1} culture filtrate. Three strains were used for the investigation of PL induction. The cells were grown in different media, enzyme production was recorded and the effect of enzymes secreted on potato cell walls was characterized.

Induction of PL: PL enzyme production was increased by adding pectin (Tab.1). The inducing effect was exceeded morefold by using plant tissue for growing the bacteria. In contrast to pectin wide differences of the *if*-values occured between the strains. On the other hand the PL *if*-value of an individual strain was affected by different plant tissue (Tab.1). The extreme increased PL production in plant tissue suggest the conclusion that biological active substances of the plant material may support additionally the inducing effect of pectic substances. These could be valuable proteins, essential amino acids, vitamins or minerals. How these processes are regulated is of interest. The *aep* A gene encoding an activator of extracellular Ec enzyme production seems to be a key factor (7).

Tissue maceration: The macerating potential of enzymes depends on the Eca strain and is affected by the Ec growth substrate (Tab. 1). Interestingly, the enzyme mixtures of strain Ec 43/15 revealed the lowest macerating potential despite of the high rate of PL production. The increased cell wall degradation caused by enzymes from the other strains was based on a higher concentration of CEL in the enzyme mixtures. The best substrate for the production of most efficient enzyme mixtures were paprica tissue slices. It can be speculated that the rate of *Erwinia* enzyme production and the spectrum of enzymes secreted is adapted to the host tissue attacked by the bacteria. The regulation of theses processes is unknown up to now. On the other hand the degree of tissue maceration by Ec enzyme mixtures and individual PL isoenzymes was dependent on the tissue species. This was confirmed for tuber tissue from six different potato varieties (v.). Non of the PL isoenzymes was as active as the enzyme mixture from Eca C18, the gene donor strain. The same was found for different combinations of PL isoenzymes using tuber tissue from v. Adretta and Desiree and potato leaf tissue from v. Adretta (data not shown). It seems that the production of the PL isoenzymes is an answer of the bacteria to the different qualities of pectins in the Ec host plants. Scanning electron microscopy confirmed the results of the macerating tests. Enzyme action impaired intercellular cohesion after 5 min of incubation, after 30 min complete membrane destruction occured using Eca enzyme mixtures, PL1 and PL2 isoenzymes. Action of PL3 was limited on degradation of middle lamellae. Cell suspensions were produced (Fig. 1). The membranes surrounding the protoplasts remained intact (Fig. 2).

PL 3 preincubation and Eca tissue maceration: Tuber tissue maceration by Ec bacteria was reduced after preincubating potato tuber disks from v. Nicola with PL 3 isoenzyme activities of ≥ 25 Uml^{-1} causing tissue maceration of 12 % after 30 min of incubation. The reducing effect on tissue maceration by Ec bacteria was joined to the cell wall degradation by PL enzyme action. Thus PL activities ≤ 12 Uml^{-1} were of no effect. Obviously the reduced tissue maceration by Ec bacteria is based on a reduced bacterial propagation due to a plant defence reaction induced by the PL reaction products (13). A similar indication for plant defence reactions induced by PL and PG action has been given with the induction of ß-1,3-glucanase expression, a pathogenesis related protein,in tabacco seedlings (8).

Table 1. PL production and enzymic maceration

	PL activity [a] (Uml^{-1}) *if*-PL value[b] Eca strain			Maceration [c] (%) Eca strain		
Growth substrate	**32/9**	**43/15**	**1/6a**	**32/9**	**43/15**	**1/6a**
	PL activity					
LB	0,6	1,5	1,0			
	***if*-PL value**					
LB+0,2% pectin	2,83	2,73	2,80			
Cucumber	38,00	21,86	3,30	25	15	24
Paprica	16,83	33,93	2,40	34	14	39
Potato tuber	26,33	46,00	16,40	28	12	23
Tomato	1,00	0,20	0,10	3	2	0

[a] PL activity was determined in the Eca culture supernatant.

[b] PL if-value was calculated using formula if = $\frac{\text{PLactivity in plant tissue (U/ml)}}{\text{PL activity in LB-medium(U/ml)}}$

[c] Enzyme solution containing 0,5 Uml^{-1} PL was infiltrated into tuber disks (v. Nicola) using 200 mbar vacuum. Maceration was measured after incubation for 30 min at 25 °C

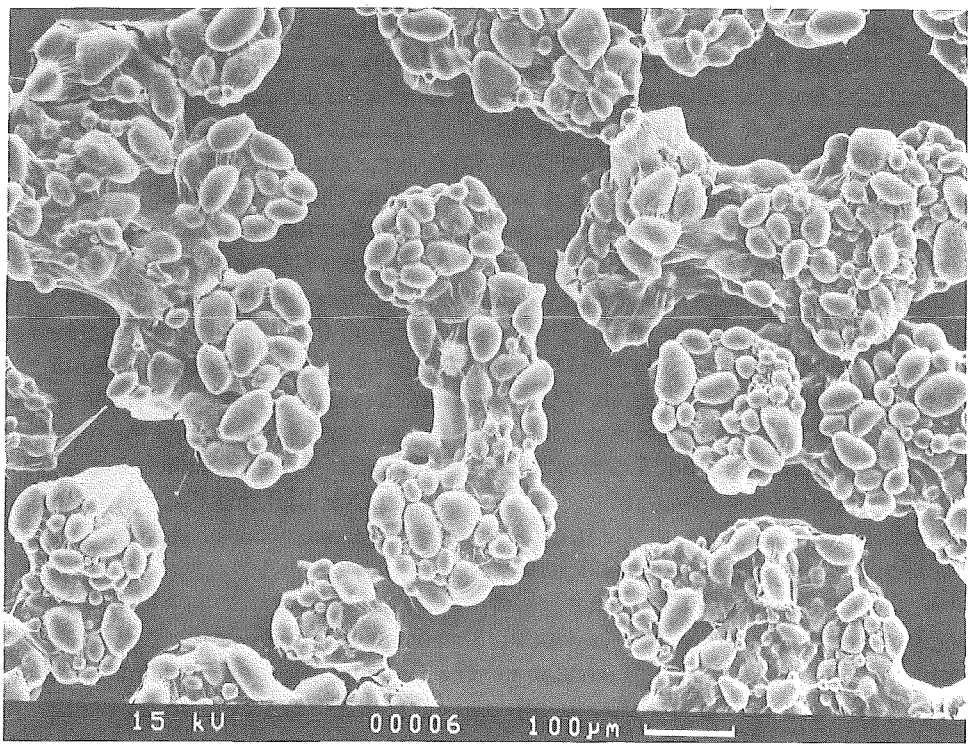

Fig. 1: Scanning electron micrograph of potato tuber tissue after incubation with PL3.

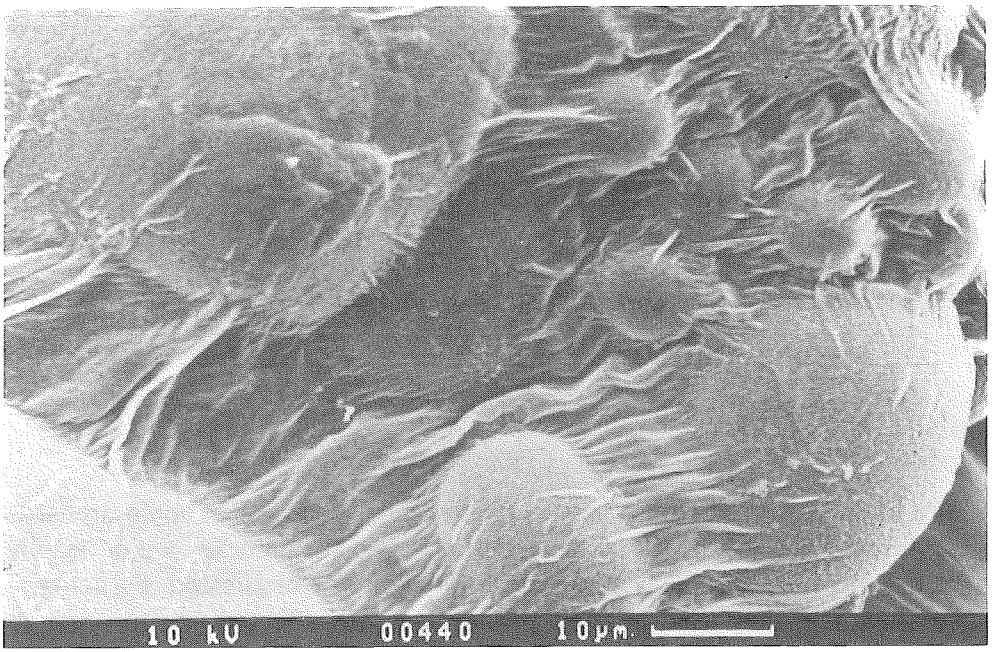

Fig. 2: Intact primary wall of a cell liberated by PL3 action from potato tuber tissue.

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STUDIES ON THE RESISTANCE OF APPLE AND PEAR ROOTSTOCKS AGAINST FIRE-BLIGHT IN GERMANY

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Introduction

During 1993, fire blight occurred with a very high incidence especially in apple orchards of South-West Germany. Beside blossom infections also rootstocks were heavily attacked for the first time, so that many apple orchards had to be eradicated. Particularly rootstock 'M9', which is mainly used in apple production, was affected (1). In order to get a survey on the resistance of the most important rootstocks of apple and pear under German conditions, an extensive assortment of rootstocks was tested against *Erwinia amylovora.*

Material and Methods

Eleven apple and three pear rootstocks were planted for screening on fire blight resistance in winter 1992 on a testplot of the Biologische Bundesanstalt in Kirschgartshausen. Studies were made in the following growing seasons 1993/1994, when shoots showed vigorous growth of 20-50 cm length (1).
Inoculation was made with a bacterial suspension of ca. 10^8 CFU / ml of a highly virulent isolate (Ea 7/74) of *Erwinia amylovora.* With a syringe 0.1 ml inoculum was injected into the shoot just above the youngest unfolded leaf; 3-5 shoots per plant were treated and 10 trees tested per cultivar. Disease assessment was calculated according the mean percentage of the current season's growth, which was necrotic.

Results

In the apple rootstocks the vigorous clones 'A2' and 'M11' showed a very high susceptibility in both years of testing, whereas in 'M25' the incidence of fire blight was considerable lower (Table 1,3). In the group of semi-vigorous clones, 'M4' and 'MM111' were grouped under the resistant types, whereas 'M2' showed a low susceptibility and 'MM106' was classified as very susceptible. Clone 'M7' was very different in its reaction from year to year. All dwarfing rootstocks ('M27', 'M9', 'B9') were rated as susceptible. Generally the infection rate was marked increased in 1994 in comparison to the year before (1).

The tested pear rootstocks showed a nearly similar reaction in both growing seasons (Table 2,4). A very high resistance was rated in clone 'OHF333', a crossing between the american pear varieties 'Old Home' and 'Farmingdale'. As in comparison to other experiments 'Quince C' and 'Quince BA29' were classified as susceptible (2, 3).

Table 1: Fire blight resistance of apple rootstocks, 1993

Apple rootstocks	No. Shoots inoculated	Shoots infected in %	Mean Length infected in %
high susceptible			
A2	35	100	63,1
M11	32	100	46,0
MM106	30	96,6	42,7
susceptible			
B9	31	90,0	17,0
M27	30	70,0	12,9
M9	30	76,6	12,7
M25	30	33,3	7,2
M2	30	56,6	6,9
resistant			
M4	30	13,3	2,0
M7	30	3	0,1
MM111	30	0	0

0-5% mean length infected: resistant, 6-30%: susceptible, >30%: highly susceptible.
Inoculation: 28 May 1993, Rating: 25 June 1993.

Table 2: Fire blight resistance of pear rootstocks, 1993

Pear rootstocks	No. Shoots inoculated	Shoots infected in %	Mean Length infected in %
susceptible			
Quince C	34	97,0	29,1
Quince BA 29	33	78,7	17,8
resistant			
OHF333	30	0	0

0-5% mean length infected: resistant, 6-30%: susceptible, >30%: highly susceptible.
Inoculation: 28 May 1993, Rating: 25 June 1993.

Table 3: Fire blight resistance of apple rootstocks, 1994

Apple rootstocks	No. Shoots inoculated	Shoots infected in %	Mean Length infected in %
high susceptible			
M11	30	100	67,6
A2	30	100	63,8
B9	27	92,5	37,7
susceptible			
M7	27	88,8	29,8
M27	29	96,5	24,3
M25	30	86,6	21,1
MM106	29	96,6	17,8
M2	30	93,3	9,5
M9	30	86,6	5,8
resistant			
M4	30	71,5	3,3
MM111	30	50	2,6

0-5% mean length infected: resistant, 6-30%: susceptible, >30%: highly susceptible.
Inoculation: 6 June 1994, Rating: 26 August 1994.

Table 4: Fire blight resistance of pear rootstocks, 1994

Pear rootstocks	No. Shoots inoculated	Shoots infected in %	Mean Length infected in %
susceptible			
Quince C	27	92,5	19,0
Quince BA 29	27	92,5	12,4
resistant			
OHF333	27	40,7	2,6

0-5% mean length infected: resistant, 6-30%: susceptible, >30%: highly susceptible.
Inoculation: 6 June 1994, Rating: 26 August 1994.

Discussion

Our results indicate that in most cases a good correlation between both test years could be observed (1). Only in three clones of apple rootstocks differences occurred ('MM106', 'M7', 'B9'). As in similar studies of other authors conditons of testing, for example weather conditions, the phenological status of the testplant and length of infection can be regarded as possible reason for these variations (4, 5). Correlation between the specific growth of rootstock clone and susceptibility for fire blight could not be drawn, which also is in accordance to other studies (6, 7).

In conclusion after two years testing on fire blight resistance in the pear rootstocks 'OHF 333' can be recommended as an alternative to the quince rootstocks to the growers. A similar recommendation cannot be given for the apple rootstocks. Although the clones 'M4' and 'MM111' were rated as resistant, because of cultural reasons they cannot be proposed to the growers as an alternative to the mainly used rootstock 'M9'.

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Fungal diseases of plants

COMPARATIVE STUDIES OF EUROPEAN AND CANADIAN POPULATIONS OF PYRENOPHORA TERES F. SP. TERES DRECHS

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INTRODUCTION

An International set of Differentials to characterize populations of Pyrenophora teres f.sp. teres Drechs.was created as a result of cooperation between All-Russian Institute for Plant Protection (Sankt-Petersburg, Russia), Institute of Phytopathology (dr.H.Hartleb, Aschersleben, Germany), Cereal Reserch Institute (V. Minarikova, Kromeriz, Czechia), Plant Industry Institute (dr. M. Janosheva, Piestany, Slovakia). On the base of investigation 11 pathogen populations from different regions of Europe on the 38 barley genotypes with different level of resistance were chozen 12 genotypes which showed a good differentiating abilities. They are : CI 2750, CI 4929, C 8755, C 20019, CI 4207, CI 739, CI 4407-1, C 29192,CI 9825, CI 5791, CI 9820, CI 9819 (CI - cereal investigation number, C - number of VIR catalog). The last six genotypes were the most resistant in our experiments as well as in another investigations. The use of them allows to differentiate the isolates with rare virulence in different populations.
The purpose of our reserch was to study the possibilities of differentials using in Europe and North America.

MATERIAL and METHODS

Barley leaves with natural infection from Canada was kindly provided by dr. A. Tekauz, from Czechia by V. Minarikova, from Poland by dr. J. Butrimovich, from Belorussia by dr. A. Zubcovich. Authors wish to thank these colleagues for there help. Isolation of P. teres from plant tissue was carried out on the CLM media (1). Sporulation from leaf tissue and on the medium near the leaf fragments occured within 4-5 days after transfer under continuous light at 20-24 C. Suspensions of conidia from each isolate were filtered through 2 layers of sterile cheesecloth and a drop of each suspension was placed on the same CLM medium but with 0,16 mg/l Triton X 100 added to limit fungul growth. Drop spread on the surface of agar medium and in 2 days the single conidia were examined under the light microscope. One colony from each isolate was transferred to CLM medium for propagation under continuous light for 10-12 days. Suspensions were prepared in destilled water with 0,01% Tween 80. Optimal concentration for inoculation tests was determined as 5000 - 10000 conidia/ml. Segments of first seedling leaves (2 - 2,5 cm length) were placed on filter paper mosted with 0,004% solution of benzimidazole in enamelled cuvets. Suspension drops (0,05 ml) of each monoconidial izolate were put on the leaf segments with a Pasteur pipet. Infection responses on the leaf segments were scored 4-5 days after inoculation by the following assessment using a 5 - point scale: 1 - highly resistant, only brown points without chlorosis;

2 - resistant, brown necrosis limited to the diameter of the nfection drop;
3 - susceptible, brown necrosis spreading on the leaf segment with chlorosis;
2,3 - intermediate type;
4 - highly susceptible, brown necrosis occupying the whole leaf egment.

93 monoconidial isolates from Canada and 59 from Europe were studied for there reactions to 12 differentials. The octal nomenklature for naming the resultant pathotypes was used in this investigation (2). The frequency of pathogen pathotypes were compared in fungus populations from different regions from Europe and Canada. Statistical index "share of identical phenotypes", analogous to the formula proposed by Givotovski (3) was used in this study: r = min (pi,qi), were pi, qi are the frequencies of the i-th phenotype in the 1st and 2nd populations, respectively.

RESULTS

The prevalent pathotype 0000 was the same in populations from Canada, Belorussia, Poland and Russia. Pathotypes 0300 and 0200 were prevalent in pathogen population from Czechia (43,7 and 31,2%, respectively), pathotype 1000 in population from Belorussia occurred with the same frequency as pathotype 0000 (34,3 and 31,4%, respectively)(Table 1).In the most cases there were the groups of pathotypes which were present in one investigated population and absence in another. Pathotypes:
2000, 6200, 6002,2002, 2040, 2242, 2042,2024, 2200, 2604,
2162, 0360, 0342,0122, 0006, 4242, 4062, 6100, 6242, 6162,
6000, 6042, 6140, 6122, 6362, 6262, 6322, 7100,7362, 7352,
7646, 7060, 7000, 7222 were revealed only in Canada and were represented by single, two or three isolates. Pathotypes 0300, 0340, 0600, 4300 were characteristic for opulation from Chechia; 1000, 5010, 1120, 1020 from Belorussia; 0400, 6400 from Poland; 6702,2602,4202,6306, 6302 from Russia.

Table 1

Frequency of P. teres race occurence in different geographical regions

Race number	Frequency of race occurrence in Canada	Czechia	Belorussia	Poland	Russia
0000	19,3	0	31,4	35,7	35,0
0100	1,1	0	2,8	7,1	0
0002	9,6	0	2,8	0	15,0
2100	1,1	0	0	21,4	0
0200	2,2	31,2	0	0	0
4000	1,1	0	0	7,1	0
6100	1,1	0	0	7,1	0
0300	0	43,7	0	0	0
0340	0	12,5	0	0	0
1000	0	0	34,3	0	0
2000	11,8	0	0	0	0
2002	7,5	0	0	0	15,0
Total number of monoconidial isolates	93	16	35	14	20

The similarity of studied populations showed in Table 2.
These results demonstrates the absence of resemblance between populations from Poland and Czechia, Czechia and Belorussia, Czechia and Russia. Very low similarity was between populations from Canada and Czechia (2,2%). Approximately similar meanings were obtained when population from Canada was compaired with populations from Poland and Belorussia (23,7 and 23,2, respectively) and when population from Russia was compaired with populations from Canada, Poland and Belorussia; Poland with Belorussia (34,2 - 39,6%).

Table 2
The similarity of P. teres populations from different geographical regions

Share of identical races (%) in populations from			
Canada and Poland	23,7	Poland and Belorussia	34,2
Canada and Czechia	2,2	Poland and Russia	35,0
Canada and Belorussia	23,2	Czechia and Belorussia	0
Canada and Russia	39,6	Czechia and Russia	0
Poland and Czechia	0	Russia and Belorussia	34,2

Discussion

High level of variability on virulence and absence or low similarity between P. teres populations were found by using the International set of differentials. The similarity lower then 50% is not considerable thus we can propose that studied populations are differ from each other. This conclusion correspond to our previous assumption about local distribution of P. teres populations (4). We propose the possibility of differentials using in Europe and North America.

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BIOCHEMICAL ASPECTS OF CICER ARIETINUM AND ASCOCHYTA RABIEI INTERACTION

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Introduction

Ascochyta blight, an important leaf spot disease of chickpea (Cicer arietinum) is caused by the necrotrophic fungus Ascochyta rabiei (teleomorph: Mycosphaerella rabiei). The plant-pathogen interaction has extensively been studied in this laboratory using resistant and susceptible plant genotypes, their heterotrophic, photomixotrophic and photoautotrophic cell suspension cultures as well as a considerable number of fungal isolates with highly different virulence . The fungus is characterized by pronounced genetic variability as shown by random amplified polymorphic DNA-techniques.

The main objectives of the research project are the identification and biochemical characterisation of chickpea resistance mechanisms, antimicrobial defense reactions and analysis of metabolic changes induced by fungal virulence/avirulence factors. In case of A. rabiei the studies aim at characterisation and biochemical identification of virulence/avirulence factors and the fungal mechanisms of pathogenicity.

This contribution deals with current investigations on pathogenesis-related proteins and rapidly started structural modifications of cell walls.

Material and Methods

All relevant methods and materials are comprehensively described in the references.

Results

During investigations on chickpea-Ascochyta rabiei interaction a number of putative fungal virulence factors have been isolated from culture filtrates, spore germination fluids or the fungal mycelium. These factors were separately tested for their reactions induced in chickpea cells. Altogether, 5 different groups of fungal components have been identified and they are listed as follows.

1. A. rabiei produces exoenzymes such as cutinase, pectin methylesterase, exo-polygalacturonase, pectin lyase, pectate lyase, xylanases, arabinosidase, cellulases, cellobiosidase, ß-galactosidase and proteases. All these enzymes are considered to be involved in fungal penetration into the plant tissue and in the digestion of plant cell walls and tissues. (1).

2. A polysaccharide elicitor which when applied to chickpea cell suspension cultures or sliced tissues leads to the expression of numerous antimicrobial defense reactions (2). The reactions are oxidative burst (formation of H_2O_2), induction of pterocarpan phytoalexins, pathogenesis - related proteins (chitinases, ß-1,3-glucanases), modification of cell walls without incorporation of phenolics and cell wall-bound glycine-rich-proteins. These

defense reactions are expressed at very different rates.

3. From fungal myceliums a glycoprotein suppressor has been isolated which inhibits the elicitor-induced phytoalexin biosynthesis by suppression of enzyme synthesis in susceptible cultivars (3).

4. In the culture filtrate an extracellular protein is accumulating which leads to the symptoms of hypersensitive response as indicated by extensive browning of cells and polyphenol incorporation in the walls of cells from resistant cultivars. (4).

5. The solanapyrone toxins A, B and C have been identified and when applied to leaf tissue the formation of necrotic spots is observed. The toxins are believed to be responsible for plant cell death as required for a necrotrophic fungus (5).

This list of putative fungal virulence factors and the various reactions induced in the plant tissue demonstrate that the pathogen possesses a number of mechanisms to interfere with plant cellular metabolism. Furthermore, the antimicrobial defense system of chickpea plants involves mechanisms which are expressed at different sites of cells and tissues. The expression of the plant antimicrobial defense reaction in most cases is by gene activation and the synthesis of enzymes occurs at highly variable rates which some reactions expressed in a few minutes and others require many hours or even days.

Pathogenesis-related proteins

Pathogenesis-related proteins (chitinases, ß-1,3-glucanases, thaumatin-like proteins, and several other groups of proteins of presently unknown biological function) have quite often been shown to be expressed as antimicrobial defense reactions of higher plants. Quite characteristically such PR-proteins occur as sets of basic or acidic isoforms (6). In general, the acidic isoforms are excreted from the producing cells into the apoplast whereas the proteins with a basic IEP are stored in vacuoles (7).

In case of the chickpea plant, previous investigations in the senior authors's laboratory described the isolation and characterisation of 2 chitinases and 1 ß-1,3-glucanase from elicited cell suspension cultures (6). The gene of a class III acidic chitinase has been cloned and sequenced .

A new approach into the detection of chickpea PR-proteins was recently started by the isolation of extracellular washing fluid (EWF) from A.rabiei infected chickpea leaves. In this compartment peptides, proteins, enzymes and phenols are being accumulated and the EWF represents an elegant method to analyse such components. Our investigations on the constituents of EWF have clearly shown that the protein material in this compartment accumulates at comparatively high concentrations but when compared with the great amount of protein in a total extract of leaves the apoplastic components are of minor quantity. Therefore, preparation of EWF leads to specific antimicrobial defense compounds.

The protein pattern of the EWF (approx. 20-22 bands) was completely different from the pattern obtained with total protein extracts from the leaves. Quantitative analyses of EWF revealed that approximately 14 proteins (molecular mass from 15 kDa to 110 kDa) showed considerable changes in their quantity. In most cases these proteins appeared as new bands approximately 2-4 days after infection. Analyses, revealed that an acidic chitinase previously isolated from elicited cell suspension cultures (6) was a prominent component in the EWF.

The current investigations showed a new basic ß-1,3-glucanase (MR 34 kDa), a new acidic chitinase (Mr 38 kDA) and a thaumatin-like protein (Mr 15 kDa). In addition to protein material various phenolic components are co-isolated with the EWF and their structural elucidation will hopefully lead to new information on chickpea antimicrobial reactions.

Structural modification of plant cell walls

The plant cell wall with its complex pattern of various carbohydrates and proteins is a major site for the expression of antimicrobial defense reactions. Such reinforcement reactions of cell wall structures may comprise several different reactions (8) of which oxidative cross-linking of protein components is of special interest. Such modification reactions of cell walls result in the formation of much more rigid structures which are more resistant against the penetration of pathogens or against enzymatic hydrolysis by microbial exoenzymes (8).

In case of chickpea cell suspension cultures a hydroxyproline-rich glycoprotein (HRGP) and a proline-rich protein (PRP) are soluble cell wall proteins in unstressed cells. The HRGP (Mr 190 kDa) and the PRP (Mr 80 kDa) were shown to become insoluble upon elicitation of the cells. The insolubilisation of the 2 proteins is a polymerization process with a progressive increase of inter-and intramolecular cross-links and this process occurs within 5-10 minutes after application of the elicitor. The process is thought to involve the formation of isodityrosine structures which are formed by an oxidative coupling of 2 tyrosine residues. One very obvious result of such an oxidative cross-linking is the observation that the cell wall may no longer be removed by enzymatic hydrolysis and these cells can practically no longer be turned into protoplasts (8).

Oxidative cross-linking of phenolic residues requires the presence of H_2O_2 and peroxidase. Our investigations with elicited chickpea cells indeed provided evidence that H_2O_2 is very rapidly synthesized *de novo* within 1-2 minutes after elicitor application; formation of H_2O_2 has been measured using chemiluminescence assays. Exogenous addition of H_2O_2 without elicitation also results in the aforementioned insolubilization of the HRGP and PRP and cocommittant addition of either catalase or peroxidase inhibitors prevents the protein polymerization process.

Discussion

Our present knowledge of antimicrobial defense reactions of chickpea documents that an impressive number of responses are being expressed. The responses comprise different cellular and extracellular sites and occur with very different rates. In some cases (oxidative burst with H_2O_2 formation, polymerisation of cell wall structural proteins) the responses occur so rapidly that constitutively formed enzymes must be involved. In other responses (formation of pterocarpan phytoalexins and their biosynthetic enzymes, PR-proteins) gene activation and new synthesis of biosynthetic proteins have clearly been shown. In current and future investigations great emphasis will be placed on a detailed comparison of the reactivity of resistant and susceptible cultivars. A very interesting aspect will be the observed rapid formation of H_2O_2 and the role of this oxygen species not only in cell wall modification but also as a component of a signal transduction chain which conveys the elicitor signal to the nucleus.

Acknowledgement

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INTERNATIONAL TESTING SOURCES OF RESISTANCE TO PUCCINIA RECONDITA F. SP. TRITICI

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Introduction

The best method of control rust pathogens is a network of international cooperative studies which would cover large epidemiological areas. Cooperative research of yellow rust of wheat for Europe had been organized in Netherlands, for stem rust in Italy, and for leaf rust in Yugoslavia (1). Leaf rust of wheat caused by **Puccinia recondita** Rob. ex. Desm f.s p. **tritici** has posed a great problem in normal wheat production, as the most widespread wheat diseases in the world. In European-Mediterranean analysis of **P. recondita tritici** population single resistant Lr genes used have not shown satisfactory efficiency (2; 3). It was become clear that these regions needed new more efficient resistance genes and large testings followed by crossing program started in that time. Later on, in order to get sources of resistance with accumulation of resistant genes farther breeding program contained the crossings of the best previous hybrid lines with the strong Lr resistant genes, Lr9, Lr19 and Lr24. The best hybrid winter wheat resistance lines and some selected resistance spring wheat lines were tested for a years in European Leaf Rust of Wheat field Nurseries (ELRWN).

Material and Methods

The first group for international testing where fifteen selected resistance winter wheat hybrid lines from our breeding program, and fifteen selected resistance spring wheat genotypes from International Rusts Nurseries. Besides in ELRWN were included three backcross-derived, near-isogenice lines in a Thatcher background, namely strong genes Lr9, Lr19 and Lr24. The line Lr18 were included, as differentiation resistant, and Lr14a as a susceptible control line. In each year ELRWN entries were tested with the most virulent cultures of the pathogen in the seedling stage. In the begining winter wheat hybrid lines originated that sixteen selected donors resistant lines had been crossed with recurente parents varieties Princ and Starke as a background (4; 5). As was mentioned, fifteen specially selected genetically different winter wheat lines from this program have been tested in ELRWN during three years (1987-1989), as well as in the seedling stage ELRWN in each year have been tested to the virulent international cultures of **P. recondita tritici.**

Second group of sources of resistance where the best sixteen hybrid lines containing the most interesting donors (66, 77, 26, 32, 37, 46, 74 and 146) and crossed with only effective genes Lr9, Lr19 and Lr24. In addition four other winter wheat hybrid lines where selected from the first cross, as control lines of the second crossings. Within spring wheat lines where eleven genetically different spring genotypes, and control lines Lr9,

Lr19, Lr24, Lr18 and Lr14a, as in a previous nurseries. Only in one year twenty of these winter wheat hybrid lines and sixteen spring wheat lines have been tested in ELRWN of thirteen countries. Cooperative seedling tests to different cultures have been realized in eight of those countries.

In the field nurseries are recorded; Disease Severity, using the modified Cobb scale, and Host Response, the type of infections observed (R-resistant, I-intermediate and S-susceptible). For greenhouse seedling tests reaction types were classified as R, I and S comprising types 1, 2, 3, 4 (0, 0; 1, 2^-), then 5, 6, (X^-, X^+) and types 7, 8 and 9 (3, 3^+, 4) respectively. The segregation was designated by coma (,).

Results

In a three years period (1987-1989) of testings the first group in ELRWN, fifteen winter wheat hybrid lines have shown very good resistance in the seedling stage to different cultures and in the adult stage in the nurseries on the large epidemiological territory. Parcial susceptibility have had only two hybrids Sava/1 x Princ and Sava/2 x Princ, mostly in segregation to susceptible and resistant plants. From the spring wheat lines of the first group the best results have shown only the lines: 647-CMA-14793, 417-ND-600, 81-ND-582 and Verry 722.

Summary results of the seedligs and field response in ELRWN 1989/90. of selected winter wheat hybrid lines (20 entries) and 16 selected spring wheat lines is presented in the Table.

The first 16 winter wheat hybrid lines originated from the second crossings with Lr9, Lr19 and Lr24 and the other four hybrid lines were, as control lines from the first crossings. Seedlings testings of ELRWN 1989/90. composed by 20 winter wheat breeding lines and 16 spring wheat resistant lines were realized in eight cooperative countries using 24 different cultures of **P. recondita tritici**. Only two winter wheat lines, NS-32-24/3 and NS-37-9/8, and two spring wheat lines 647-CMA-14793 and 26-TH-ESWYT-10 have shown complete homozygus highly resistant reactions. The field reactions of ELRWN 1989/90. have been evaluated in thirteen countries. Complete resistance to **P. recondita tritici** were registered in five winter and four spring wheat lines. The best evaluated lines for leaf rust in the in the seedling and adult stage, were, winter wheat lines: NS-66-9/2, NS-94-19/2, NS-94-24/1, NS-77-19/4, NS-32-9/4, NS-24/3 and NS-146-19/5 and spring wheat lines: 647-CMA-14793, 11-TH-ESWYT-25, 26-TH-ESWYT-3, 26-TH-ESWYT-10 and 26-TH-ESWYT-49.

Discussion

Parcial susceptibility of two hybrids (Sava/1 x Pr. and Sava/2 x Pr.) from the first group origanted from the weak leaf rust gene Lr3 found in our variety Sava (6). From the same first group good resistance of the variety Verry 722 is dialing with Lr26 gene associated with a translocation between the short arm of rye chromosome 1R and long arm of wheat (**Triticum aestivum** L.) chromosome 1B (7).

Seedling and field response of ELRWN 1989/90.
to **P. recondita tritici**

Winter wheat hybrid lines	Cooperative seedling tests: Reactions to 24 cultures			Field response: Reactions in 13 nurs.	
	R	Seg.	S	R	S
NS-66-9/2	19	4	1	13	/
NS-66-24/5	16	3	5	10	3
NS-77-19/4	18	4	2	11	2
NS-77-24/3	17	5	2	10	3
NS-26-9/6	16	5	3	11	2
NS-26-24/9	12	4	8	8	5
NS-32-9/4	16	4	4	12	1
NS-32-24/3	23	1	/	13	/
NS-37-9/8	24	/	/	13	/
NS-37-24/5	7	3	14	6	7
NS-46-9/7	12	5	7	11	2
NS-46-19/6	12	6	6	4	9
NS-94-19/2	19	3	2	12	1
NS-94-24/1	18	3	3	12	1
NS-146-9/8	12	10	2	8	4
NS-146-19/5	18	5	1	11	2
NS-32/2	19	3	2	11	2
NS-26/1	8	7	9	10	3
NS-82/1	15	2	7	9	4
NS-66/2	15	4	5	8	5
Spring wheat lines					
81-ND-582	13	1	10	6	7
417-ND-660	5	3	16	7	6
647-CMA-14793	23	1	-	9	4
11-TH-ESWYT-20	18	3	3	12	1
11-TH-ESWYT-25	17	4	3	11	2
11-TH-ESWYT-30	6	9	9	7	6
26-TH-ESWYT-3	18	3	3	11	2
26-TH-ESWYT-10	20	3	1	10	3
26-TH-ESWYT-36	14	4	6	5	8
26-TH-ESWYT-49	18	3	3	9	4
26-TH-ESWYT-50	11	7	6	7	6
Lr9	3	8	13	4	9
Lr18	3	4	17	7	6
Lr19	15	5	4	11	2
Lr24	16	5	3	7	6
Lr14a	4	2	18	2	11

Legend: R-resistant; Seg.-Segregation; S-susceptible.

Within the best evaluated nine hybrid lines better combining abilities for resistance have shown donors 66, 37 and 32 with Lr9, than 77, 94 and 146 with Lr19 and 32 and 94 with Lr24. Some other authors found corresponding differences in the use different donors of resistance in order to get better combining abilities (8; 9).

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IMMUNOLOGICAL METHODS FOR THE DETECTION OF *VERTICILLIUM DAHLIAE* IN OILSEED RAPE FOR RESISTANCE BREEDING

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Introduction

Verticillium dahliae is an important plant pathogen causing vascular wilts in a wide range of dicotyledonous crops and wild plant species (7). The fungus survives in soil as microsclerotia, initially embedded in fragments of plant tissue. After germination it penetrates directly into the vascular system of the plant. Verticillium wilt of oilseed rape (*Brassica napus* var. *oleifera* L.) is a serious problem in some regions with intensive cultivation (6).
The development and use of resistant cultivars remains the most effective, practical and economical method for controlling this disease. The current standard techniques used to screen oilseed rape populations for resistant genotypes are based on isolation of the fungus and optical disease assessement. The quantitative analysis of fungal biomass of vascular wilt diseases has been particulary intractable using standard methods. These are laborious and time consuming. An alternative approach are serological procedures such as enzyme-linked immunosorbent assay (ELISA). ELISA assays have found widespread application in plant pathology. This assay is routinely used for detection, identification and quantification purposes. Several polyclonal and monoclonal antibody-based immunoassays for *Verticillium* have been developed (4), (5), (9). These antibodies were used to compare *Verticillium* isolates and to determine the antigenic similarities between different *Verticillium* species. We developed a sensitive immunological assay to detect *V. dahliae* in infected oilseed rape plants from greenhouse and field experiments and in crop debris.

Materials and Methods

Antigen preparation. *Verticillium dahliae*, isolated from oilseed rape, was cultivated on polysulphone membranes in petri dishes on Czapek Dox culture media. After incubation (4 days), the fungal mycelium was separated from the growth media. The tissue was ground in an ice-cooled mortar and pestle with phosphate-buffered saline, pH 7.4 (2). The suspension was centrifuged at 20,000 g for 20 min and at 75,000 g for 60 min at 4°C. The culture filtrate was concentrated by lyophylisation, resuspended in PBS (PBS= 8 g NaCl + 0.2 g KH_2PO_4 + 2.9 g $Na_2HPO_4*12H_2O$ + 0.2 g KCl ad 1,000 ml, pH= 7.4) and dialysed against three changes of PBS. The protein concentration of the samples was determined with the method of Bradford (1). Protein extracts were stored at -20°C until needed.

Antiserum preparation. Soluble protein solutions from mycelium (1.0 mg) or culture filtrates (0.5 mg) were injected 3 times in 2 week-intervals intramusculary into rabbits. Freund's complete (first injection) and incomplete (subsequent injections) adjuvant were used. The serum used in this study was collected after the third booster injection. Antisera were stored frozen at -20°C until purification.

Purification of IgG. The antisera were prepared as described earlier (3). The immunoglobulin fraction was purified in 1/2 PBS using Fractogel TSK DEAE 650 (M) (Merck). The optical density at 280 nm was used to calculate the concentration of IgG.

Biotinylation of IgG. The Purified IgG fraction was dialysed against carbonate buffer (10 g NaCl, 10 g $NaHCO_3$ ad 1,000 ml, pH 7.5) overnight. 50 µl Biotin X-NHS (1 mg/ml, Sigma B-2643) in dimethylformamide solution was added for 30 min. The reaction was stopped with Tris/HCl (1M, pH 7.4) and the product was dialysed against three changes of 0.85% NaCl overnight.

ELISA. The B-A ELISA (=DAS ELISA using biotinylated detection-antibodies and steptavidin-alkaline phosphatase) was conducted in microtitre plates (Immulon F, Dynatech) using the following procedure: 100 µl of IgG in coating buffer (2) (1 µg/ml) were incubated overnight. After every step the plates were rinsed 3 times with 1/2 PBST (PBST = PBS+ 0.05% Tween 20). The plates were blocked by adding 200 µl coating buffer with 0.2% BSA for 2 hours at room temperature. 100 µl of prepared samples (plant material were homogenized in PBST + 0.2% PVP buffer, centrifuged, supernatant was used) were incubated overnight at 4°C. 100 µl biotinylated IgG fraction (0.1 µg/ml) were incubated overnight at 4°C or for 4 hours at 37°C. Streptavidin-alkaline-phosphatase conjugate (Fa. Biomol, 0.1 µg/ml) was added and incubated for 0.5 hour at 37°C. 100 µl substrate solution [*p*-nitrophenyl phosphate, 1 mg/ml in substrate buffer (2)] were added. After incubation for 2 hours at room temperature, the reaction were quantified by measuring the absorbance of each well at λ=405 nm in a Spectra plate reader (SLT, Austria).

Results

The lowest level for the detection of *V. dahliae* could be detected was 2 ng fungal protein. In the B-A ELISA, the antiserum did not cross-react with mycelial proteins from other fungal pathogens, e.g. *Phoma lingam*, *Sclerotinia sclerotiorum*, *Fusarium* spp., *Alternaria* spp., respectively (Fig. 1). Cross reactivity was detected with *Verticillium albo-atrum*, *V. tricorpus*, *V. tenerum*, but not with *Verticillium nigrescens*.

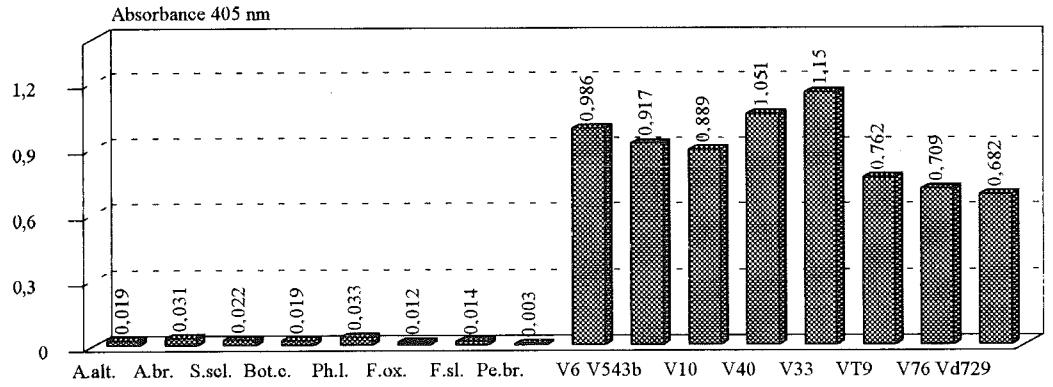

Fig 1. B-A ELISA with protein from pathogenic fungi of oilseed rape and from *V. dahliae* isolates. Protein concentration of the samples was 1 µg/ml (A.alt.= Alternaria alternata; A.bras.= Alternaria brassicae; S.scl.= Sclerotinia sclerotiorum; Bot.c.= Botrytis cinerea; Ph.l.= Phoma lingam; F.ox.= Fusarium oxysporum; F.sl.= Fusarium solani; Pe.br.= Penicillium brevicorinae; V6, V543b, V10, V40, V33= *V. dahliae* isolates from oilseed rape; VT9, V76= *V. dahliae* from cotton; Vd729= *V. dahliae* from tomato).

The B-A ELISA was used to detect *Verticillium dahliae* in artificially inoculated oilseed plants. Roots, stems and leaves of susceptible and resistant cultivars were prepared to follow the spread of the fungus through the plant (Fig. 2). All roots and stems were highely infected. The differences between cultivars were found in the oldest leaves.

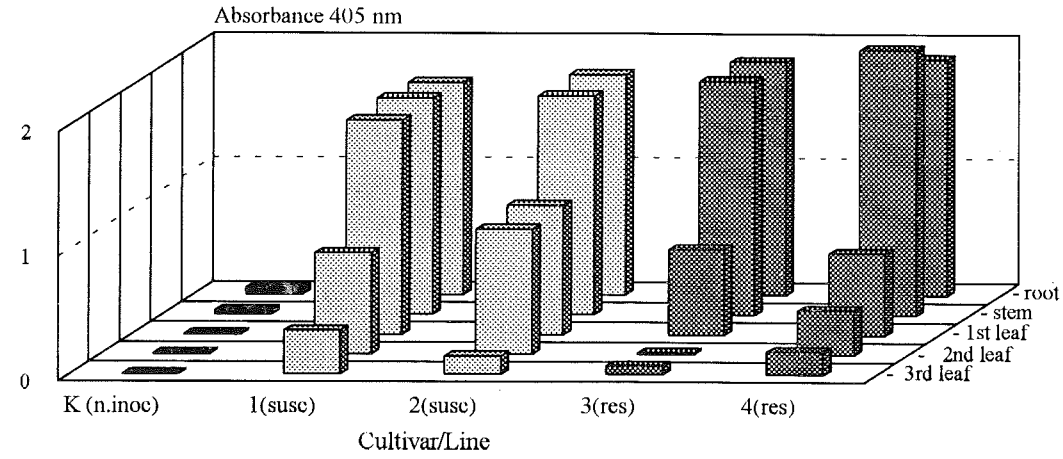

Fig 2. B-A ELISA with non inoculated and artificially inoculated susceptible and resistant oilseed rape cultivars, 35 dpi; (1st leaf= oldest leaf; K= non inoculated, susc.= susceptible, res.= resistant). Plant preparation with extraction buffer 1:5.

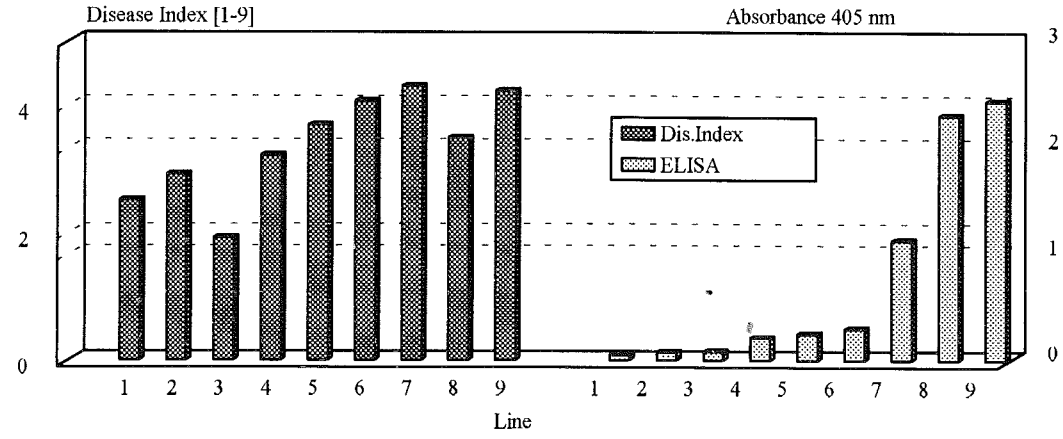

Fig. 3. B-A ELISA and optical disease assessment of different artificially inoculated susceptible oilseed rape lines. Plant preparation with extraction buffer 1:5. The plants were artificially inoculated and evaluated by Fa. KWS, Einbeck.

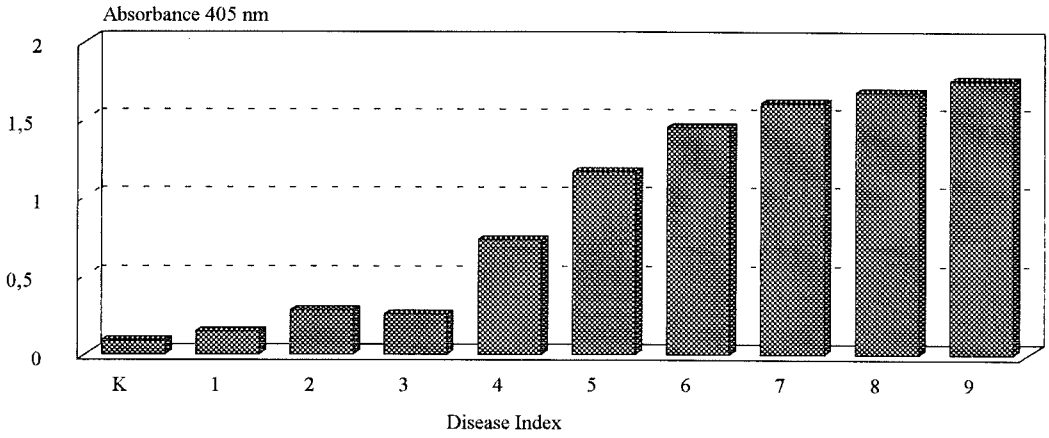

Fig. 4. B-A ELISA and optical disease assessement with crop debris (K=non inoculated greenhouse plant, 1=microsclerotia were not observed; extraction with PBST buffer 1:320).

We tested leaves of different lines with both ELISA and optical disease assessment (Fig. 3) to investigate different resistance to *Verticillium dahliae* between genotypes. The differences between oilseed rape lines measured by optical disease assessment were small. In contrast to results from disease index, the cultivars/lines investigated by ELISA resulted in different sequence.

The disease severity of crop debris were evaluated with optical disease assessment by microsclerotia quantity in the plant tissue. The stems were divided into incidence class from 1 to 9 (1= no microsclerotia) and investigated simultaneously by B-A ELISA (Fig. 4). To control non specific reactions with other pathogens, we tested also healthy plant tissue from greenhouse experiment.

Discussion

B-A ELISA enabled the identification and quantification of *Verticillium dahliae* in infected plants from greenhouse and field experiments. The antisera developed from other authors (4), (5), (9) were used to compare *Verticillium* isolates or had cross-reactions with healthy plants (8). We did not get cross-reactions with oilssed rape fungal pathogens and healthy plant material. It is possible to detect *V. dahliae* already 14 days past inoculation. The highest antigen titer was found in roots and stems. In contrast to optical disease assessment which observed the plant reaction, ELISA detect the real mycelium weight.

Regarding resistance to *Verticillium dahliae*, small differences between genotypes could be detected by this method. We found a high correlation between results obtained by B-A ELISA and optical detection of microsclerotia on oilseed rape stems.

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USING GENETIC RESOURCES OF *MALUS* FOR THE PILLNITZ APPLE BREEDING PROGRAMME

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The Fruit Genebank Dresden-Pillnitz has the **task, to conserve and to evaluate the genetic resources of pome, stone, small and wild fruit and to support projects for breeding, fruit growing, landscape shaping, pomology, taxonomy and phytopathology**. Apart from tasks in landscape development in conservation of old German cultivars and indigenous wild fruit species the collections of the genebank serve as a stock and a source of basis material for fruit breeding. At present, special importance is attached to the finding and preservation of resistance donors.

The **direct use of the results of the evaluations of collections of the Fruit Genebank** led to the edition of **numerous new cultivars by the breeding** institutions, so among others the **apple cultivars** of the **Pi-series** ('Piros', 'Pinova', 'Pilot', 'Pikant', 'Pirol' and others), the resistant apple cultivars named **Re-cultivars®** ('Retina', 'Remo', 'Reglindis', 'Reanda', 'Renora', 'Rewena' and others), the **apple rootstocks** 'Pillnitzer Supporter' 1, 2 and 3, the **sweet cherry cultivars** of the Na-series ('Nadino', 'Nalina', 'Namare', 'Namosa' and others), the **sour cherry cultivars** 'Karneol', 'Korund', 'Topas' and others and the **strawberry cultivars** 'Fratina' and 'Fracunda', suitable for mechanical harvesting. Additional breeding material stands just before the release.

In the **apple breeding** programme the aim is the **combination of different kinds of resistance and good fruit quality** in two lines: dessert cultivars and cultivars for processing. The best of these cultivars enclose resistance to scab (*Venturia inaequalis*), mildew (*Podosphaera leucotricha*), fire blight (*Erwinia amylovora*), bacterial cancer (*Pseudomonas syringae*), red spider mite (*Panonychus ulmi*), winterfrost and good fruit quality. Different scab resistance sources (Vf, Vr, VA) are used and will be combined in the next series of new cultivars.

In cross combination programmes the following apple cultivars were used as **donors**:
- sources for high yield and fruit quality:
  in the first step: 'Cox Orange', 'Oldenburg', 'Baumann Renette', 'Hammerstein', 'Northern Spy' and others,
  in the next steps: 'Alkmene', 'Auralia', 'Clivia', 'Elstar', 'Golden Delicious', 'Helios', 'Jonathan', 'Pilot', 'Pinova', 'Piros', 'Undine' and others;
- sources for scab resistance:
  in the first step: 'Steinantonovka' (VA),
  in the next steps: *Malus x floribunda (Vf), M. x micromalus (Vm), M. x atrosanguinea (Vf ?), M. pumila (Vr),* and the cultivars 'James Grieve', 'Cox Orange', 'Oldenburg' and the other quality-sources;
- sources for mildew resistance:
  Malus x zumi 'Calocarpa', *M. x robusta* 'Persicifolia', *M. x floribunda, M. x micromalus,* and the cultivars 'Dülmener Rosen', 'James Grieve', 'Helios', 'Alkmene', 'Lord Lambourne', 'Worcester Parmain' and others
- sources for fire blight resistance:
  M. x robusta 'Persicifolia', *M. x sublobata, M. x floribunda, M. prunifolia, M. fusca,* and a resistant clone from Pillnitz 'Pi-A 44,14', in the last time also the cultivars 'Remo', 'Rewena', 'Reanda'.

In long-term combination breeding programmes (diallel, topcross and others) all different sources were combined. All seedlings had been screened in early selection tests against scab, fire blight and mildew in the first three years. The first fruit quality test takes place after

a special management in a generation acceleration programme on M9/'Hibernal' rootstock/interstem in the field after 4...5 years after crossing.

Resistant cultivars exist for all ripening times. Especially for the Re-cultivars® it is a high benefit because a plantation only with resistant cultivars is possible under all farm conditions. The advantage of the saving of fungicides can be effective.

In the first step of the resistance breeding work the selected cultivars possess only one resistance source. Very important is the fact, that under the first varieties of the Re-series are cultivars with different bases of scab resistance :

Vf: 'Remo', 'Retina', 'Rewena', 'Rene', 'Reanda', 'Relinda', 'Releika', 'Resi', 'Renora';
Vr: 'Realka', 'Releta', 'Remura', 'Reka';
VA: 'Reglindis'

In the following steps the first high quality clones with two scab resistance sources are in the last field testing:

VA + Vf: 'Pi-AS 5,157', 'Pi-AS 5,169'.

Some scab resistant cultivars especially with the Vf gene, proved to be mildew resistant. These are 'Remo', 'Rewena', 'Reanda' and 'Resi'. Other named cultivars are only weakly susceptible in different degrees. Breeding material with mildew resistance obtained from both oligogenic and polygenic sources (*M. x robusta* 'Persicifolia', *M. x zumi* 'Calocarpa', cultivars) are in the field testing at present. Problematically we found the Vr-cultivars, many of which were susceptible to mildew. - The same problems with mildew in the field we found in 'Jonafree', 'Freedom', 'Liberty' and 'Priscilla'. - Commercial trials carried out over 12 years without fungicidal sprays have demonstrated, that the Pillnitz resistant cultivars have up to now a durable resistance to scab and good levels of resistance to mildew. It was very encouraging to note that diseases caused by other fungi were also absent in these trials.

Fire blight resistance is very important because there are no efficient bactericides registered for use in orchards. Donors of alleles for resistance have been found in progenies involving *M. x floribunda* and some cultivars. Progenies of 'Clivia', 'Golden Delicious', 'Alkmene' and 'Pi-A 44,14' produce a good percentage of seedlings with a high level of resistance. Fire blight resistant varieties are 'Remo', 'Rewena', 'Rene', 'Reanda' and 'Realka'. Triple resistant selections with resistance to scab, mildew and fire blight are the cultivars 'Remo', 'Rewena' and 'Reanda'. Parents with triple resistance transmit a high degree of resistance to their progenies.

The Pillnitz apple resistance breeding programme has produced a number of desirable quality selections with resistance to economically important diseases. Selection efficiency is high if both parents have high levels of resistance combined with good fruit quality. The best Pillnitz resistant clones and cultivars have been tested in commercial trials in several European countries - being exposed to a wide range of environmental conditions. They demonstrated their ability to maintain their resistance and provide fruit either suitable for table use and / or for processing. With their resistance properties they are suitable for ecological and integrated fruit production. The multiple resistant dessert cultivars are 'Reka', 'Retina', 'Realka', 'Releta', 'Remura', 'Releika', 'Resi', 'Rea', and 'Regine'. Special resistant cultivars for processing (canning) are 'Remo', 'Rewena' and 'Relinda'. Alternate resistant cultivars for both dessert and processing are 'Reglindis', 'Reanda', 'Renora' and 'Rene'.

With the Re-cultivars® we offer a concept for a direct using of genetic resources of *Malus* and on the other hand with the new cultivars a concept for a new growing management. Only in an integrated system of different resistant cultivars in one orchard the advantages of resistance will be noticeable. Different resistance sources can be combined with different maturing periods of cultivars. In this way a complete cross pollination and fruit set of the resistant cultivars is guaranteed. The combination of different cultivars for different production aims - table fruits, processing, landscape conservation - is possible.

Trials of the durability of the resistance obtained in the Pillnitz breeding programme need to be continually assessed under different climatic conditions over a number of years. Results so far show that a very significant reduction of fungicidal sprays is satisfactory achieved without risk of attacks by scab and mildew. In years of high risk for the two

diseases, sprays can be reduced to 2 - 3 treatments at the beginning of the vegetation period, while in years of low risk for some cultivars the fungicidal sprays can be eliminated completely.

For the durability of scab resistance in the field we recommend
- no "monocultur" with Vf-cultivars,
- tolerance of a slight leaf infection on polygenic resistant cultivars to preserve the stability of the host - pathogen system (VA - cultivars like 'Reglindis' or 'Freedom'),
- promoting the breeding of cultivars with two or more resistance sources (Vf + VA, Vf + Vr and so on).

The new **resistant Pillnitz apple cultivars (Re-cultivars®) guarantee the possibility to reduce 80 % and more of fungicides** in fruit growing and are very suitable for ecological and integrated fruit production.

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Multiple resistance in the Pillnitz Re-cultivars®

Re-cultivar®	Resistance to scab	res.-source	mildew	fireblight	bact.-cancer	red sp. mite	spring frosts	winter frost
REALKA	x	Vr	#	x	o	o	#	o
REANDA	x	Vf	x	x	o	#	x	o
REGLINDIS	x	VA	(x)	(x)	o	x		x
REKA	x	Vr	(x)	o	x	#	#	(x)
RELEIKA	x	Vf	o	(x)	x	x		
RELETA	x	Vr	#	o	x	o	o	o
RELINDA	x	Vf	(x)	o	x	#		
REMO	x	Vf	x	x	o	o	x	x
REMURA	x	Vr	o	o	o	#	o	x
RENE	x	Vf	o	x	(x)	#	x	o
RENORA	x	Vf	(x)	o	o	o	x	(x)
RESI	x	Vf	x	o	x	#		
RETINA	x	Vf	(x)	o	o	(x)	x	#
REWENA	x	Vf	x	x	x	o	x	o
Pi-AS-5,157	x	VA+Vf	(x)	o	x	x		
Pi-AS-5,169	x	VA+Vf	x	(x)	x	x		

x: resistant
(x): moderately resistant
o: moderately susceptible
#: susceptible
without: no investigated

DEVELOPMENT OF A METHOD TO TEST THE RESISTANCE OF LAMB'S LETTUCE (VALERIANELLA LOCUSTA L.) TO DOWNY MILDEW (PERONOSPORA VALERIANELLAE)

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Introduction

Downy mildew (Peronospora valerianellae) is an important fungal disease of lamb's lettuce. In the dark time of winter, and particular at humid weather and with dense plant growth, the fungus finds good conditions to develop. With the pathogen spreading, marketable yields of lamb's lettuce may decrease considerably. For keeping the crop healthy, it is an efficient measure to grow resistant varieties. At present, there are a number of varieties on the market which are generally considered to be resistant to downy mildew. However, reliable results as to their susceptibility or findings regarding differences by degree of their susceptibility are not yet on hand. For a determination of such differences in a safe and reliable manner, it is necessary to develop a specific method related to the host-parasite relationship.

The evaluation of variety's susceptibility was very complicated in previous investigations (1, 2) because of the different sporulating ability of the pathogen and the development of different symptoms of disease on the varieties. Despite controlled conditions in the test system, the pathogen did not sporulate in each test. For a clear evaluation of variety's susceptibility by means of sporulation on the plant, it is necessary to optimize the parameters in the test system. Results of first investigations are demonstrated in this paper.

Method

The method comprises a test using artificial infection under controlled conditions in climate chambers. At the 3 to 4 leaf stage, the lettuce plants were inoculated by spraying a defined quantity of spore suspension having a density of 10^4 spores per ml. To find out the optimum conditions for infection and sporulation on the host the inoculated plants were kept under various environmental conditions. In first investigations, the plants were held at three different temperatures (10°C, 12°C and 15°C), in darkness or under comperatively shady conditions during the first 48 hours after inoculation, and afterwards at 6000 lux of light intensity during 14 hours per day. To provide a high air humidity and free moisture on the host surface, which is essential for the establishment of infection, the plant were grown under plastic bags. The severity of disease was assessed using the attached key including five categories of symptom expression.

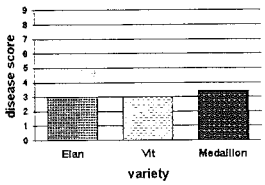
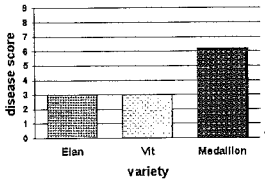
key for the assessment of the disease

1	no infection no symptoms visible on the leaves
3	low infection symptoms visible as leaf deformations and swollen cells of the lower epidermis, but no sporulation of the fungus
5	medium infection symptoms visible as leaf deformations and yellowing of the lower leaves, first appearance of conidial formation covering up to 10% of the foliage
7	strong infection symptoms visible as leaf deformations and yellowing of the lower leaves, intensive sporulation of the fungus covering up to 30% of the foliage
9	extreme infection intensive sporulation of the fungus on more than 30% of the foliage

Results

The results of artificial infection given in Table 1 demonstrate that the most intensive sporulation of the fungus was reached at 12°C on the variety 'Medaillon'. A dark period of 48 hours immedatly after inoculation stimulated the infection. The first formation of spores was apparent after 10 days and an abundant sporulation on the susceptible variety 'Medaillon' after 14 days. At 15°C, especially under shady conditions after plant inoculation, formation of oospores was often detected in the leaf tissue. Under these conditions, spores can also be formed, but only after a long incubation period of three weeks.

Tab. 1 Results of artificial infection with P. valerianellae on lamb's lettuce under various test conditions 14 days after inoculation

10°C dark period after inoculation	12°C dark period after inoculation	15°C after inoculation	
		dark period	shady conditions
only little sporulation of downy mildew fungus on the variety 'Medaillon' (disease score from 3 to 7) 	medium to intensive sporulation of the fungus on the variety 'Medaillon' (disease score from 5 to 9) 	sporadic sporulation of downy mildew fungus on the variety 'Medaillon' (the test result was influenced by the occurence of Phoma valerianellae)	no sporulation of the downy mildew fungus, but often formation of oospores in the leaf tissue (the test result was influenced by the occurence of Phoma valerianellae)

Proceeding from the results of artificial infection, we changed the parameters of the method used in previous investigations, accordingly. The test of susceptibility of varieties was carried out at 12°C and with darkness for 48 hours immedatly after inoculation. The results of the test including 16 lamb's lettuce varieties are illustrated in Figures 1 and 2.

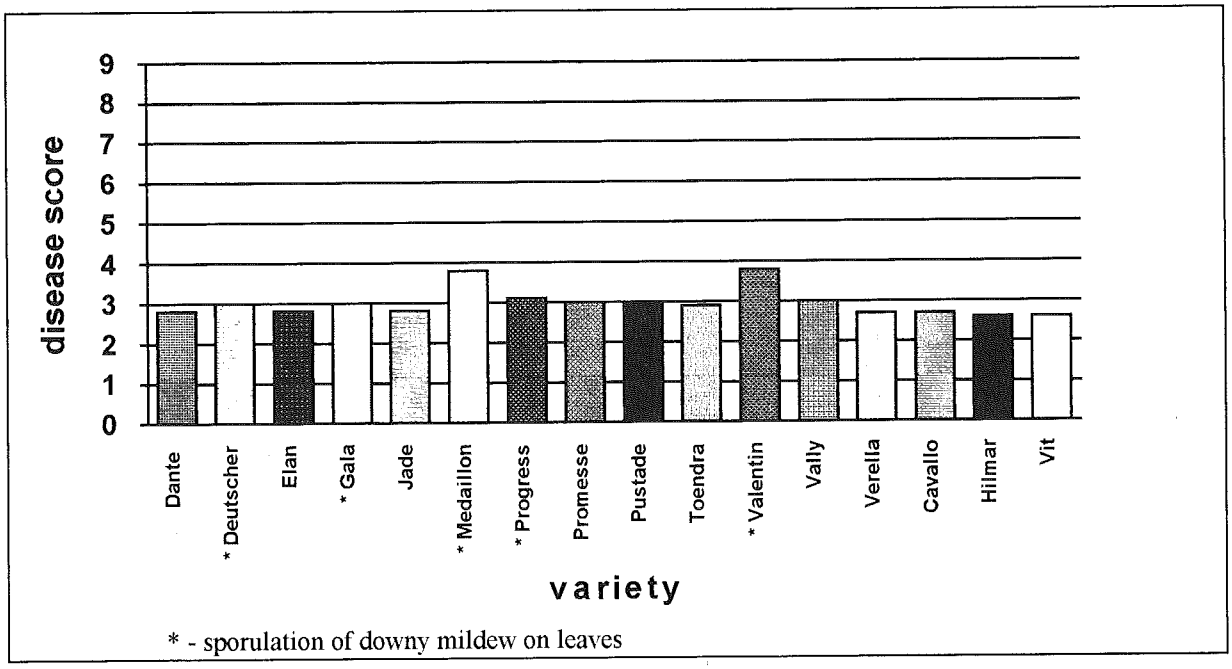


Fig. 1 First results of the susceptibility of lamb's lettuce varieties to downy mildew (P. valerianellae) using the improved method - evaluation of the varieties 14 days after inoculation

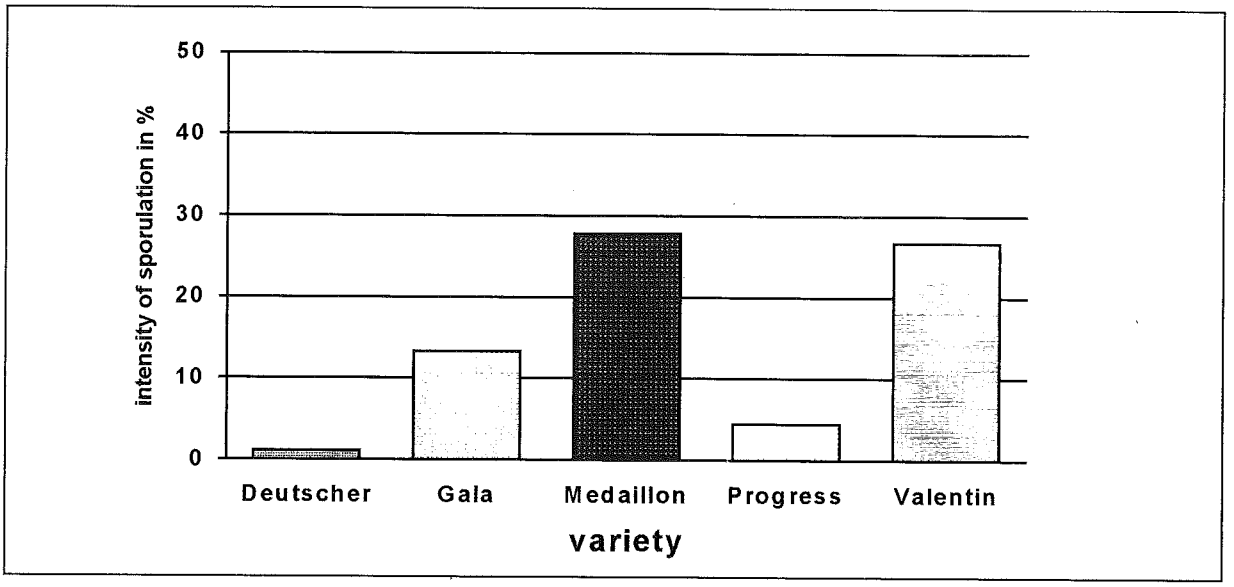


Fig. 2 Percentage of infected plants with sporulation of downy mildew 14 days after inoculation

The level of infection with downy mildew was not very high. The results were influenced by the occurence of Phoma valerianellae contained in a great quantity in the leaf material used for the inoculation. Despite this disturbing factor the test showed that the varieties 'Medaillon' and 'Valentin' are susceptible to downy mildew. Sporulation of the fungus appeared also on the varieties 'Gala', 'Progress' and 'Deutscher'. In previous tests, the varieties 'Gala' and 'Deutscher' have never been shown to be susceptible to downy mildew. It is supposed that the pathogen P. valerianellae differentiates in races like other downy mildew fungi do.

Discussion

To gain a reliable evaluation of the varieties, the parameters in the test system are optimized to guarantee a high sporulating ability of the fungus which is the basis for disease assessment. Thus it is necessary to intensify the investigations into the correlation of various factors such as temperature, light and humidity and their influence on the infection behaviour of the pathogen, the length of the incubation period and the development of sexual and asexual organs of the fungus. Furthermore it is of great importance to eliminate disturbing influences by other fungi, especially other pathogens of lamb's lettuce like Phoma valerianellae. The test with defined pathogen material requires to find out optimum conditions for the maintenance of the obligate fungus Peronospora valerianellae.

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POTATO BIOTECHNOLOGY FOR PRODUCING DISEASE RESISTANT VARIETIES

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Introduction.

The control of pest and disease in agriculture today is heavily dependent on use of chemists. Thus even during a favorable season potato crops may be sprayed ten times for protection against late blight. The growing of resistant varieties is the most effective way to make agriculture ecologically friendly. Selection of resistant plants it is a long process but it can be accelerated by the use of biotechnological methods. Our research is concerned with obtaining potato forms resistant to the late blight (Phytophthora infestans), black leg (Erwinia caro tovora var.atroseptica). Studies are being carried out in three fields:

1. Using of somaclonal variation phenomenon;
2. Somatic hybridization
3. Gene engineering

Materials and Methods.

The following potato varieties and forms were used in our expe riments:Adretta, White spring , Stolovy 19 Snow-White varieties, dihaploid M2HH 372 from the institute of Genetics collection (Germany), non tuber bearing species Solanun brevidens Phil. and S. etu berosum Lind.Aseptically plants were maintain in tubes by micro propagation on medium MS (1),containing 0.2 g/l casein gydro lizate, 1 mg/l adenine and 1 mg/l glycine at 25 C and 16-hour photoperiod.For plant and tuber inoculation was used a synthe tic population of complicated and wide spread strains of Phytoph thora infestans from Leningrad and Sakhalin regions and C-5 strain of first serotype of Erwinia carotovora var. atroseptica Collection of strains were maintained in vitro on ryesucrose medium (20g/l sucrose, 200g/l rye seeds, 20g/l agar). For plant transformation we use Agrobacterial tumefaciens strain pGV3850tt with genes of endotoxin from Bacillus thuringiensis and neomycinphosphotransferase synthesis(2).This strain is a gift of Dr.Lutova (St-Petersburg State University). Protoplasts were isolated from leaves of tube plants using 0.4Onozuka R-10 and 0.3fusion protoplasts and fusants were cultivated for 3-4 weeks in liquid medium and then microcally were transferred to solidified mediums for shooting.Hybrid plants initially were identified by morphological peculiarities and then by calculation of chromo somes. A hybrid

nature was proved by universally primes polymerase chain reaction. A laboratory model system "potato-drosophila" which was developed at the Department of Genetics, St.Petersburg State University was used for estimation of relative resistance to insects(4).

Results.

While using somaclonal variation phenomenon we obtained a run of plant regenerants, both via calli and straight on the explants of Adretta and White spring varieties. We chose forms with greater resistance than that of the resistance of original variety. One of the forms of the " Adretta" variety, similar to the initial form in morphology and yield, showed a considerable increase in resistance to the disease both in laboratory and the field.We identified forms with resistance to both the late blight and black leg. We obtained some somatic hybrids between wild nontyber bearing potato species and Stolovy 19, Snow-White varieties, M2HH 372 digaploid. Among them we selected plants with resistance to one or both diseases. Some of them can produce tubers in vitro. We obtained a run of regenerants of " White Spring" variety whose explants were transformed by the agrobacterial vector strain pGV3850tt with gene of endotoxin from Bacillus turingiensis,they were resistant to kanamycin, which was a marker of transformation. Some of them have a modified morphology. Plants were tested in a laboratory model system "potato-drosophila". One form dramatically decreased the fecundity of insects.This regenerant also shows the increasing of resistance to both late blight and black leg.

Discussion.

The use of somaclonal variation phenomenon is designed not only to obtain new resistant forms but also to improve existing varieties. Cultivation of plants on the cell or tissue level produces a wide range of genetic diversity, from which we can pick up forms with the required qualities. In the case of resistance to late blight there is no appropriate toxin which would be responsible for disease development. For example how it is in the case of Fusarium.Moreover Phytophthora infestans - the causal agent of the late blight,induces a hypersensitive reaction in the plant tissues. Therefore, we cannot search for resistance on calli or cell stages because the resistant cells are the first that perish. So the first stage when we can investigate the resistance of the obtained material is micropropagated in vitro plants.But if you have a reliable testing system the use of somaclonal variation phenomenon ia a suitable way to obtain late blight resistant forms. Success in obtaining somatic hybrids resistant to late blight and black lag confirm that somatic fusion can be a useful way to overcome the sexual incompatibility as well as to get new positive genetic modifications. Modified morfology and resistance to kanamycin of regenerants, obtained by transformation of potato explants by the agrobacterial vector strain pGV3850tt with genes of endotoxin from Bacillus turingiensis partially confirm their transgenic nature. Laboratory model "potato- drosophila" is rather artificial system, but it can be successfully used on fist stages of potato selection to insect pests.(4).The form that dramatically decreased the fecundity of drosophila will be also tested in bioassay with colorado beetle.The increased resistance to late blight and black lag of this regenerant can probably be

explained by a spontaneous mutation caused by the integration of foreign genetic material in the plant genome.

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Screening shoot cultures of *Malus* for apple powdery mildew infection by in vitro inoculation

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Introduction

Apple powdery mildew, caused by the fungus *Podosphaera leucotricha* (Ell. et Ev.) Salm. is one of the most serious diseases that affects apple trees.
To reduce the application of fungicides and the environmental pollution the only alternative way is to utilize resistant cultivars. Complete resistance to powdery mildew was found in wild *Malus*-species (1) but the use of such species in a breeding programme requires several generations of backcrossing before a high level of fruit quality is obtained. Many fruit breeders throughout the world are currently exploring cell and tissue culture techniques for the genetic improvement: to overcome the limitations of the sexual system by permitting the introduction of foreign genes. The application of the new techniques, like protoplast fusion, induction of somaclonal variation, gene transformation, utilization of dihaploid plants, needs a screening system to assess resistance and susceptibility of plants regenerated from somatic or gametic tissue before transfer to soil. In vitro screening systems for disease resistance were developed in apple and pear for *E. amylovora* (2, 3) and *P. leucotricha* (4), in peach for *X. campestris* pv. pruni (5) and in apple for cedar-apple rust (6).
The aim of this study was to develop a simple and effective inoculation technique for screening shoot cultures of *Malus* for resistance to *P. leucotricha* and to test the reliability of this method to study genetic variability.

Material and Methods

Shoot cultures

Seeds of two apple crossing populations (Idared x Helios) x (Golden Delicious x Alkmene) and (Idared x Helios) x (Idared x Golden Delicious) were collected from 100-day-old fruits, surface sterilized in a 0,1%-$HgCl_2$ -solution for 2 min and rinsed three times with sterilized destilled water. The two seed coats were removed aseptically and the embryo-axis was excised. The cotyledons were cultured with adaxial surface in contact with the medium.

Cotyledons were cultured on two different adventitious shoot proliferation media containing a half-strength of Murashige and Skoog (1962) salts and complemented with 4,0 mg/l BAP (7) or 0,1 mg/l TDZ + 0,1 mg/l IBA (8).
The cotyledons of each zygotic embryo were placed in a single well of a 12 multiwell-dish. The explants were kept in darkness for 8 days and then transferred into light (16/8 h photoperiod, 2000 lux) at 23 + 2 °C. Well-developed adventitious shoots were excised from the cotyledon explant and transferred onto a axillary shoot proliferation medium containing MS macro- and microelements, vitamins and supplemented with 30 g/l sucrose and 0,5 mg/l BAP, 0,2 mg/l IBA and 0,5 mg/l GA_3 (9). The shoots were placed into culture jars, kept in a culture room under the light conditions described and subcultured every 4 weeks.
The procedure of inoculation and selection is shown in figure 1.

IN VITRO CULTURE

Collection of different isolates of *Podosphaera leucotricha* with a determined geographic origin

Maintenance of a dual culture: host - susceptible *Malus*-genotype parasite - *P. leucotricha*

Preparation of a mixture from different isolates in a separate culture vessel to use as inoculum

Preparation of seeds from crosses of the breeding programme

Regeneration of adventitious shoots from cotyledons

Proliferation of the regenerated shoots

Inoculation of 3 detached fully expanded terminal leaves from in vitro shoots slippin off the conidia mechanically
3 replicated experiments for every genotype at different time

Incubation of the inoculated leaves on medium in 12-wells in a culture room at 20...24°C under conditions without condensation

4 days p.i. →
fixation (ethanol : chloroform : trichloracetic acid = 7 : 3 : 0,1) and staining (coomassie brilliant blue) of the infected leaves

Determination of the number of conidia per conidiophore microscopically
40 random counts per leaf with 3 replicated experiments per genotype

GREENHOUSE

Transfer of rooted plants to soil

Application of a conidia suspension to potted ex vitro plants

Assessment of the whole plant 3 and 6 weeks after inoculation

1	No visible symptoms (immunity)
3	No sporulation (hypersenstivity). Chlorosis often present. Necrotic flecks, usually minute. (highly resistant).
5	Scarce sporulation. Necrotic spots, often with chlorosis. (resistant - moderate resistant).
7	Moderate sporulation. Small necrotic areas, frequent chlorosis. (moderate susceptible - susceptible).
9	Profuse sporulation of well developed colonies. No necrosis, sometimes limited chlorosis. (highly susceptible).

Transfer of the plants to the open ground and assessment in the field

Figure 1: The procedure of in vitro- and greenhouse-screening for resistance to *Podosphaera leucotricha* on regenerated from cotyledons shoots

Results and Discussion

Cotyledon culture

The capacity to form adventitious shoots on cotyledonary explants depends from the progeny and was higher on media containing TDZ than on BAP-media (Fig.2a). The regenaration ability was better on the proximal than on the distal part of the cotyledons.

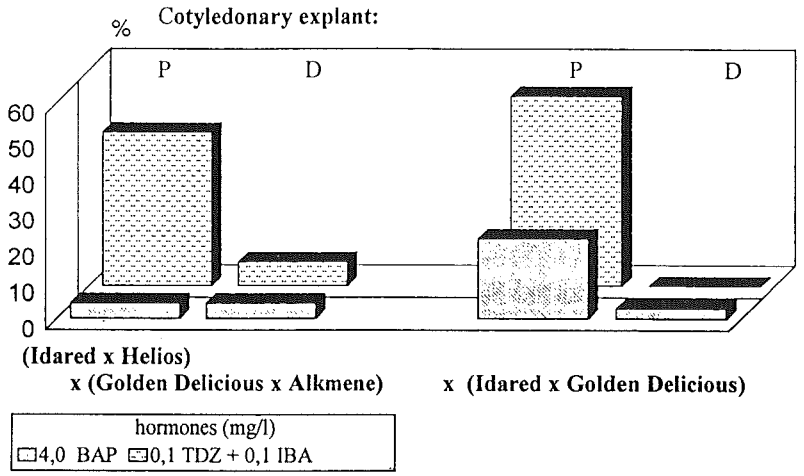

Figure 2a: Cotyledon culture: Percentage of regenerating cotyledons in dependence from the progeny, the kind of explant (P = 1/3 part proximal; D = 2/3 parts distal) and the induction medium

Caused by the high cytokinin level of the induction media multiple shoot complexes were preferably formed and the yield of well developed microshoots was reduced.

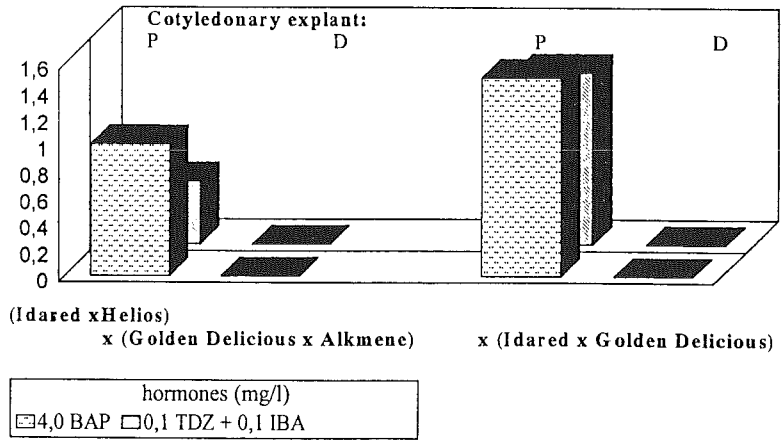

Figure 2b: Cotyledon culture: Number of well developed shoots per regenerating explant in dependence from progeny, the kind of explant and the induction medium

In vitro screening

The genotypes of the progenies showed significant differences in their susceptibility in vitro to *Podosphaera leucotricha* (genotype 2 - highly susceptible; genotype 30 - highly resistant) (Fig. 3).

High correlations were obtained between the mean value of the number of conidia/conidiophore and the data of three replicated experiments (r = -0,86; -0,84; -0,82). The most susceptible genotypes showed the lowest deviation from the mean value of the number of conidia/conidiophore in 3 experiments.
The more resistant the genotype the less counts of conidia/ conidiophore are possible due to the reduced development of conidiophores on the infected leaf.

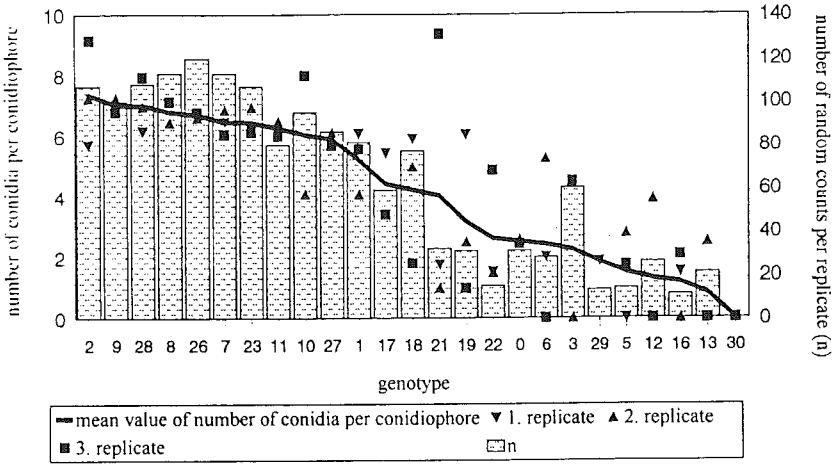

Figure 3: Screening for resistance to *Podosphaera leucotricha* in vitro: Infection on detached leaves of regenerated shoots from cotyledon culture

If the susceptibility of genotypes in vitro is compared by the susceptibility in the greenhouse it can be seen that independent from the time of assessment the two replicates in the greenhouse do not significantly differ for the examined genotype (Figure 4).
The assessment in the greenhouse do not correlate with the data obtained in vitro. This underlines the difficulty of preselection in the greenhouse in one-year-investigations.
The variability of the genotypic reaction to mildew observed in the greenhouse was low. Resistant or moderate susceptible genotypes could not be found. In order to find exact results about the susceptibility or resistance of genotypes, long-time-experiments are necessary in the field (10, 11).

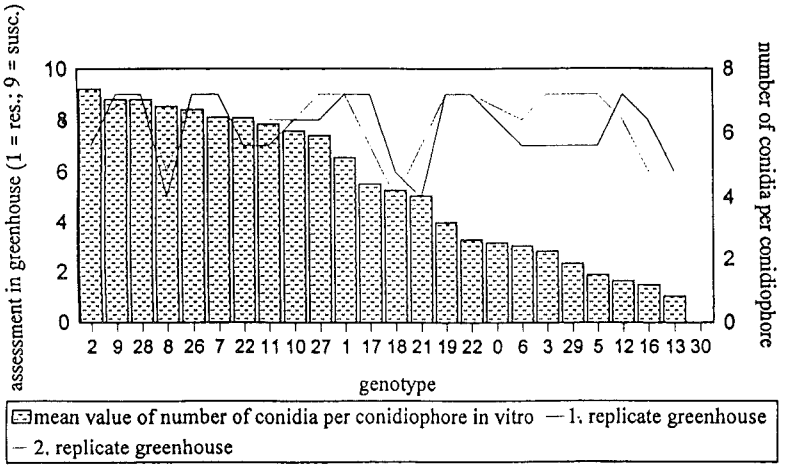

Figure 4: Screening for resistance to *Podosphaera leucotricha*:
in vitro - Infection on detached leaves
greenhouse - Infection on potted plants

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PRACTICAL UTILIZATION OF DISEASE RESISTANCE TO MINIMIZE THE APPLICATION OF FUNGICIDES TO CONTROL LEAF RUST OF WHEAT AND BROWN RUST OF BARLEY IN THE DRY-REGION OF SACHSEN-ANHALT; GERMANY

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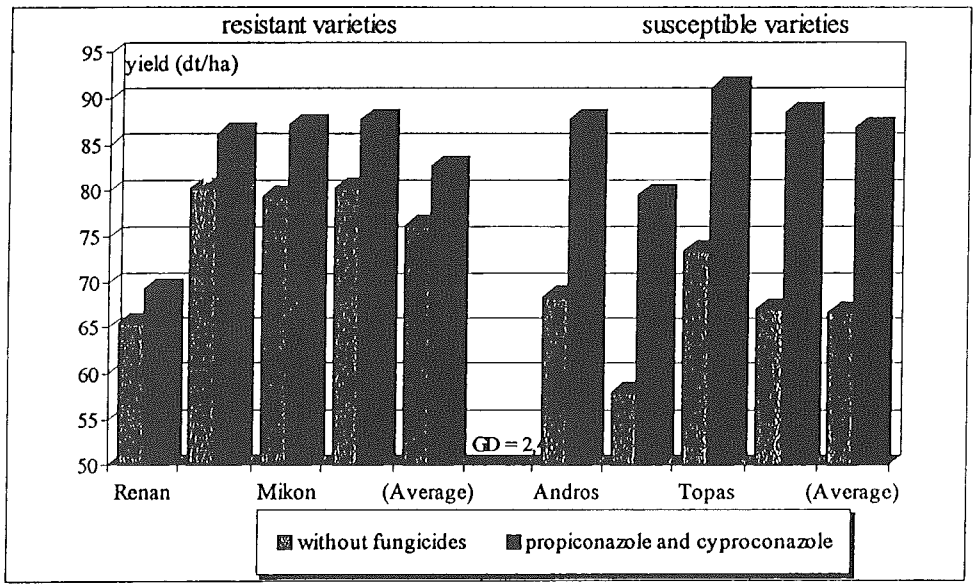
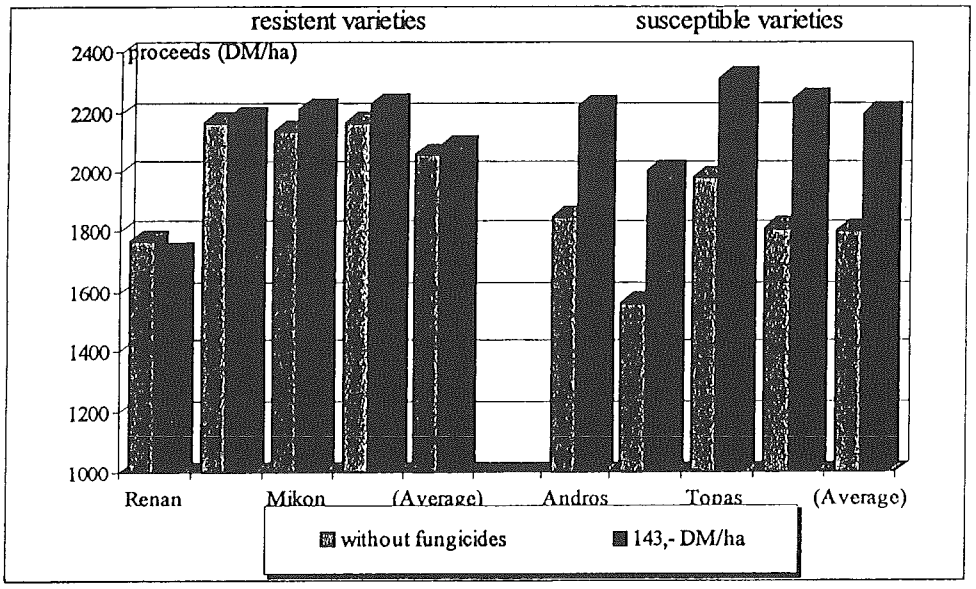
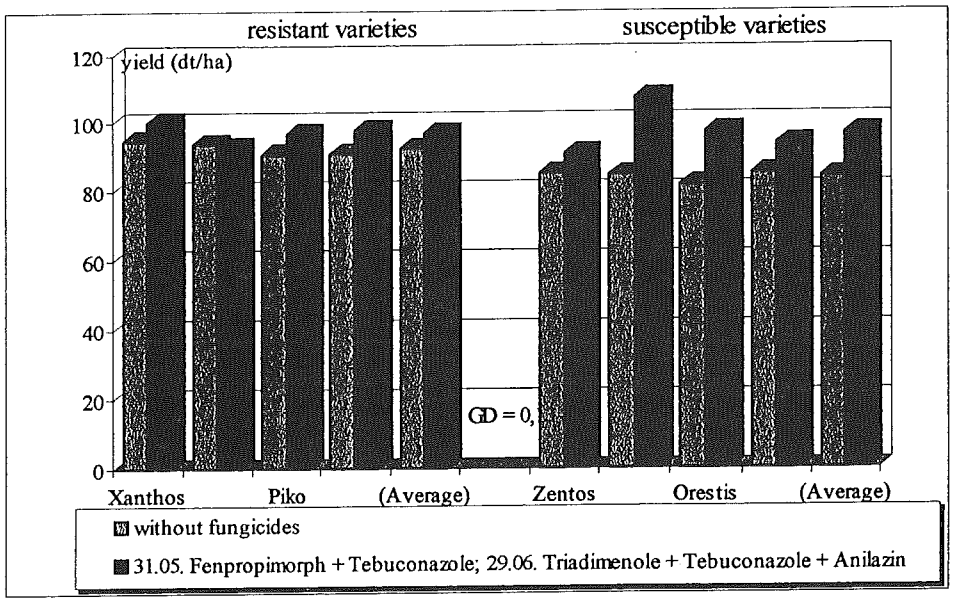
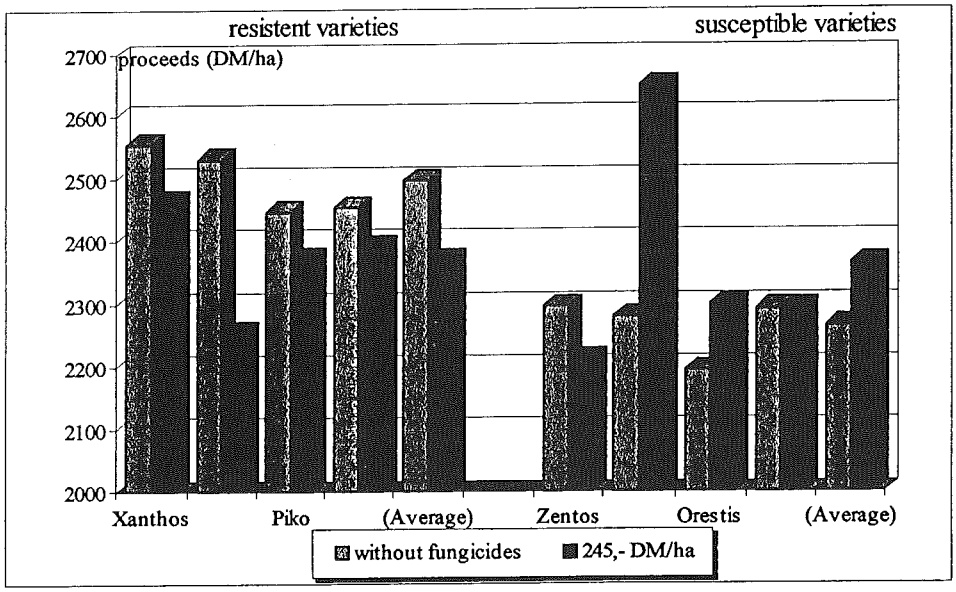
Leaf rust of wheat (*Puccinia recondita* Rob. ex Desm.) and brown rust of barley (*Puccinia hordei* Otth) caused the greatest yield losses of all leaf spot diseases in the dry-region of Sachsen-Anhalt in the last 3 years.
Field trials carried out in 1993 with both pathogens all over the country indicated yield losses on wheat and barley of more than 20% and the average thousand kernel weight reduced up to 17%.



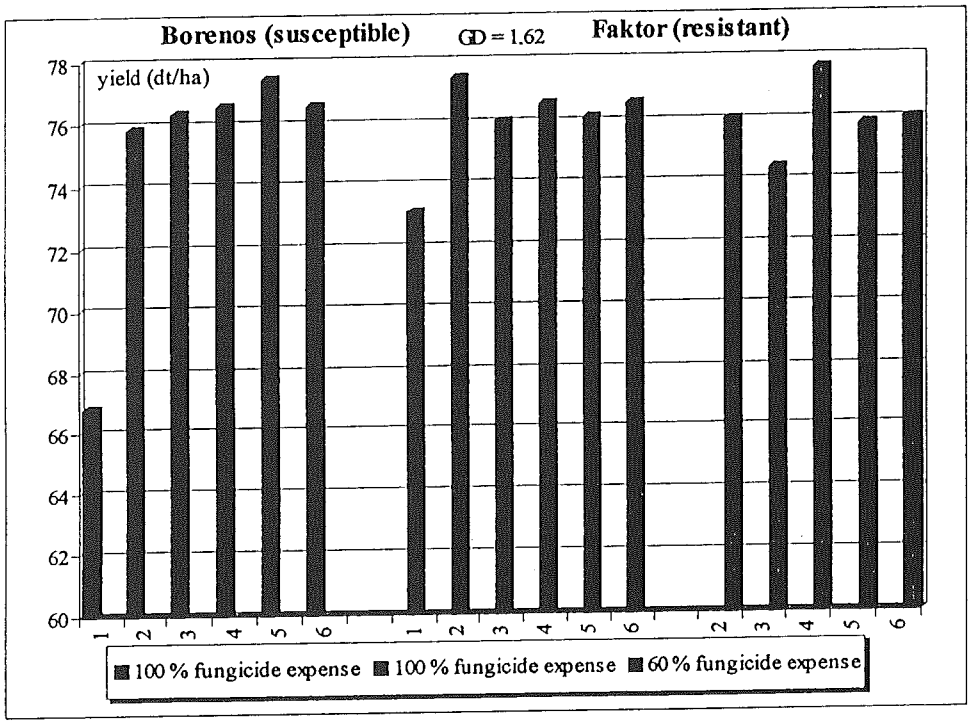


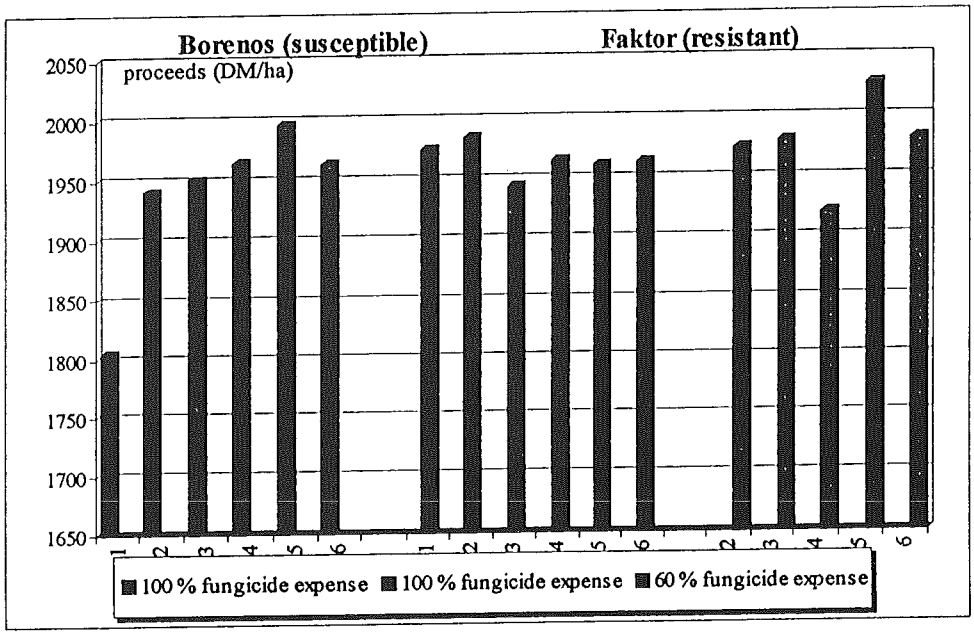
- Group of susceptible varieties with an average of 7.3 on the score scale (1= resistant; 9= susceptible) reacts to a fungicide application with an increase in yield by 30% and the proceeds by 22%.
- The resistant varieties (code= 2.5) increased the yield after fungicide application only by 8% while the proceeds were on the same level.
- The proceeds realized by the susceptible varieties with fungicide application were 7% higher than that of the resistant varieties without fungicide treatment.





- Susceptible varieties (code 6.8) resulted in 15 and 4% increases in yield and proceeds, respectively, after fungicide application. The variety „Borenos", widely cultivated in the dry region of Sachsen-Anhalt, reacted with a higher yield increase.
- Resistant varieties (code 2.3) led to only 5% yield increase and a decrease of 5% in proceeds after fungicide application.
- The proceeds realized by the susceptible varieties with fungicide application were 6% lower than that of the resistant varieties without fungicide treatment.





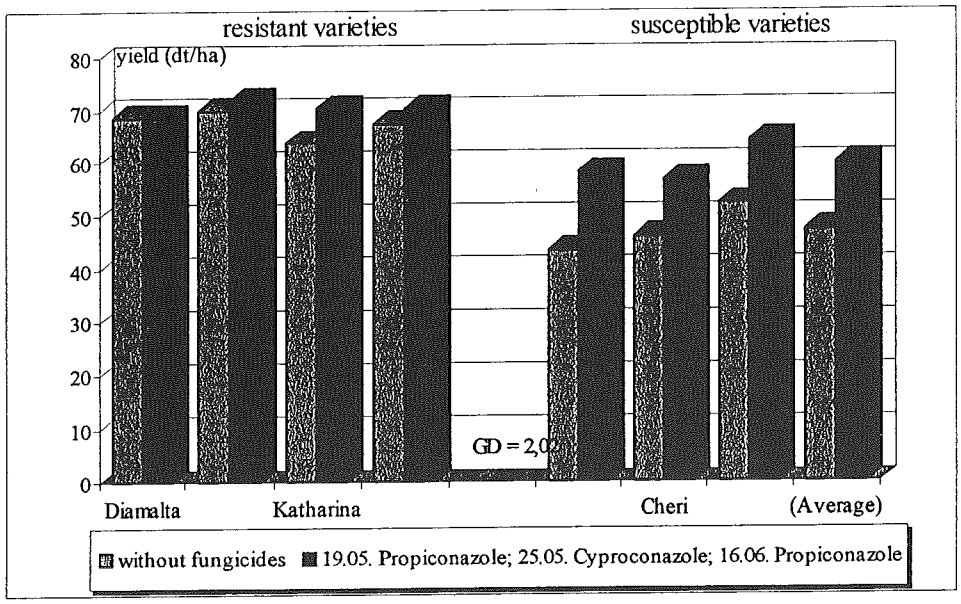
- The susceptible variety „Borenos" (27% leaf rust in DC 75) needs protection by fungicides to reach the genetically fixed yield potential. The fungicide treatment in this variety led to 14,5% and 8,8% increases in yield and proceeds, respectively.

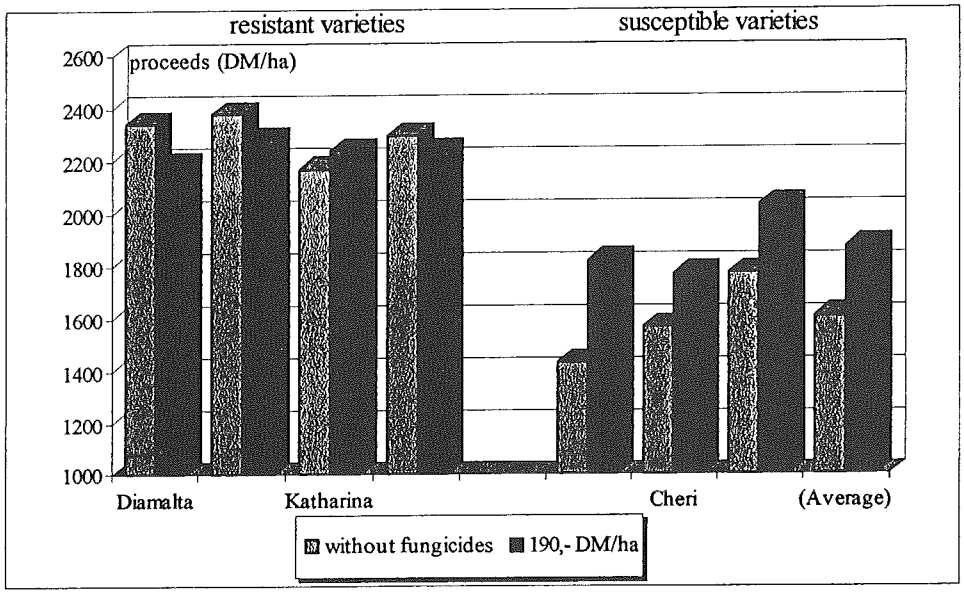
- The resistant variety „Faktor" (6% leaf rust in DC 75) showed 4,5% yield reduction without fungicides compared to „Borenos" with fungicide. However, the proceeds were the same after fungicide treatment with these susceptible varietiy.

- The yield level of the resistant variety was enhanced by application of 100% and 60% fungicide expense only 4,7% and 4,0%, respectively.

- No further increase in the level of proceeds of the resistant variety was recorded by application of 100% and 60% fungicide expense, respectively.

1 - without fungicides
2 - prochloraz + cyproconazole (DC 61)
3 - cyproconazole + chlorthalonil (DC 61)
4 - difenoconazole + propiconazole (DC 61)
5 - triadimenole + tebuconazole (DC 61)
6 - average (2...5)





- The proceeds obtained by growing the resistant varieties (code 3.5) without fungicide application were 12% higher than that of the susceptible varieties (code 6.5) given after fungicide treatment. The reason for this positive effect is the combination of a high yield level with an effective resistance to rust resulting from a consequent resistance breeding.
- If there is a special reason for growing susceptible cultivars (for reason of better brewing or malting attributes) they must be protected by fungicide application to ensure yield and quality parameters.

Conclusions

Advances in resistance breeding and/or qualified application of fungicides enable an effective control of rusts by using suitable varieties and principles at the present time.

The results of our experiments at several localities demonstrate the possibility to reduce the amount of fungicide application by conscious use of rust resistant varieties.

In some cases highly resistant varieties of winter wheat and spring barley without fingicide application realized the same proceeds as susceptible varieties treated with fungicides. Susceptible varieties need under the given regional conditions treatment with fungicides to reach the genetically fixed yield potential.

ASSESSMENT OF *VERTICILLIUM DAHLIAE* KLEB. IN SOIL AND ITS INOCULUM POTENTIAL FOR OILSEED RAPE BY AGAR PLATE ASSAY AND ELISA

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Introduction

In Germany, premature ripening of oilseed rape mainly caused by *V. dahliae* has become more significant in the last two decades (1, 2). The fungus persists in soil as microsclerotia for many years. The inoculum level of *V. dahliae* in soil plays a critical role in disease development (3,4). For oilseed rape, the relationship between soil inoculum densities of *V. dahliae* and the disease severity or yield losses are unknown. To address this question we designed greenhouse and microplot experiments with artificially infested soil.
For the detection of *V. dahliae* in soil divers techniques like baiting and agar plate assays have been developed (5,6). These conventional methods require long incubation periods and mycological expertise. We developed an enzyme-linked-immunosorbent assay for the assessment of *V. dahliae* in soil as a faster and easier detection method.

Material and Methods

Production of antiserum. Antiserum was raised in a rabbit against soluble proteins from microsclerotia of *V. dahliae* (isolate V 10 from K. Zeise). The immunization was done in the BAZ by the Institute for Pathogen Diagnostics. We islolated and purified the IgG as described by Clark and Adams (7).
Double-antibody sandwich (DAS) ELISA. All working volumes were 100 µl per well, except blocking and washing (200 µl/well). Between incubation, all wells were washed three times with PBS containing 0.05 % Tween 20. The biotinylation of the IgG was done as described by Neugebauer (8). Microtiter plates were incubated successively with specific IgG (1µg/ml in carbonate buffer pH 9.6) for 4 h at 37°C, with block reagent (0.2 % BSA/ml carbonate buffer) for 2 h at 37 °C, with soil extract samples overnight at 4 °C, with biotin labelled specific IgG (1µg/ml in PBS containing 0.2 % BSA) for 4 h at 37 °C, with streptavidin-alkaline phosphatase conjugate (Boehringer Mannheim; 1:8000 in PBS containing 0.2 % BSA) and with substrate buffer (9.7 % diethanolamine, pH 9.8 containing 1.0 mg p-nitrophenyl phosphate) at room temperature. The colour development was measured after 2 h at λ=405 nm.
Soil sample preparation for ELISA and agar plate assay. The wet- sieving and agar plate assay were based on the method of Harris (9). A soil suspension (50 g sieved soil/100 ml destilled water) was prepared by shaking 1 h at 270 oscillation/min. The slurry was wet sieved through nested 1000, 250, 125 and 20 µm sieves with tap water. The 20 to 125 µm soil fraction was recovered.
Procedure for the ELISA. Water was filtered and the residues dried for 12 h. After sonication of the residues in an extraction buffer (100 mM Tris, 2 M NaCl, 20 mM ETDA, 0.1 % Triton-X-100, 1 % Casein pH 7.4) for 30 sec the slurry was centrifuged. The supernatant was used for ELISA.
Procedure for the agar plate assay. The 20 to 125 µm soil fraction was made up to 100 ml with destilled water. Under stirring aliquots of 0.5 ml were transferred on a semiselektive

medium after Huisman (10). After incubation (22 °C) for 4 weeks, we scanned the plates for colonies of *V. dahliae* using a dissecting microscope.

Disease severity. Quantification of disease at harvest was done as described by Holtschulte (2).

Results

Antiserum specificity. The extract (2.0 µg protein/ml) of eight *V. dahliae* isolates, five from oilseed rape and three from other hosts, gave an absorbance value > 2.0 when tested in DAS-ELISA, except for an aggressive strain from cotton (A_{405}: 0.77).Extracts of other *Verticillium* species reacted less intense (A_{405}: 1.00-1.43), but there was no reaction with *V. cinnabarinum*. No cross-reactivity with eleven other soil fungi, except *Rhizoctonia. solani* (A_{405}: 0.41) could be observed.

Determination of *V. dahliae* in artificially infested soils by ELISA and agar plate assay. The relationship between the inoculum density (mg microsclerotia/g soil) and the absorbance was linear after logarithmic transformation. The ELISA has a detection limit of 2.40 µg microsclerotia / g soil, which is about 1 to 2 cfu / g soil. A concentration of 0.24 mg microsclerotia showed readings of 0.89 (Fig.1).

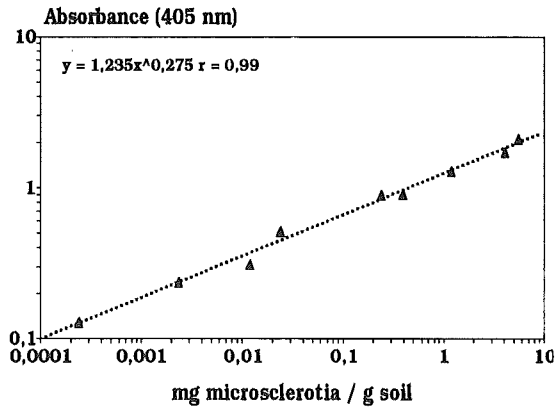

Fig. 1. Relationship of absorbance (DAS-ELISA) and inoculum density of *V. dahliae* in artificially infested soil

The detection of the pathogen under field condition by agar plate assay and ELISA 1 and 90 days after infestation of the soil is demonstrated in Figure 2. The detection limit was again 2.40 µg microsclerotia / g soil. Concentrations of 0.24 mg resulted in readings of 0.61, corresponding to 19 cfu. 90 days after infestation an increase of *V. dahliae* in soil can be determined with the agar plate method. This phenomenon could hardly be detected with the ELISA.

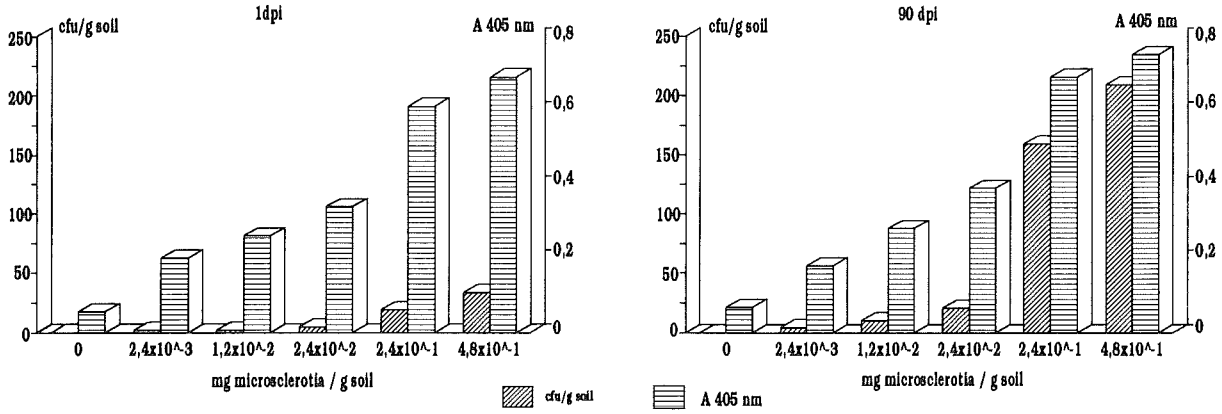

Fig. 2. Detection of different inoculum densities of *V. dahliae* in artificially infested field soil (microplots 1 m^2) by agar plate assay and ELISA 1 and 90 days after infestation

Relationship between inoculum density of *V. dahliae* in soil and disease severity as well as yield losses. No effect up to a concentration of 2.40 µg microsclerotia/g soil on disease severity could be detected in greenhouse experiments (Fig. 3) and field microplots (Fig 4, A). Higher concentrations led to significantly enhanced disease symptoms, but no yield losses. The rapid-cycling population of *Brassica napus* showed a maximum disease severity at 2.40 mg microsclerotia / g soil (Fig. 3). Yield reduction of the susceptible oilseed cultivar „Ceres" was significant at a concentrations of 0.24 mg microsclerotia/g (Fig. 4, B). The tolerant cultivar Express showed no yield reduction under these conditions.

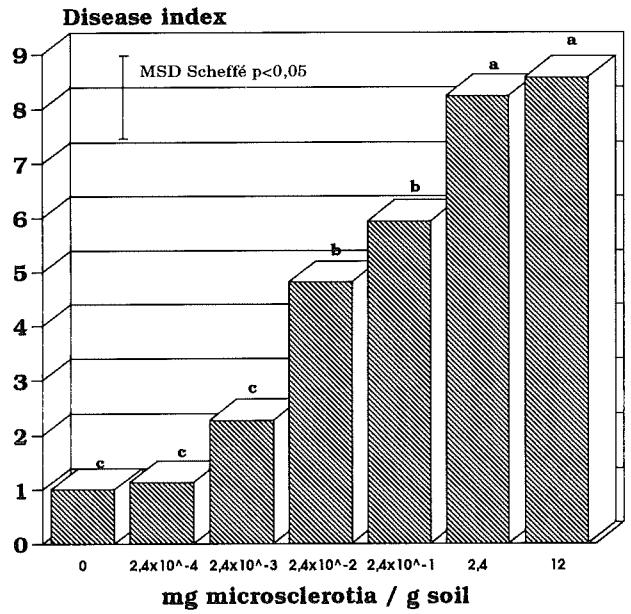

Fig. 3. Relationship between the inoculum density of *V. dahliae* in artificially infested soil and the observed disease severity on rapid-cycling *Brassica napus*

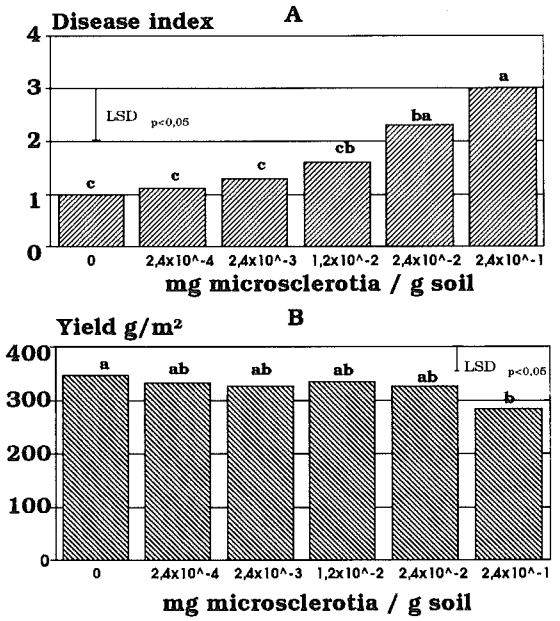

Fig. 4. Relationship between the inoculum density of *V. dahliae* in artificially infested field soil (microplot 1 m²) and the observed disease severity (A) and yield (B); cultivar „Ceres"

Occurrence of *V. dahliae* in naturally infested soils in FRG estimated by ELISA and agar plate assay. The concentration of *V. dahliae* estimated with the two methods was relatively low, except for the field site Dollern. On the other hand, disease severity was low in all cases, even at Dollern (Table 1). The correlation between the absorbance of the ELISA and the cfu of the agar plate assay was high (r=0.93) and the relationship was linear after logarithmic transformation of the data.

Table 1. Inoculum densities of *V. dahliae* in naturally infested soils of different districts in FRG estimated by agar plate assay (cfu/g soil) and ELISA (A_{405}nm) before seeding of oilseed rape (28.8.1993) and disease severity at harvest of the cultivar „Express"

Field location	Inoculum density (cfu/g soil)	Absorbance (λ = 405 nm)	Disease index cv. Express GS 92
Altjellingsdorf	**0.45**	**0.248**	**1.5**
Intschede	**1.49**	**0.365**	**2.9**
Oederquart	**0.23**	**0.167**	**-**
Dollern	**74.39**	**0.778**	**1.0**

Discussion

The polyclonal antiserum was highly specific for oilseed rape isolates of *V. dahliae*. The low cross-reactivity with *V. albo-atrum, V. nigrescens, V. tricorpus* and *R. solani* can be neglected as soil preparation by wet-sieving was optimized for *V. dahliae*. Thornton et al. (11) were not able to detect *R. solani* in soils. For the antigen extraction they incubated the samples in a semiselectiv medium. Our results show that after wet-sieving of the soil and sonication in an extraction buffer *V. dahliae* can be detected in soils.
We determined an increase of *V. dahliae* in soil three months after seeding of oilseed rape by the agar plate assay, hardly by ELISA. A possible explanation for the multiplication of *V. dahliae* are secondary, smaller microsclerotia (12), which may have a lower amount of antigen. In naturally infested soils we found a good correlation between the DAS-ELISA and the agar plate assay.
Ashworth et al. (13) reported for cotton that the threshold of microsclerotia required for infection was 0.03 / g soil and 0.5 units *V. dahliae* / g soil for strawberry wilt (14). In our experiments with oilseed rape with the susceptible cv. „Ceres" infection occurred in soils containing more than 2.40 µg microsclerotia / g soil (ca. 1-2 cfu/g soil). For a tolerant cultivar „Express" the threshold was higher (0.24 mg microsclerotia/g soil; ca. 19 cfu/g soil). Microsclerotia at concentrations of 74 cfu / g soil in a naturally infested field site resulted in no disease symptoms on different oilseed cultivars. Factors like soil type and soil physical properties can directly or indirectly influence the biological activity of *V. dahliae*. Due to the preceeding crop potato we suspect that we determined the haploid type of *V. dahliae*, that is non-pathogenic to oilseed rape. Several workers have described this phenomenon (15). This problem can be solved with a specific antiserum to distinguish haploid and diploid types of *V. dahliae*. Our results show that the ELISA can provide a basis for the determination of *V. dahliae* in soil.

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RAPD and ARDRA Studies on *Pseudocercosporella*

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Pseudocercosporella herpotrichoides (Fron) Deighton causes eyespot disease, an economically important stem base rot of cereals (Scott et al., 1975). Two varieties, *P. herpotrichoides* var. *herpotrichoides* Nirenberg corresponding to wheat-pathotype (W) and *P. herpotrichoides* var. *acuformis* Nirenberg also known as rye-pathotype (R), are widespread in temperate cereal-growing zones. *P. herpotrichoides* var. *herpotrichoides* isolates are highly pathogenic on wheat but are only slightly pathogenic on rye, whereas isolates of *P. herpotrichoides* var. *acuformis* are generally equally pathogenic on wheat and rye (Cavelier et al., 1991). Two other species *P. aestiva* Nirenberg and *P. anguioides* Nirenberg occur often in cereal-farmland. These species are known as non-pathogenic or antagonistic against *P. herpotrichoides*, respectively. When cultured in vitro, morphological differences of the conidia commonly permit the differentiation of the two varieties of *P. herpotrichoides*. Some isolates exhibit intermediate phenotypes and cannot be identified without physiological and plant pathological tests. *P. aestiva* with shorter and *P. anguioides* with longer conidia can be clearly separated from *P. herpotrichoides*-isolates Nirenberg, 1981, 1984, 1985).

In this study we wanted to determine the relationships between the two varieties of *P. herpotrichoides* and the non-pathogenic species. Two separate PCR-techniques were used which reveal banding patterns for characterisation of the isolates. One of it, the Random Amplified Polymorphic DNA - RAPD - (Williams et al., 1990; Welsh & McClelland, 1990), can differentiate closely related taxa, races or pathotypes (Fig. 1; Hering et al., 1992, 1993, 1994a). On the other side amplified ribosomal internal transcribed spacer (ITS) regions were subjected to restriction digestion. This method (Fig. 3) called Amplification Ribosomal DNA Restriction Analysis - ARDRA - (; Vaneechoutte et al., 1992; Claeys et al., 1993) revealed banding patterns that generally show no or slightly intraspecific variation (Hering et al., 1994a, 1994b). Some authors believe that different banding patterns reflect different species.

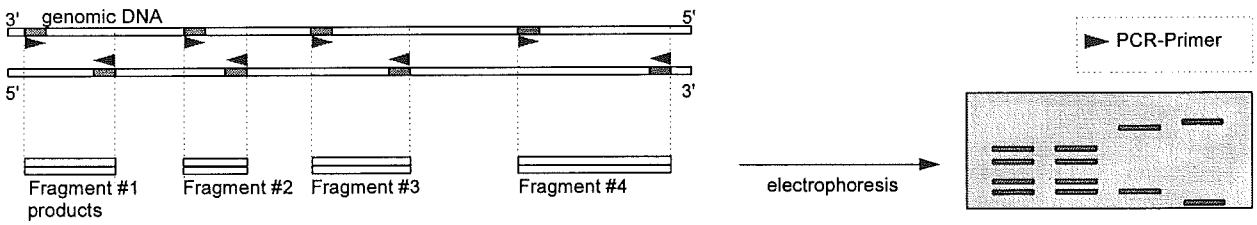

Fig. 1. Principles of Random Amplified Polymorphic DNA (RAPD)

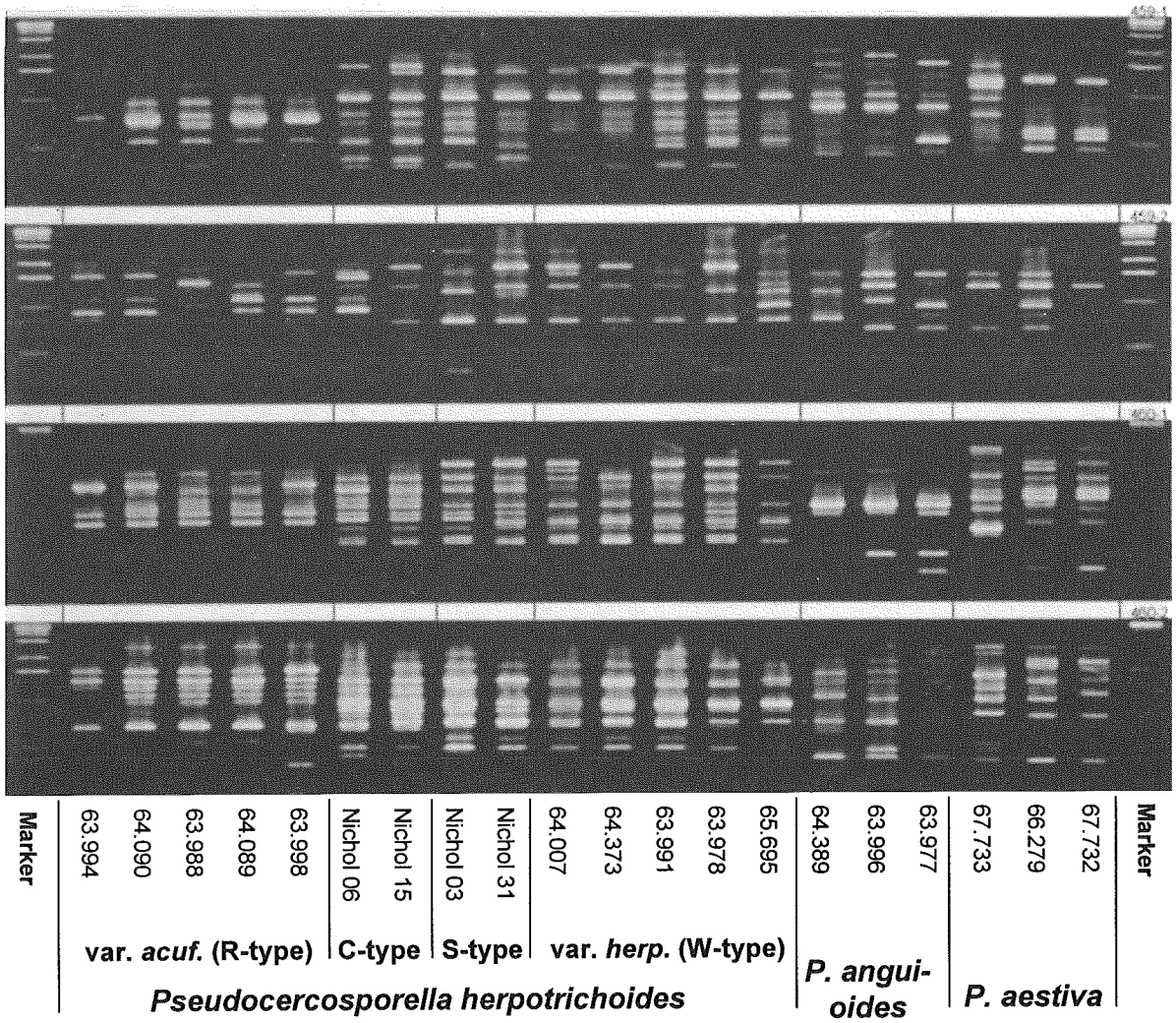

Fig. 2. RAPD Patterns. PCR conditions: 50 µl reactions containing 0.5 U Taq-Polymerase (Stratagene, Heidelberg), 200 µM dNTP (Böhringer, Mannheim), 1 µM primer, 4.5 mM $MgCl_2$, 5 µl PCR buffer (10x), 50 µl light mineral oil (Sigma). Thermocycler programming: initial denaturation for 60s at 95 °C, 37 cycles of denaturation for 45 s at 95 °C, annealing for 60 s at 40 °C (primer MB27, MB28) or 50 °C (primer MB01, MB16), extension for 90 s at 75 °C. Used Primer: a) MB05 (5'-gAgggTggCggTTCT); b) MB28 (5'-ggCATCggCC); c) MB06 (5'-gACAgACaGACAgACA); d) MB16 (5'-TCCTCCTCCTCCTCC). Electrophoresis for 2 h at 4 V/cm in 1.5 % agarosegel. Marker: 1 kb ladder (Gibco BRL).

The RAPD patterns (Fig. 2) obtained by oligonucleotide primers allowed the differentiation of the two varieties of *P. herpotrichoides*. Some monomorphic bands support their close relationship. Isolates of *P. herpotrichoides* var. *herpotrichoides* showing generally a higher degree of variability in their patterns than isolates of variety *acuformis* with nearly homogenous banding patterns. The occurrence of a sexual stage (Wallwork and Spooner, 1988) in *P. herpotrichoides* var. *herpotrichoides* – *Tapesia yallundae* Wallwork & Spooner – may be responsible for the greater variation due to recombination. C- and S-types, pathogenic on grasses, could not be clearly differentiated from each other and from the isolates of *P. herpotrichoides* var. *herpotrichoides*. *P. aestiva* and *P. anguioides* could definitely be distinguished by their banding patterns.

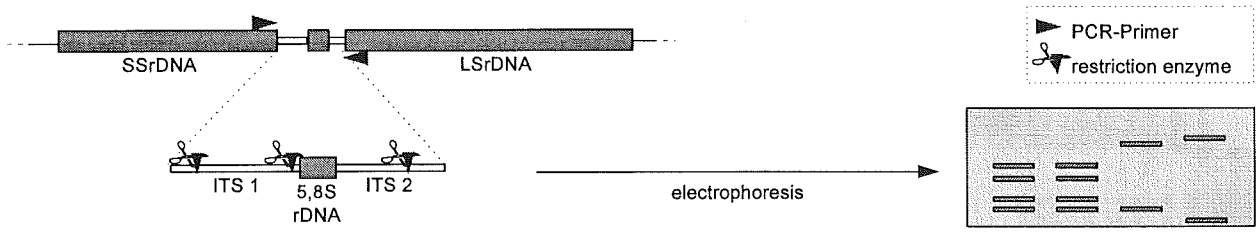

Fig. 4. Principles of Amplified rDNA Restriction Analysis (ARDRA)

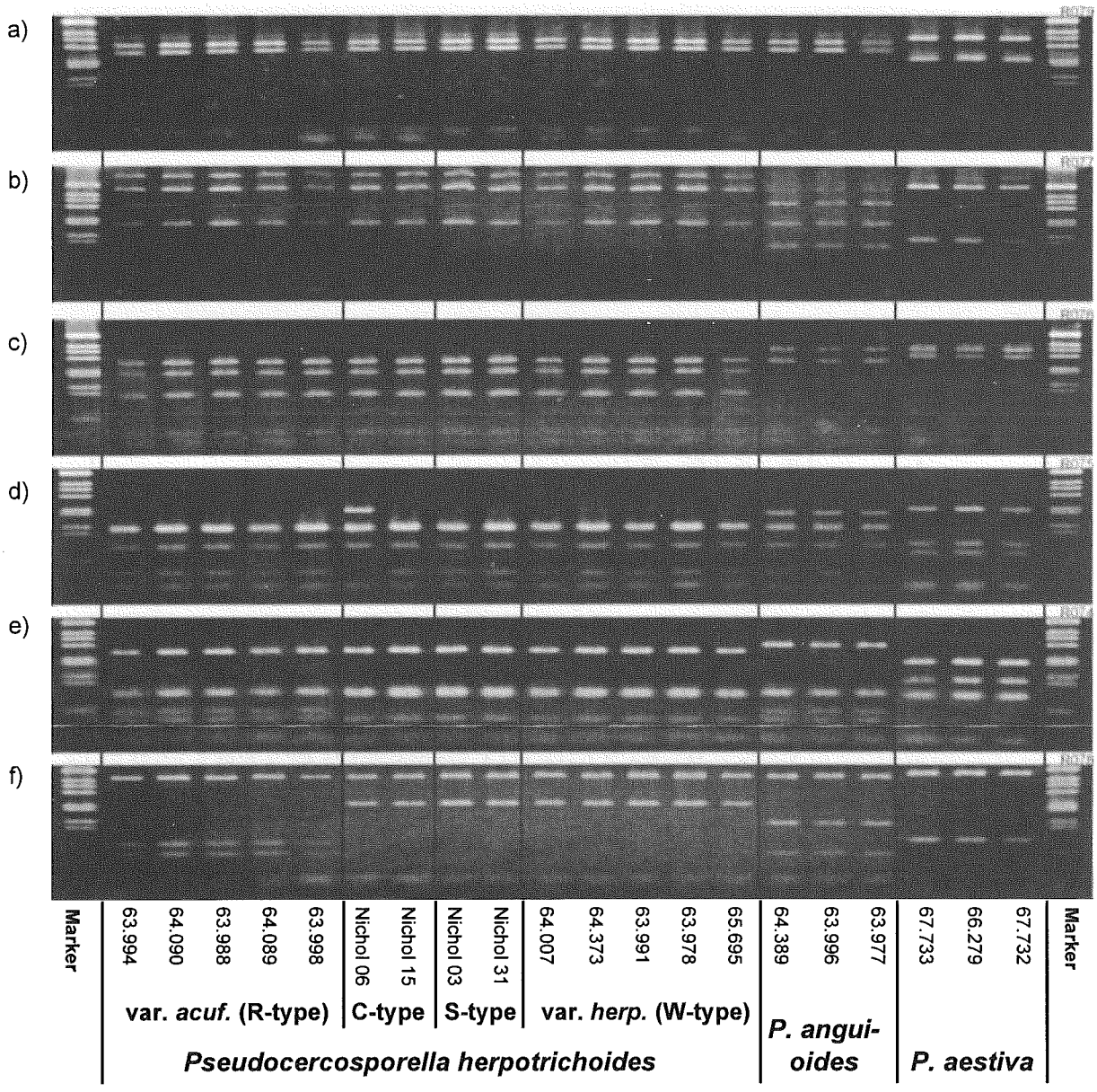

Fig. 3. ARDRA-Patterns. Double digestion of amplified ITS-region with 2 U of each enzyme (Gibco BRL) for 90 min at 37°C. Electrophoresis for 2.5 h at 4 V/cm in 4 % agarosegel. Marker: 1 kb ladder (Gibco BRL). Digestion with a) Bsp 143 I, b) Alu I, c) Hha I and Taq I, d) Msp I and Rsa I, e) Hae III and Hinf I, f) Bsi Z I.

Investigations with ARDRA (Fig. 4) lead to similar restriction patterns for *P. herpotrichoides* var. *herpotrichoides* and var. *acuformis*. The use of Hae I and Hinf I in double digestion or Bsi Z I singly allows the delimitation of the variety *acuformis* by additional restriction fragments. The banding patterns scored for *P. anguioides* showed some identical fragments with those of *P. herpotrichoides*. With the exception for the C-type strain "Nicholson 6" by Msp I + Rsa I digestion all enzymes for C- and S-types tested showing identical restriction patterns to *P. herpotrichoides* var. *herpotrichoides*.

The results suggest the close relationship within the varieties of *P. herpotrichoides* and a similarity of them to *P. anguioides*. *P. aestiva* can be differentiated from the others by all enzymes tested. This underlines the assumption of Nirenberg that *P. aestiva* actually belongs to a different genus. The present study demonstrates the usefulness of PCR-based techniques for determining genetic variability and for the classification of fungal isolates.

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Selective Amplification of rDNA Internal Transcribed Spacer Regions to Detect *Fusarium graminearum, F. cerealis* and *F. culmorum*

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Fusarium graminearum Schwabe [Teleomorph: *Gibberella zeae* (Schw.) Petch], *F. culmorum* (W. G. Smith) Saccardo and *F. cerealis* (Cooke) Saccardo (= *F. crookwellense* Burgess et al.) cause head blight or ear rot (Logrieco et al., 1990; Atanassov et al., 1994; Kovacs et al., 1994; Miller, 1994). These fungi are known to be closely related species that produce mycotoxins (Szecsi and Dobrovolszky, 1985; Hagen and Hagen, 1991; Lepschy, 1992; Suigura et al., 1993; Altpeter and Posselt, 1994; Miller, 1994; Beck, 1995; Lew, 1995). The safe identification is possible using the conidial morphology of strains cultivated in the dark on SNA (Nirenberg, 1990)

The banding pattern obtained from isolates of these species by Amplified Ribosomal DNA Restriction Analysis - ARDRA (Vaneechoutte et al., 1992; Claeys et al., 1993) - of the internal transcribed spacer (ITS) regions is identical underlining their close relationship (Fig. 1).

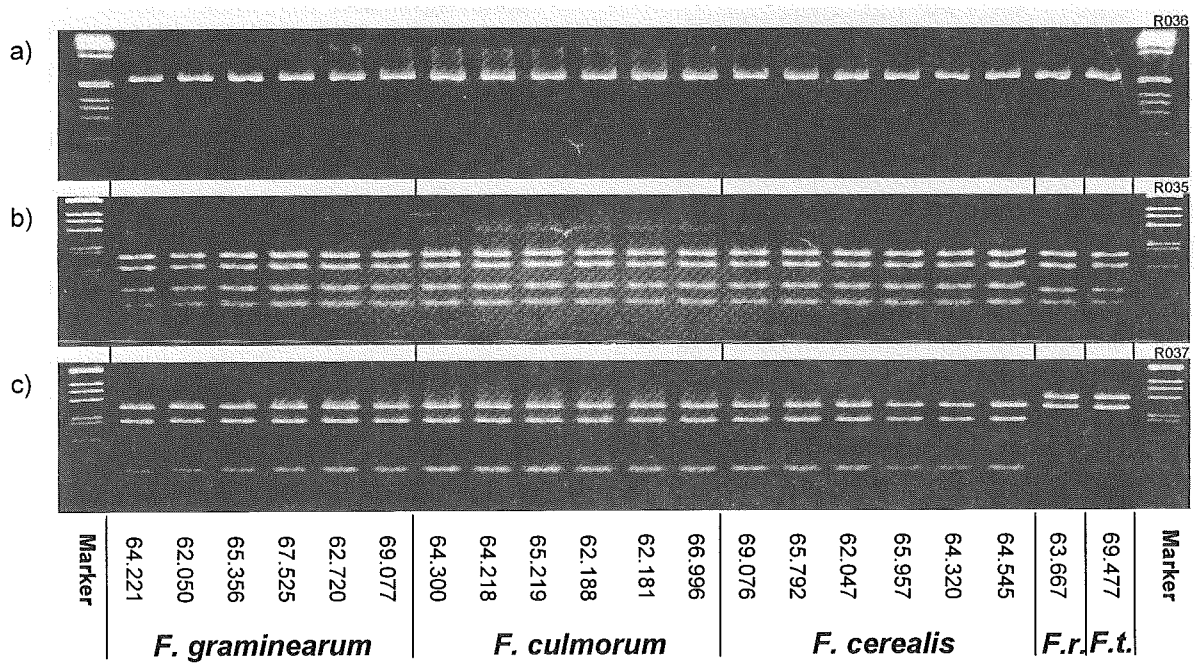

Fig. 1. ARDRA-Patterns. Double digestion of amplified ITS-region with 2 U of each enzyme (Gibco BRL) for 90 min at 37°C. Electrophoresis for 2.5 h at 4 V/cm in 4% agarosegel. Marker: 1 kb ladder (Gibco BRL). Double digestion with a) Msp I and Rsa I, b) Hae III and Hinf I, c) Hha I and Taq I.

Therefore additional studies with Random Amplified Polymorphic DNA - RAPD (Williams et al., 1990; Welsh & McClelland 1990) - were conducted. Although the species show similarities in their RAPD banding-patterns, they can be distinguished and identified by characteristic bands (Fig. 2).

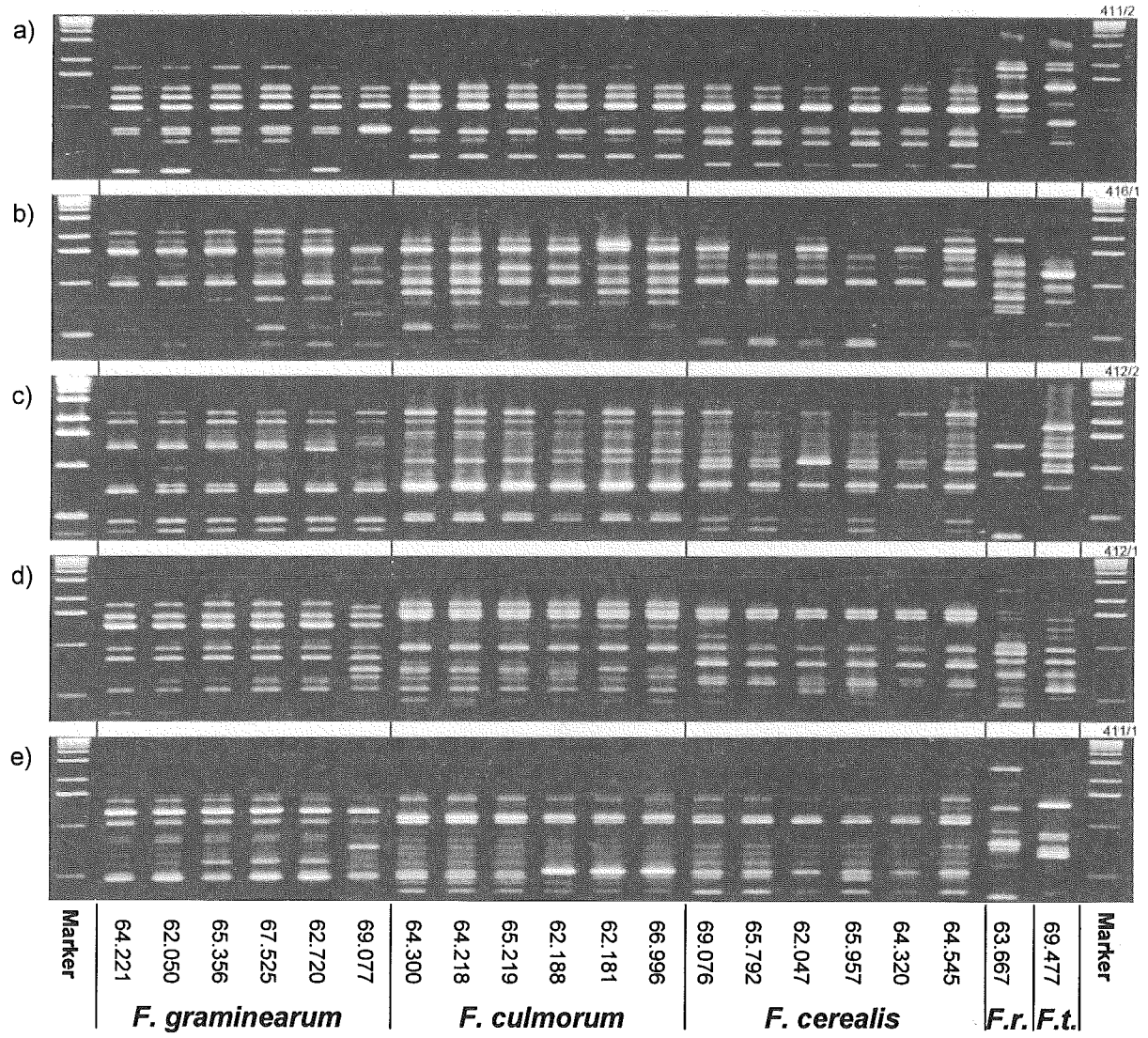

Fig. 2. RAPD Patterns. PCR conditions: 50 µl reactions containing 0.5 U Taq-Polymerase (Stratagene, Heidelberg), 200 µM dNTP (Böhringer, Mannheim), 1 µM primer, 4.5 mM $MgCl_2$, 5 µl PCR buffer (10x), 50 µl light mineral oil (Sigma). Thermocycler programming: initial denaturation for 60s at 95°C, 37 cycles of denaturation for 45s at 95°C, annealing for 60s at 40°C (primer MB27) or 50°C (other primers), extension for 90s at 75°C. Used Primer: a) MB05 (5'-gAgggTggCggTTCT); b) MB27 (5'-ACggTCTTgg); c) MB16 (5'-TCCTCCTCCTCCTCC); d) MB15 (5'-gTCgTCgTCgTCgTC); e) MB06 (5'-gACAgACaGACAgACA). Electrophoresis for 2 h at 4 V/cm in 1.5 % agarosegel. Marker: 1 kb ladder (Gibco BRL).

For the rapid diagnosis of infected plants or grains we have developed specific primers for the detection of fusarium head blight pathogens. The ITS regions of morphologically and molecularly identified isolates of head blight pathogens and other fusaria as controls were amplified using universal primers described by White et al. (1990). These amplicons, containing the transcribed 5.8S rDNA, were sequenced (sequences shown on Fig. 3). Specific primers (MB41: 5'-CgCgCCCCgTAAAAA; MB42: 5'-TTTggggAgTgCAgC) were selected from homogenous nucleotide-sequences for *F. graminearum, F. cerealis* and *F. culmorum*. The primers were tested on genomic DNA from *F. tumidum* (*F.t.*)', *F. robustum* (*F.r.*) as well as *F. avenaceum, F. sambucinum, F. venenatum, Pseudocercosporella herpotrichoides* and *Drechslera* spp. (not shown). Amplifications of genomic DNA from *F. graminearum, F. cerealis* or *F. culmorum* always resulted in a 300 bp fragment (Fig. 4). No characteristic 300 bp fragment was produced by amplification of the DNA from the other pathogens tested. On the basis of these results we shall test this diagnosis system on infected plants and grains.

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    0         1         2         3         4         5         6         7         8         9
    123456789012345678901234567890123456789012345678901234567890123456789012345678901234567890

040 TGAGGATTACCGAGTTTACAGGGTGCATTACCGAGTTTACAACTCCCAAACCCCTGTGAACATACCT-TATGTTGCCTCGGCGGATCAGCCCGCGCCCCG
002 ----------------------.A.G.....................................*.....................
526 ----------------------..A.....................................*.....................
073 ----------------------.A.G.....................................C.....................-----TT
036 -------------------------.....................................A..C...................
187 -------------------------.....................................TA.....................T.....

040 TAAAAAGGGACGGCCCGCCGCAGGAACCCTAAACTC---TGTTT-TTAGTGGAACTTCTGAGTATAAAAAACAAATAAATCAAAACTTTCAACAACGGAT
002 ......................................................................................
526 ......................................................................................
073 CCTC.C..A.............C.....................................A.....-...................
036 .....C.............CG...*.....................................A..C...................
187 .....C.............AG....*...-......TAA.....C...T..T...........A..C...................

040 CTCTTGGTTCTGGCATCGATGAAGAACGCAGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC
002 ......................................................................................
526 ......................................................................................
073 ......................................................................................
036 ......................................................................................
187 ......................................................................................

040 CGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCCAG---CTTGGTGTTGGG-AGCT-GCAGZCCTGCTGCACTCCCCAAA
002 ..........................................................CT..........................
526 ......................................................................................
073 ..........................................................................GTTTTAGT.AA--..........
036 ..................................................T.....T.............-..C..G..AAAC.GCG-T.........
187 ...TG......C.....................................X..CCGGGT...........G.--.C.GCTCT..CTCA.GGCGG.AGCC

040 TACATTGGCGGG-CACGTCGAGCTTCCATAGCGTAGTAATTTACATA-TCGTTACT-GGTAA-TCGGCGCGGG-CACGCCGCTAAAACCC-AACTTCCTG
002 .........N..N.....................Y.W.................T.........N..G..-........T....C..........T..
526 ..........N.....................................N.....N...T.....A......G...........T....N.......N....N
073 .TG........N.....................................V.....N.............C.ACGC..C.......T....C.............
036 .CG.....................................Y.....CACACACC...C..-.T........NT.N.N.......N.CN....N...C......TGN
187 CCT---------.-----------------------------------.--------.------.----------.----------------.---------
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Fig. 3. DNA sequences (5' to 3') of the ITS1, 5.8SrDNA, and parts of the ITS2-region. 040 *F. culmorum* (BBA 64.218), 002 *F. culmorum* (BBA 64.300), 526 *F. cerealis* (BBA 64.545), 073 *F. venenatum* (BBA 65.030), 036 *F. semitectum* (BBA 64.351), 187 *F. lateritium* (BBA 64.225). Selected primer sequences are underlined.

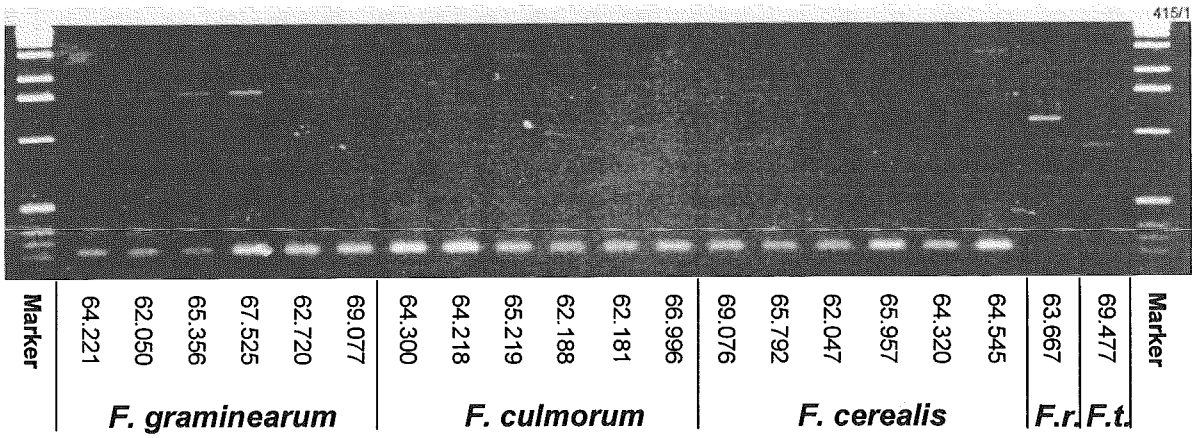

Fig. 4. Selective amplification of a rDNA fragment for detection of *F. graminearum*, *F. culmorum* and *F. cerealis*. PCR conditions: 50 µl reactions containing 0.5 U Taq-Polymerase (Stratagene, Heidelberg), 200 µM dNTP (Böhringer, Mannheim), 0.5 µM each primer (MB41: 5'-CgCgCCCCgTAAAA and MB42: 5'-TTTggggAgTgCAgC), 1.5 mM $MgCl_2$, 5 µl PCR buffer (10x); overlayed with 50 µl light mineral oil (Sigma). Thermocycler programming: initial denaturation for 60 s at 95 °C, 37 cycles of denaturation for 45 s at 95 °C, annealing for 60 s at 60 °C, extension for 90 s at 75 °C. Electrophoresis for 2 h at 4 V/cm in 1.5 % agarosegel

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SIDE-EFFECTS OF HERBICIDES ON THE RESISTANCE OF BARLEY TO MILDEW

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Introduction

Pesticides do not hit the target object only. They can exert an influence on many other species inclusive crop plants, their diseases, pests and their counterparts. Importance of pesticides side-effects has increased in the systems of integrated crop protection dealing with all the factors that could interfere with the diseases and pests incidence. Unfavourable pesticides side-effects can be reduced by carefully planned compensating measures (1).

Possible effects of herbicides on plant diseases were divided in five groups: inhibition of biochemical plant resistance mechanism, increased root exudates usually resulting in an increased damping-off, direct stimulation of the fungus (especially in the saprophytic phase), increased virulence or pathogenicity of the fungus and indirect stimulation of the pathogen by inhibition of its competitors (2).

Surprisingly many data are available on the effect of herbicides on the soil borne pathogenic fungi. First papers studied the direct inhibition of growth and spore production of fungi by herbicides (3, 4). Effect of herbicides on root rotting fungi was reviewed several times (5, 6, 7).

On the contrary only very limited number of papers is dealing with the effect of herbicides on the leaf diseases. Most extensive work on the side effects of herbicides on fungal diseases, especially mildew on wheat, was done by Heitefuss and his co-workers. Mildew development on spring wheat was inhibited immediately after the application of urea- and triazine-derivatives. Later the stimulation of mildew was observed. In field trials the yield of spring wheat was increased by the herbicides only if simultaneously mildew was controled by fungicides (8). Individual herbicides had dissimilar effect on different diseases of winter wheat (9, 10).

Increased mildew incidence on barley treated by the growth herbicides (11) and decreased one after dinosebacetate had been seen in herbicide-trials since fifties very often but without more precise assessment (Zemánek, pers. comm.,). Difenzoquat is known to possess some fungicidal activity against mildew. Milgo and Fademorf just compensated mildew increase caused by the growth herbicides (12, 13). No accurate data were available on practical importance of herbicides side-effect on barley mildew and on interactions of barley, varieties and annual conditions.

Material and Methods

Effect of the herbicides on barley mildew was studied in 1985-94 in field trials in randomized blocks and 4 replication of plot size 125 x 135 cm. Trials were sown in Ruzyně. Mildew

incidence was estimated 2-3 times between treatment and senescence on the upper fourth and third leaves. Analyses of variance and Duncan s test were calculated after angular transformation. In results original percentage data are presented. Herbicides were applied in highest recommended dosses. They contained following a.i.: mecoprop, MCPA, MCPA + flurenol + dicamba, difenzoquat, bentazone, chlorfenpropmethyl, chlorsulfuron, chlorsulfuron + thifensulfuron, barban, bromofenoxim. tribenuron, thifensulfuron, dinosebacetate, tralkoxydim, fluroxypyr and flamprop-M-isopropyl.

Results

To demonstrate the side-effect of herbicides on barley mildew we present the last assessment of the trial in 1985 in the stage 59 of decimal code. Very sensitive variety was "Slovenský dunajský trh", sensitive one "Favorit" intermediate and resistant varieties are represented by the mean of seven varieties in each case.

Tab. 1.

Effect of herbicides on the mildew severity
(second assessment 1.7. 1985)

variety- herbicide	very sensitive	sensitive	intermediate	resistant	mean
MCPA	26.7_a	13.5_a	6.8_a	3.2_a	6.9_a
mecoprop	23.6_b	8.1_a	5.4_{ab}	3.0_a	5.6_b
control	18.1_c	5.7_b	4.2_b	2.5_a	4.4_c
dinosebacetate	11.6_d	4.4_b	3.4_b	2.1_a	3.4_d.

The numbers followed by the same letter are not significantly different at P=O,O5

The conclusions of the experimental work 1985-1994:
- Dinosebacetate decreased mildew very remarkably, but its approval was canceled in the meantime and no other herbicide possesses comparable activity.
- Difenzoquat was less effective.
- The effect of other herbicides that control wild oats varied, usually it was statistically not significant.
- Sulfonylurea herbicides were without any effect but a more detailed studies are needed.
- Mildew was increased notably by the growth herbicides.
- Highest effect was observed in the combined herbicides (MCPA + dicamba + flurenol), little bit lower effect have MCPA and even lower mecoprop.
- Growth herbicides increased mildew incidence to the highest degree in the variety without any gene of resistance (Slovenský dunajský trh) and in other susceptible varieties.
- The effect in intermediate resistant varieties was often not

statistically significant, but the strong tendency for an increase remained.
- Growth herbicides increased mildew in the new highly resistant varieties too, but their effect was not statistically significant.
- Similar results were found on the differential varieties.
- Negative effect of the growth herbicides was very strong and long lasting. Mildew incidence was increasing till the senescence in the years with continuous development of the disease.
- No effect of herbicides was found in the years in which the plants had high field resistance from the onset of tillering and mildew appeared again till during senescence.
- In the greenhouse trials MCPA drastically increased mildew incidence on the third to fifth leaves already resistant in the untreated control.
- Growth herbicides never substantially increased the mildew incidence on varieties with resistance gene still effective, highest increase was found in varieties which possess only unspecific resistance and especially in young plants with beginning "adult plant resistance". For that, we came to the preliminary conclusion that growth herbicides do not affect the resistance controlled by specific resistance genes, but can substantially decrease the field resistance (adult plant resistance).
- The adverse effect of growth herbicides is very important in varieties with decreased monogenic resistance but still used on a large scale. In them the disease incidence was increased to the same level as that of very susceptible varieties treated with dinosebacetate.

Discussion

Results presented above just raise but don t answer several questions concerning the effect of the growth herbicides on the barley resistance to mildew:
- Do growth herbicides affect really only the non-specific part of the resistance and don t interfere with the gene-for-gene relationship?
- If growth herbicides don t affect the major gene, have not they any effect on minor genes too? Do they affect the mildew incidence on varieties with detected partial resistance? They affect adult plant resistance of very susceptible varieties. Is there any link between those two types of the plant resistance?
- If growth herbicides decrease the non specific part of the resistance, the mildew control depends just on the major gene. Does it result in an increased selection pressure and faster loss of the efficiency of major gene?
- Is the long lasting effect of growth herbicides the result of such a long lasting biochemical changes in the treated plants, or just the result of a short effect which increase mildew incidence and as a consequence the further development of the epidemic is faster?
- If the growth herbicides decrease the non-specific resistance, do they increase the display of the specific resistance more visible? Could they help to reveal specific resistance?
- Decreased resistance of barley against mildew seems to be a result of non-specific plant response. Is the barley resistance against other diseases also decreased?

- Is the principle of described phenomena of induced sensitivity analogous to that of activation of induced resistance in plants, but just in a negative sense?

Because of limited choice of the herbicides available this side-effect was sooner accepted without more attention of practical farming. Nowadays many herbicides are on the market and the cost of the treatment does not differ so much as used to be, so the undiserable side-effect of growth herbicides should be an important decision factor in weed control. Growth herbicides are recommended for the highly resistant varieties only.

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ACCUMULATION OF GENES FOR ENHACEMENT OF INCOMPLETE RESISTANCE TO *PUCCINIA RECONDITA TRITICI*

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Introduction

Results of breeding for the resistance altered according to possibilities, mostly connected with choice of the resistant parents (alien species, resistant varieties out of production and fortunately very often widespread varieties) (6). Pyramiding, or accumulating genes for the specific resistance was not successful because of practical reasons: how to select in progenies and to keep all genes from the initial parents? Transmission of genes from alien species, mostly causes expression of negative characteristics controlled by linked genes. Determination and transmission of genes in the interaction until now is most successful from the aspect of durability and long term protection level (4). Nowadays results suggest the more accurate way: combination of the parents with high level of incomplete resistance to increase that characteristics or to get the complete resistance to the present population of pathogen. It agrees with the most important point in breeding for the resistance: durability (2). High level of incomplete resistance is sufficient to avoid epidemics development of pathogens specially in semiarid regions (3).

This paper demonstrates the accumulation of genes which control this characteristics, by the testing of lines created from the progenies of the same combinations but partially in opposite direction from the future one.

Material and Methods

Lines from the progenies of complex crossings using the lines and varieties with different levels of incomplete resistance in field, and complete or incomplete resistance in the seedling stage under controlled conditions (Table 1) were studied for estimating the effect of gene accumulation.

Novosadska rana 2 was chosen as susceptible control widespread variety (sometimes it covers over 60 % of the area under the wheat in Yugoslavia), which had remarkable influence on the creating of the present *Puccinia recondita* population.

Tab. 1

Genealogies
series 1
**
(Loznička x Nova posavka) x Rana niska) x (NS 2853 x Zg-1-628-/77)
series 2
*
(Tobari 66 x Kavkaz) x (Nova banatka x NS 3143) x Rana niska) x (NS 2568/6 x MV 08/78)

Repeated estimation of the infection intensity according to Cobb scale (1963) in the comparative trials (1x5 m^2 in four replications) were the basis for AUDPC values counting (1) in 1993 and 1994, while maximal intensities were determined in 1991 and 1992.

Under the controlled conditions (15 °C day and 15 °C night, with constant relative humidity of the air of 60 %) the length of the latent period 50 and infection efficiency were estimated in the seedling stage (3). Inoculation was performed with the culture in the type of the race 77 which is virulent to all series of Lr genes except Lr 9, Lr 19 and Lr 24.

Results and Discussion

The lines from the same combination of the first series, expressed higher resistance level in the field conditions in comparison with those of the series two. In 1992 and 1993 these differences were not exposed because of inadequate conditions for development of *Puccinia recondita.* In 1994, 59 days from March to middle of June were rainy so the intensity of attack was stronger, material was differentiated according to the resistance. If we assume that mos susceptible lines in field conditions from series one (NS 2-2675, NS 2-2675/5) and two (NS 2-2742, NS 3160, NS 2-2740) carry only one gene for the resistance different than these in Novosadska rana 2, it is evident that the lines NS 2-2675/1, NS 2-2675/2, NS 2-2675/3, NS 2675/4 and NS 2-2741 carry at least two, and the line NS 2-2739 three additional genes (Tables 2 and 3). It is abvious that by interaction of these genes resistance is expressed in the field. As no parent line expressed such resistance in field, it is clear that we have new different (dependant of line) combinations of resistant genes. Hypersensitive reaction in the seedling stage was expressed from the interaction of genotypes NS 2-2675/3, NS 2-2675/4, NS 2739 and NS 2-2740. Lp 50 and infection efficiency values proved the higher differences between estimated lines in resistance combinations genes than can be concluded only according to field estimation data (Tab. 3). Because of insufficient number of applied isolates different according to the virulence, field susceptibility and seedling resistance of some parents, it is impossible to ascertain complete resistance from the combination of genes for the incomplete resistance (5).

Tab. 2: Infection intensities with *Puccinia recondita tritici* in 1991-1994

line/year and screening date	1994	1. 31.05. 2. 08.06. 3. 21.06.	1993	1. 01.06. 2. 07.06. 3. 14.06.	1992	1991
		1, 2, 3		1, 2, 3		
series 1 *						
NS 2-2675		T, 5, 30		0, 0, T	0	0
NS 2-2675/1		0, 0, T		0, 0, T	0	0
NS 2-2675/2		0, 0, T		0, 0, T	0	0
NS 2-2675/3		0, T, T		0, 0, T	0	0
NS 2-2675/4		0, T, T		0, 0, T	0	0
NS 2-2675/5		0, T, 20		0, 0, T	0	0
series 2 **						
NS 2-2738		0, T, 30		0, 0, T	0	T
NS 2-2739		T, 5, 5		0, 0, T	0	T
NS 2-3160		0, T, 30		0, 0, T	0	
NS 2-2740		0, T, 30		0, 0, T	0	0
NS 2-2741		T, 5, 20		0, 0, T	0	T
NS 2-2742		0, T, 40		0, 0, T	0	T
Novosadska Rana 2		5, 40, 80		T, T, T	20	35

Tab. 3: Characters of the incomplete resistance and AUDPC values in 1993 and 1994

line/parameter year	IT	LP 50	IF	AUDPC 1994	AUDPC 1993
NS 2-2675	4	9,1	5,6	254,5	3,5
NS 2-2675/1	4	10,0	3,0	7,5	3,5
NS 2-2675/2	4	10,3	6,0	7,5	3,5
NS 2-2675/3	;			17,5	3.5
NS 2-2675/4	0			17,5	3,5
NS 2-2675/5	4	8,3	3,5	141,0	3,5
NS 2-2738	4	8,5	4,0	206,0	3,5
NS 2-2739	;23	8,5	6,0	92,0	3,5
NS 2-3160	;			206,0	3,5
NS 2-2740	;			206,0	3,5
NS 2-2741	4	8,8	2,7	192,7	3,5
NS 2-2742	4			271,0	3,5

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USE OF GENETIC MARKERS TO CHARACTERIZE SELFED PROGENIES OF A *HORDEUM BULBOSUM X HORDEUM VULGARE* HYBRID

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Introduction

Cultivated barley is one of the oldest crops. One important task in barley breeding is to improve resistance towards a variety of diseases. One approach to this end is the species hybridization between tetraploid wild *(Hordeum bulbosum)* and cultivated barley *(Hordeum vulgare)*. *H. bulbosum* displays resistance to a number of important diseases which can be transferred into cultivated barley. The production of such hybrids is hampered, e.g., by the elimination of *H. bulbosum* chromosomes during embryo development and by sterility of F_1 hybrids (9). Szigat and Szigat (11) succeeded to overcome some of the difficulties and obtained tetraploid hybrids. Most of the hybrids were resistant to powdery mildew, leaf rust and viruses. These hybrids provide a basis for transferring desirable traits from wild into cultivated barley. The aim of this present project is to characterize the selfed progeny from the tetraploid *H. bulbosum x H. vulgare* hybrid in their cytology, isozyme patterns, resistance for mildew, leaf rust and viruses. In additon, isozyme markers are used to search for linkages to resistance genes.

Material and Methods

The hybrid 2BW1/4 (GB15) was derived from the cross between wild *(H.bulbosum)* and cultivated barley *(H.vulgare)*. We obtained 126 selfed progeny and these, together with `Borwina`, `Haisa` and A42` were characterized using following methods:

Cytological analysis during the mitotic metaphase were carried out in root tip cells.

Powdery mildew (*Erysiphe graminis* sp. *hordei*) was assessed by placing detached leaves onto benzimidazole agar and inoculating them with isolates kindly supplied by Fischbeck, (TU Munich and Walther, BAZ Aschersleben) using the inoculation box. The resulting infection types were scored on a 0 to 4 scale (8). `Haisa` was used as the susceptible type. The isolates were also tested in the field on older plants.

Leaf rust (*Puccinia hordei* Otth.) resistance was assessed as applied to mildew above. The detached leaves were inoculated with the isolates from Walther (BAZ Aschersleben) using a preval sprayer. The infection types are scored on a 0, 0n, oc, 1 to 4 scale (3). `Borwina` was used as a susceptible type (0n without mildew, but necrosis 0c no mildew, but chlorosis)

Virus tests for resistance to Barley Mild Mosaic Virus (BaMMV) and Barley Yellow Dwarf Virus (BaYDV) were carried out by the Institute of Epidemiology (BAZ Aschersleben). The plants were inoculated mechanically in the greenhouse as described (10).

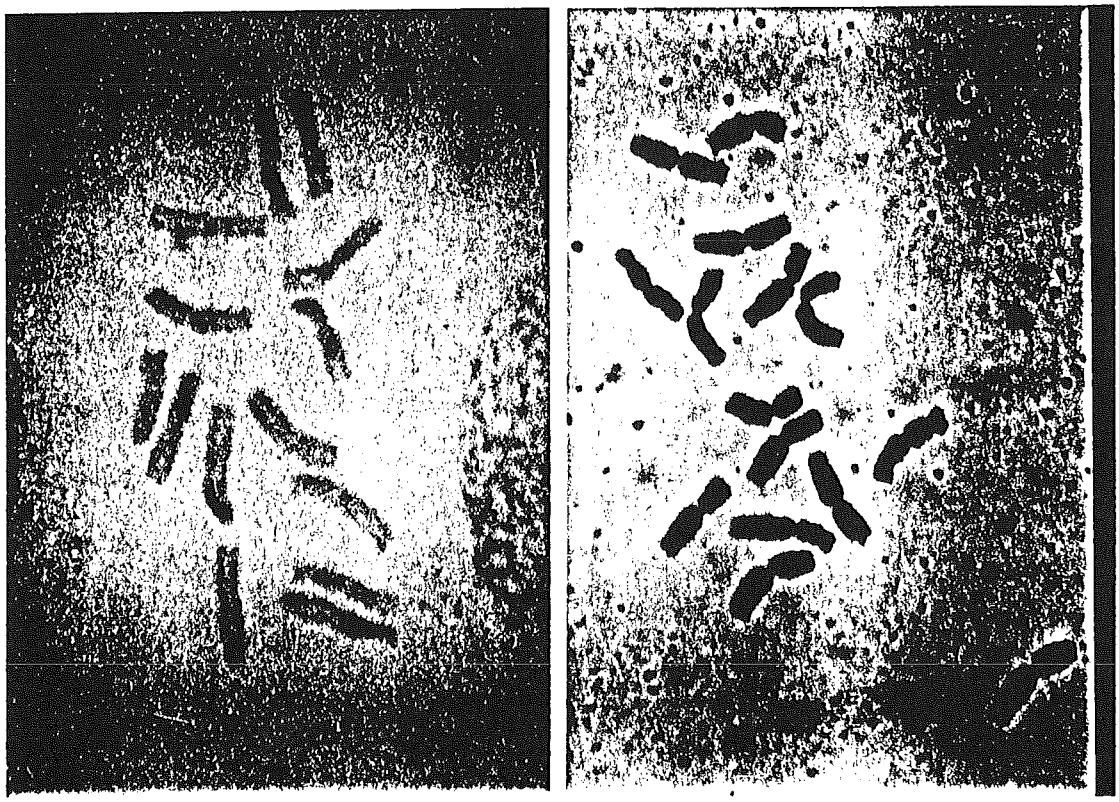
Isozyme analysis by polyacrylamide gel electrophoresis (PAGE) was carried out as described (6). Extracts of `Borwina`, `A42` and the hybrid GB15 were included in each gel as a standard to compare their bands for migration distance and enzyme activity. Four enzyme systems (glutamate oxaloacetate transaminase (GOT), 6-phosphogluconate dehydrogenase (6-PGD), alcohol dehydogenase (AADH-NAD^+) and leucine aminopeptidase (LAP) were evaluated in this study.The chromosomal localization of isozyme loci in the progeny was determined by the use of wheat-barley addition lines.

The following morphological characters were analysed trying to find morphological markers: growth habit, plant height, length of flag leaf, length of second and third leaves, length of awns, length of glume (spikelet in mid or on side of ear), and length of ear excluding awns, pith in cross section of straw (halfway between base and stem of ear) , hairiness of the flag leaf, of second and third leaves and anthocyan pigmentation of awn and of anthers.

Results and Discussion

Cytological analysis

The 126 selfed progeny were analysed as follows: 17,5% had 14 chromosomes, 65% had 15 chromosomes, 2,3% had 21 chromosomes and 15% had 28 chromosomes, (Fig. 1).



a) b)

Fig. 1 Mitotic metaphase chromosomes a) 14 chromosomes and b) 15 chromosomes

Morphological markers

Two plants possessing 14 chromosomes displayed the bulbosum type of hairiness and length of leaves. These plants were susceptible to all three pathogens tested, however, they showed presence of *H.bulbosum* genes in LAP and 6-PGD zymograms. Two other lines with 14 chromosomes also showed the presence of hairs and their growth habit was similar to that of cultivated barley. Resistance was not observed and isozyme analysis showed no difference to normal barley.

Resistance to powdery mildew, leaf rust and viruses

The results obtained using *in vitro* and field tests are presented in Table 1. Six genotypes with 14 chromosomes which were resistant to mildew using in vitro tests were susceptible in the field. By leaf rust, nine plants with 14 chromosomes were resistant (*in vitro*) whereas under field conditions only five plants remained resistant. Eight plants with 14 chromosomes were resistant to BaMMV and four plants to BaYDV (ELISA tests).

Table 1: **Testing for powdery mildew, leaf rust and viruses (*in vitro* and in the field)**

Number of plants	Number of chromosomes	Number of plants resistant to *E.graminis*		Number of plants resistant to *P.hordei*		Number of plants resistant to BaMMV		Number of plants resistant BaYDV-ELISA
		in vitro	field	*in vitro*	field	visually	ELISA	
22	14	6	0	9	5	8	8	4
82	15	25	17	15	11	15	14	9
3	21	2	0	1	2	1	1	1
19	28	19	19	16	12	13	15	6

Isozyme analysis

We have examined isozyme patterns of four enzymes involved in barley metabolism. Ten loci could be identified in the four characterized enzyme systems. Table 2 shows the results obtained.

Table 2: Isozyme systems and their diagnostic value for *H.bulbosum* chromosomes

Enzyme system	Gene	*H.bulbosum* chromosomes	Plants with 14 chr.	Plants with 15 chr.	Plants with 21 chr.	Plants with 28 chr.
Glutamate-oxalacetate	*Got 1*	$6H^{bulb}$	-	-	+	+
transaminase (GOT)	*Got 2*	$6H^{bulb}$	-	-	+	+
E.C. 2.6.1.1	*Got 3*	$3H^{bulb}$	-	-	+	+
6-Phosphogluconate	*Pgd 1*	$7H^{bulb}$	+	+	-	+
dehydrogenase (6PGD)	*Pgd 2*	$6H^{bulb}$	+	+	+	+
E.C. 1.1.1.43						
Aromatic alcohol	*Aadh 1*	$7H^{bulb}$	+	+	-	+
dehydrogenase	*Aadh 2*	$6H^{bulb}$	-	+	-	+
(AADH-NAD$^+$)	*Aadh c*	not determined	-	+	-	+
E.C. 1.1.1.90						
Leucine amino-	*Lap 1*	$6H^{bulb}$	+	+	-	+
peptidase (LAP)	*Lap 2*	$4H^{bulb}$	+	+	+	+
E.C. 3.4.1.1	*Lap 3*	$1H^{bulb}$	-	+	+	+

(+) plants showed bulbosum-spesific bands (-) plants did not show bulbosum-spesific band

The data obtained from the progeny of 2BW1/4 revealed that, besides entire chromosomes, chromosomal segments can be transferred from *H.bulbosum* into cultivated barley. In addtion, we have demonstrated that resistance to BaMMV and BaYDV was introgressed from *H. bulbosum* into *H. vulgare* (Table 1). The *Aadh c* was detected , but the chromosomal localization of these could not be determined. On the basis of homoeological relationships between *Triticeae* species markers such as isozyme loci allow the identification of specific chromosomal segments which have been transferred from wild species into cultivated crops. Once the homoeology between *H. bulbosum* and *H. vulgare* chromosomes and the syntheny of genes has been specified a detailed characterization of *H .bulbosum / H. vulgare* hybrids in terms of transferred chromosomal segments will be feasible. Our results demonstrate that the identification of such segments by genetic markers may provide a valuable tool in introgression genetics in barley.

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MOLECULAR ASPECTS OF THE GENE-FOR-GENE-TYPE INTERACTION OF BARLEY AND *RHYNCHOSPORIUM SECALIS*

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Introduction

The imperfect fungus, *Rhynchosporium secalis* (Oudem.) J.J. Davis, is the causal agent of a foliar disease of barley. In the host plant, several major genes for resistance were detected one of which, *Rrs1*, was mapped to chromosome 3 (1). This gene controls the interaction with different fungal races postulated to possess the complementary avirulence gene, *AvrRrs1*. An elucidation of the molecular basis of this gene-for-gene-type interaction is the prerequisite to understand the recognition and signal transduction processes leading to resistance of barley to *R. secalis* and to develop new strategies to improve resistance in transgenic plants.

A common model presumes that an avirulence gene controls the synthesis of a race-specific elicitor that triggers plant defense reactions only in host cultivars expressing the corresponding elicitor receptor. This receptor (or, alternatively, a downstream component of the signal transduction pathway) is anticipated to be encoded by the complementary resistance gene (specific elicitor/receptor model of the gene-for-gene hypothesis; 2). Therefore, the strategy followed to isolate the *AvrRrs1* gene from *R. secalis* aims at the identification of a fungal elicitor with activity specifically on barley cultivars carrying resistance gene *Rrs1* (3).

R. secalis grows extracellularly in the subcuticular region of barley leaves. Collapsing epidermis cells are the earliest disease symptoms that become visible during the first days post inoculation on resistant as well as susceptible plants. While fungal development is stopped by unknown mechanisms on resistant plants, fungal mycelia spread on susceptible plants to form a dense stroma. After about 2 weeks post inoculation necrotic lesions develop due to the collapse of mesophyll cells (4). The fungus does not significantly degrade plant cell walls. Therefore, diffusible toxins were supposed to be involved in the killing of cells in order to release nutrients from the plant. Fungal race US238.1 is avirulent on barley cultivars carrying resistance gene *Rrs1*. From culture filtrates of this race, a small family of nonspecific toxic proteins (necrosis inducing proteins NIP1, NIP2, NIP3; $M_r < 10$ kDa) had been isolated (5) two of which (NIP1, NIP3) are stimulators of the plant plasmalemma H^+-ATPase (6). In addition, NIP1 was found to elicit defense responses, the accumulation of mRNAs encoding peroxidase and PR proteins of the PR-5 class (thaumatin-like), specifically in *Rrs1*-barley cultivars (7). Therefore, NIP1 was postulated to be the candidate for the product of *AvrRrs1*.

Material and Methods

The experimental system consists of 4 barley cultivars and 12 races of *R. secalis*. Cv. Hannchen lacks any known resistance genes against *R. secalis* while cv. Turk possesses resistance genes *Rrs1*, *Rrs5* and *rrs6* (1). Cv. Atlas carries resistance gene *Rrs2* and the near-isogenic cultivar Atlas 46, in addition, *Rrs1*, which was introgressed from cv. Turk into the genetic background of cv. Atlas by a crossing and backcrossing program (8). Six fungal races (US238.1, AU1, AU4, AU5, UK7 and CV3) are avirulent on the *Rrs1*-barley cultivars and therefore presumed to carry the avirulence gene, *AvrRrs1*. Five fungal races (US262, AU2, UK5, UK8 and CV1) are virulent on all 4 barley cultivars. One race (AU3) is avirulent on

Rrs1-barley and cv. Atlas. Therefore, its interaction with the host appears to be not or not only controlled by resistance gene, *Rrs1* (9).

Based on the N-terminal amino acid sequence of NIP1 from race US238.1, degenerated oligonucleotides were synthesized and used to isolate *nip1* genomic and cDNA clones (9, 10). Three different approaches were followed to characterize the role of the *nip1* gene in the pathogen/plant interaction: physiologic complementation by mixing purified NIP1 with spores of a virulent fungal race prior to inoculation, genetic complementation by transforming a virulent fungal race with the *nip1* gene and gene replacement *via* homologous recombination by transforming an avirulent fungal race with an inactivated *nip1* gene.

Results

The *nip1* gene was found to be present in a single copy in the genome of *R. secalis*. The gene consists of two exons and a 65 bp intron and encodes an 82 amino acid preprotein. Comparison of the deduced amino acid sequence with the N-terminal sequence of the purified protein revealed a 22 amino acid secretory signal peptide. The mature protein has 60 amino acids, 10 of which are cysteines. While the protein sequence does not show similarity to other proteins in the databases, the cysteine pattern is related to another group of fungal proteins, the hydrophobins (9, 10).

The *nip1* cDNA from race US238.1 was used as a probe to analyze genomic DNA as well as RNA from all other fungal races. It turned out that all races that are avirulent on *Rrs1* barley cultivars carry and express the *nip1* gene, while all races lacking the gene are virulent. In addition, the presence of NIP1 in culture filtrates of all avirulent races was demonstrated using an antiserum raised against NIP1 from race US238.1. However, the *nip1* gene was also present and expressed in the virulent fungal race AU2. When NIP1 was isolated from all *nip1*-carrying races and tested for elicitor activity, the protein from races AU2 and AU3 proved to be elicitor-inactive. The protein from all other races induced PR protein mRNA accumulation indicating that virulence of *R. secalis* on *Rrs1* barley plants is achieved either by complete deletion of the *nip1* gene or by synthesis of an elicitor-inactive gene product (10).

Cloning of the *nip1* gene from races AU2 and comparison with the gene from race US238.1 showed strong sequence conservation. Over a stretch of 1.6 kb only 8 nucleotide differences were detected, 3 of which lay in the 5' untranslated region, 4 in the coding region and 1 in the 3' untranslated region. Therefore, the *nip1* coding region was analyzed in detail by direct sequencing of PCR-amplified DNA from all *nip1*-carrying races. As a result, 4 groups of alleles were identified. The *nip1* genes from races US238.1 and UK7 are identical. The allele present in races AU1, AU4, AU5 and CV3 differs from the former at 3 nucleotide positions which all lead to the incorporation of a different amino acid into the gene product without affecting its elicitor activity. The alleles encoding elicitor-inactive proteins each contain an additional fourth nucleotide alteration. These lead to a Ser→Pro exchange at position 45 of NIP1 from race AU3 and to a Gly→Arg exchange at position 67 of NIP1 from race AU2, respectively (10).

An avirulence gene and its product control the expression of incompatibility in interactions with the complementary plant resistance gene. In order to demonstrate this function, purified NIP1 from race US238.1 was mixed with spores of the virulent race AU2 and the avirulent race US238.1 prior to inoculation (physiological complementation). Only the originally compatible interaction of race AU2 with *Rrs1* plants, not however with *rrs1* plants, was shifted to incompatibility in the presence of NIP1 during infection (10). This result was corroborated after a transformation system had been established for *R. secalis* using as selection marker the hygromycin resistance (*hph*) gene from *E. coli* under the control of promotor and termination sequences from *Aspergillus nidulans* genes (11). When race AU2 was transformed with the *nip1* gene from race US238.1 (genetic complementation),

transformants were isolated that are avirulent on *Rrs1* plants while their virulent phenotype on *rrs1* plants remained unaffected (10). Both complementation experiments demonstrated that the *nip1* gene and its product, respectively, are sufficient for the expression of the avirulence phenotype on plants carrying resistance gene *Rrs1*.

For gene replacement studies, the *nip1* gene from US238.1 was inactivated by inserting the *hph* gene into a restriction site within the *nip1* coding sequence. By homologous recombination this construct was introduced into the genome of the avirulent race UK7 and a transformant was isolated that is virulent, both on *Rrs1* and *rrs1* plants (12). This demonstrated that the *nip1* gene is not only sufficient but also necessary for the expression of the avirulence phenoytpe and that it is identical with avirulence gene, *AvrRrs1*. In addition, preliminary results indicate that the virulence of these transformants is reduced compared to the UK7 wildtype. This is to be expected if the *nip1* gene has also an important function in virulence expression as presumed from its role as a toxin.

Discussion

The *nip1* gene from the barley pathogen, *R. secalis*, is the avirulence gene complementary to resistance gene, *Rrs1*. The gene product, NIP1, has two functions in the interaction with the host. It is a non-specific toxin on all host cultivars and a race-specific elicitor in those barley cultivars carrying the resistance gene. This means that a fungal virulence factor is utilized by the host to recognize the pathogen. From the data presented it can be concluded that the fungus has two possibilies to avoid recognition, complete deletion of the *nip1* gene or point mutation of the gene to produce an elicitor-inactive protein. It will be interesting to analyze whether these mutated proteins still retain their toxic activity.

Complete deletion was also found for the avirulence gene *Avr9* from the tomato pathogen *Cladosporium fulvum*. All races virulent on plants carrying resistance gene *Cf9* lack this gene (13, 14). In comparison, for the *Avr4* gene from *C. fulvum* a number of alleles were identified that differ in single nucleotide positions which render the gene products elicitor-inactive (15, 16). However, in contrast to the *nip1* gene from *R. secalis* the avirulence genes from *C. fulvum* appear to be dispensable. No differences in growth and development of the fungus could be detected in *Avr9* gene disruption transformants or in a strain carrying a natural frame shift mutation in the *Avr4* allele. For both genes, their intrinsic function is unknown. The cultivar-specific avirulence gene *AVR2-YAMO* from the rice pathogen *Magnaporthe grisea* encodes a protein with a sequence motif found in neutral Zn^{2+} proteases from different organisms (16). Although biochemical proof for the enzyme activity is still missing, mutations in the protease motif lead to a loss of the avirulence function. This indicates that the enzyme activity rather than the structure of the gene product may be necessary and possibly involved in the formation of a still unknown elicitor molecule.

In future experiments, two different strategies will be followed to identify and isolate the NIP1 receptor from barley. Binding studies using the elicitor as a ligand are anticipated to reveal the number of NIP1 receptor types and, thus, to answer the question whether the elicitor activity and the H^+-ATPase stimulatory activity of NIP1 are mediated *via* the same membrane protein. Isolation of the receptor(s) can then be achieved by affinity chromatography after chemical cross-linking using a NIP1-derivative carrying an affinity ligand such as biotin. Alternatively, expression cloning of a barley cDNA library in mammalian COS cells (17) is expected to lead to the isolation of a NIP1 receptor cDNA clone. Expression of this cDNA in transgenic plants will then prove whether the receptor is encoded by resistance gene *Rrs1*.

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INVESTIGATION OF ENZYME ACTIVITIES - A POSSIBILITY TO DETERMINE THE RESISTANCE OF BARLEY GENOTYPES TO *DRECHSLERA TERES* (SACC.) SHOEM.

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Introduction

Net blotch of barley caused by the fungus *Drechslera teres* (Sacc.) Shoem., is spread worldwide and has become increasingly important in cereal production over the past 15 years. Since the resistance level of barley cultivars grown presently is very low, it is urgently required to search for new and effective ways to improve resistance.
The control of this pathogen is considerably affected by the appearance of different races. There are two forms of the pathogen with different symptoms, *D. teres* forma *teres* (net-type) and *D. teres* forma *maculata* (spot-type).
Therefore, it is essential that a simple and precise test system suitable for the comprehensive characterization of isolates and evaluation of the resistance of cultivars is developed. Due to the widely varying symptoms caused by *Drechslera teres*, net blotch is frequently mistaken for other pathogenous or genetically caused leaf spotting diseases.

The following questions were studied in our research project:

1. Does the enzyme activity determined in vitro or in vivo allow a characterization of the available *Drechslera teres* isolates?
2. In how far do fast and simple methods to determine the extracellular enzyme activity of the pathogen allow an objective assessment of the resistance of barley genotypes?
3. Does this method also allow to distinguish the pathogenous symptoms from the physiological leaf spots occurring frequently under field conditions?

Material and Methods

Different *D. teres* isolates were used for the studies, including three spot types *(D.t. 85; D.t. 140; D.t. 230)* and three net types *(Re Am; Re 100; D.t. 265a)*.
The cellulase and xylanase activities were measured both in vitro (filtrate of barley leaf extract) and in vivo (detached leaf test). The aggressiveness of the isolates was determined on summer barley (cultivar 'Karat') on the basis of the intensity of attack (Tab. 1).
The assessment of the resistance of various barley genotypes was performed in vivo on detached leaves in accordance with HARTLEB et al.(1). The leaf pieces were inoculated by spraying with a conidial suspension of the aggressive isolate *Re Am* (density of 3000 conidia per ml). After six days the leaves were scored and then crushed in a mortar on ice using an extraction buffer and the enzyme activity measured in microtitre plates according to the method described by WIRT and WOLF (2). The absorbance was assayed spectrophotometrically at 600 nm. Infected leaves from a cultivar trial on the Aschersleben site which were assessed according to the TEKAUZ (3) scoring scale,were also used as test material.

Infected plants from the institute's nurseries in Aschersleben were used for diagnostic studies. After visual assessment an attempt was made to attribute the different symptoms to pathogens or physiological causes. During the 1994 vegetation period we repeatedly took samples and determined their enzyme activity after suitable processing.

Results and Discussion

The results indicate a close relation between the enzyme activity and the intensity of attack for the *Drechslera teres* isolates tested. The isolates selected represent three different levels of aggressiveness (Tab.1).

Table 1: Using isolates, their taxonomical classification, their origin and aggressiveness

Isolates	Taxonomical Classification	Origin of Isolates	Aggressiveness[2)]
Re Am E.1.3.	*D. teres* forma *teres*	Rostock (wb)[1)]	32,0 (high)
Re 96 E.1.3.	*D. teres* forma *teres*	Aschersleben ('Trumpf')[1]	14,9 (moderate)
Re 265a E.1.3.	*D. teres* forma *teres*	Aschersleben (wb)[1)]	13,5 (moderate)
Re 235 E.1.3.	*D. teres* forma *teres*	Aschersleben (wb)[1)]	4,0 (low)
Re 69 E.1.3.	*D. teres* forma *teres*	Aschersleben (volunteer)[1]	4,2 (low)
Re 100 E.1.3.	*D. teres* forma *teres*	Aschersleben ('Nebi')[1)]	1,3 (avirulent)
Re 230 E.1.3.	*D. teres* forma *maculata*	Aschersleben (wb)[1)]	10,2 (moderate)
D.t. 140	*D. teres* forma *maculata*	Göttingen	17,5 (moderate)
D.t. 85	*D. teres* forma *maculata*	Göttingen	2,1 (low)

[1)] isolate from winter barley (wb) and given genotypes
[2)] percentage of leaf area covered with chloroses and necroses in the detached leaf test on the genotype 'Karat'
We are grateful to Dr. Sachs (BBA Kleinmachnow) for supplying the isolates.

As clear from Figure 1 illustrating the example of xylanase, there is a close correlation between the enzyme activity and the leaf surface infested ($r = 0.73$).
However, unambiguous characterization of the spot type isolates after 6 dpi is not yet possible. Previous investigations show that the enzyme production of the aggressive isolates reaches its maximum performance only at a later time -10 to 14 dpi - (4).
For a group of selected genotypes of varying susceptibility the sequence in cellulase activity observed was identical with that of the attack score. We have observed that breeding line 4046 has a higher enzyme activity when compared to the CI 4976 genotype classified as resistant. Subsequent studies performed on these genotypes under standardized conditions during the leaf segment test revealed the same grading with regard to the assessment of resistance (infestation %) and the xylanase activity.
Hence, it could be proved that the assessment of resistance based on enzyme activity does not depend primarily on standardized conditions, but can be performed also on material of different stages of development both of the host plant and the pathogen.

High cellulase and xylanase activities were continuously measured on all leaf samples with necroses which, on the basis of visual assessment, were clearly caused by the pathogens *Drechslera teres* and *Rhynchosporium secalis* (Fig. 2).

Figure 1: Relation between enzyme activities and disease intensity of different *D. teres* isolates (6 days after inoculation)

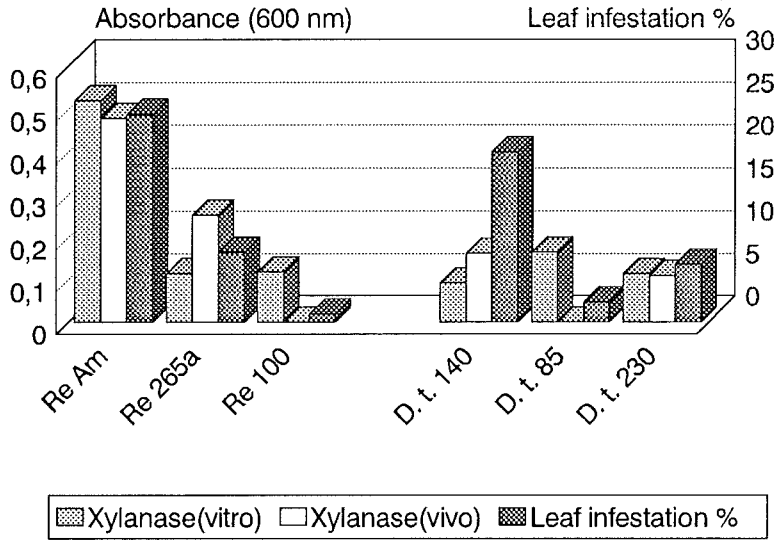
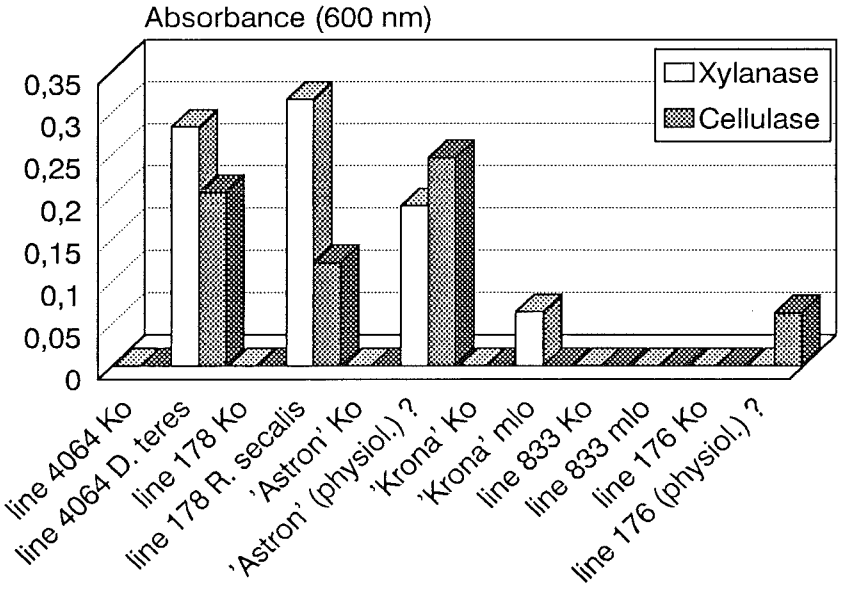


Figure 2: Enzyme activities in leaf material grown under field conditions



The leaf necroses observed on the 'Astron' cultivar and breeding line 176 could not be attributed to a cereal pathogen. The enzyme activities determined (Fig. 2) indicated for the 'Astron' cultivar a pathogen attack which was verified by the isolation of *Septoria tritici*. The analyzed leaf spots of the 'Krona' cultivar and the breeding lines 833 and 176 showed no enzyme activity at all or only a very low activity which suggests physiological or genetic causes.

These results suggest that the enzym activities determined in vitro or in vivo allow the characterization of various *Drechslera teres* isolates. Furthermore the determination of extracellular enzyme activities of the pathogen in infested leaf samples allows an objective assessment of the resistance of barley genotypes to *Drechslera teres.* This method allows a clear differentiation between physiologically or genetically caused leaf spots and the symptoms caused by pathogens.

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THE INFLUENCE OF AGRICULTURAL FACTORS ON INCIDENCE OF STEM BASE DISEASES ON WHEAT

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Introduction

The main object of this investigation was to estimate the occurrence of stem base diseases of winter wheat in Wielkopolska region as influenced by agricultural factors.

Method

The experimental material was collected from Wielkopolska region from 32 localities. Eleven cultivars were tested. (Almari, Emika, Gama, Grana, Jawa, Koda, Lanca, Nike, Orestis, Panda and Rada). Winter wheat samples were taken from commercial fields in plant growth stages from tillering to shooting, each sample of 100 plants from one field of about 1 ha area. Before mailing to the Department of Fungal Diseases of Institute for Plant Protection plants were air dried. The information about weather and soil conditions, cultural practices, fertilization and forecrop was also supplied. In the Institute the occurrence of pathogens was assessed, and results obtained were calculated statistically.

Results

In all samples from 32 places located in 7 district areas we stated the infection of plant stem bases with *Fusarium* spp., *Pseudocercosporella herpotrichoides* and *Rhizoctonia* spp. The occurrence of diseases was different in different places and it was clearly related to agricultural conditions. A majority of diseased plants were infected by *Fusarium* spp., *F. nivale*, *F. culmorum*, *F. poae* and *F. avenaceum*. The level of infection by *Fusarium* spp. ranged from 3 to 95%, in dependence on agricultural factors taken under consideration. A mean value for infection was 13,6%. The highest percentage of affected plants (95%0 was observed in sample from a field with pea forecrop. Cultural factors favouring increased level of infestation were: medium level of rainfall (18,8%); growth stage (tillering 14,4%, shooting 12,8%) and locality (Kalisz district 29,4%). The fungus *P. herpotrichoides* was the second most frequent pathogen on winter wheat stem base. The incidence of eyespot ranged from 0 to 12%. Earlier sowing dates favoured the increase of incidence of this fungus. This being confirmed by a characteristic pattern of infection at different growth stages (tillering 2,8%, first node 9,3%, shooting 10,6%), in contrast to a lower level of infection for later sowing dates. Other factors modifying the infection by *P. herportichoides* were: the amount of rainfall, forecrop and varietal susceptibility. Higher rainfall caused an increase in the number of infected plants(8,3%). In the fields with potato and pea forecrop we observed a lower incidence of the disease (0,7 and 1,0% respectively), as compared to other forecrops. The level of infection of cv. Koda and Orestis was equal to 12% and on cv. Panda (in Leszno district) the occurrence of the pathogen was not observed. Only traces infection with *Rhizoctonia* spp. were noted. The level of infection ranged from 0 to 5%. An increased number of infected plants was observed in fields with forecrops peas (5,0%) and potatoes (4,0%). A low amount of rainfall favoured the incidence of *Rhizoctonia*; other cultural factors had no evident effect on the disease occurrence.

Conclusions

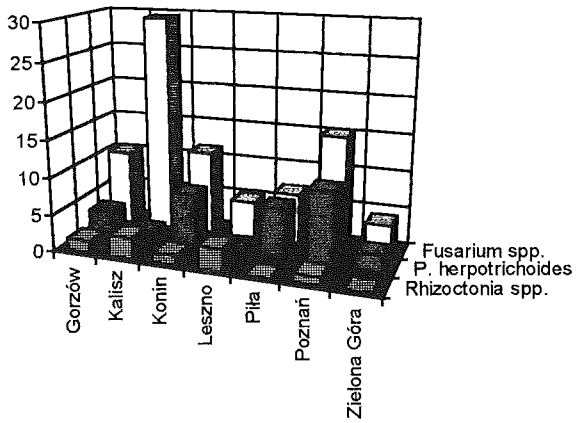
1. Main pathogens of stem base pathogens of winter wheat in Wielkopolska in 1994 were *Fusarium* spp., *Pseudocercosporella herpotrichoides* and *Rhizoctonia* spp.
2. The incidence of these pathogenic fungi was different and dependent on agricultural factors.
3. Peas as a forecrop had a greatest influence on the increase of *Fusarium* species on stem bases.
4. The development of *P. herpotrichoides* in stem bases was mainly related to an increased amount of rainfall.
5. Little influence of agricultural factors on the incidence of *Rhizoctonia* spp. on stem bases of wheat was observed.

Summary

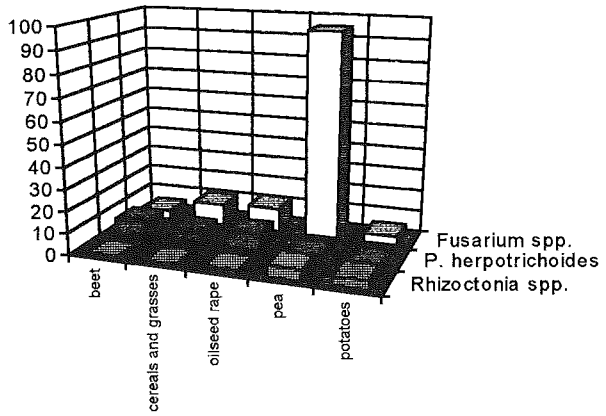
The purpose of the investigation presented in this paper to determine the occurrence of stem base diseases on winter wheat in 1994 as related to agricultural factors. Samples were taken from commercial fields in western part of Poland (Wielkopolska region). From infected plant material we isolated species of *Fusarium* (*F. nivale*, *F. culmorum*, *F. poae*, *F. avenaceum*), *Pseudocercosporella herpotrichoides* and *Rhozoctonia* spp. The occurrence of *Fusarium* spp. was influenced by: forecrop (pea 95%), rainfall (18,8%), locality (29,4%) and growth stages. The occurrence of *P. herpotrichoides* depended on: growth stage (tillering 2,8%; first node 9,3%; shooting 10,8%) and the amount of rainfall. No distinct influence of agricultural factors on occurrence of *Rhizoctonia* spp. was observed.

The influence of agricultural factors on the occurrence of stem base diseases on winter wheat in 1994

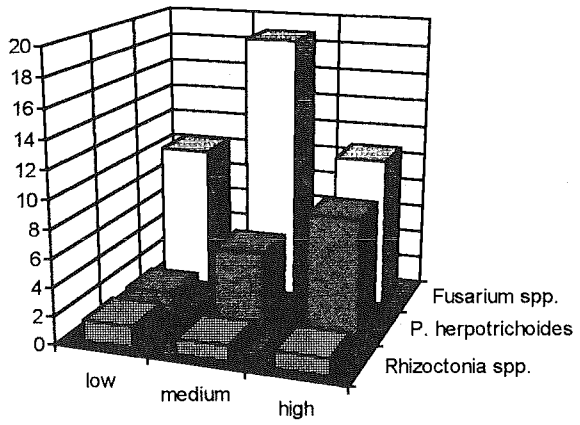
locality



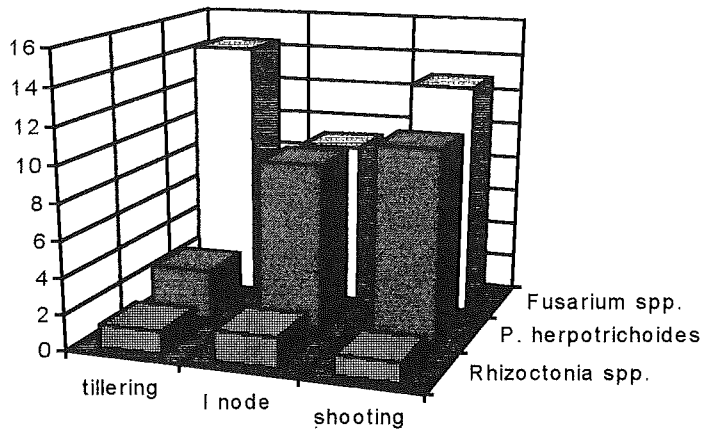
forecrop



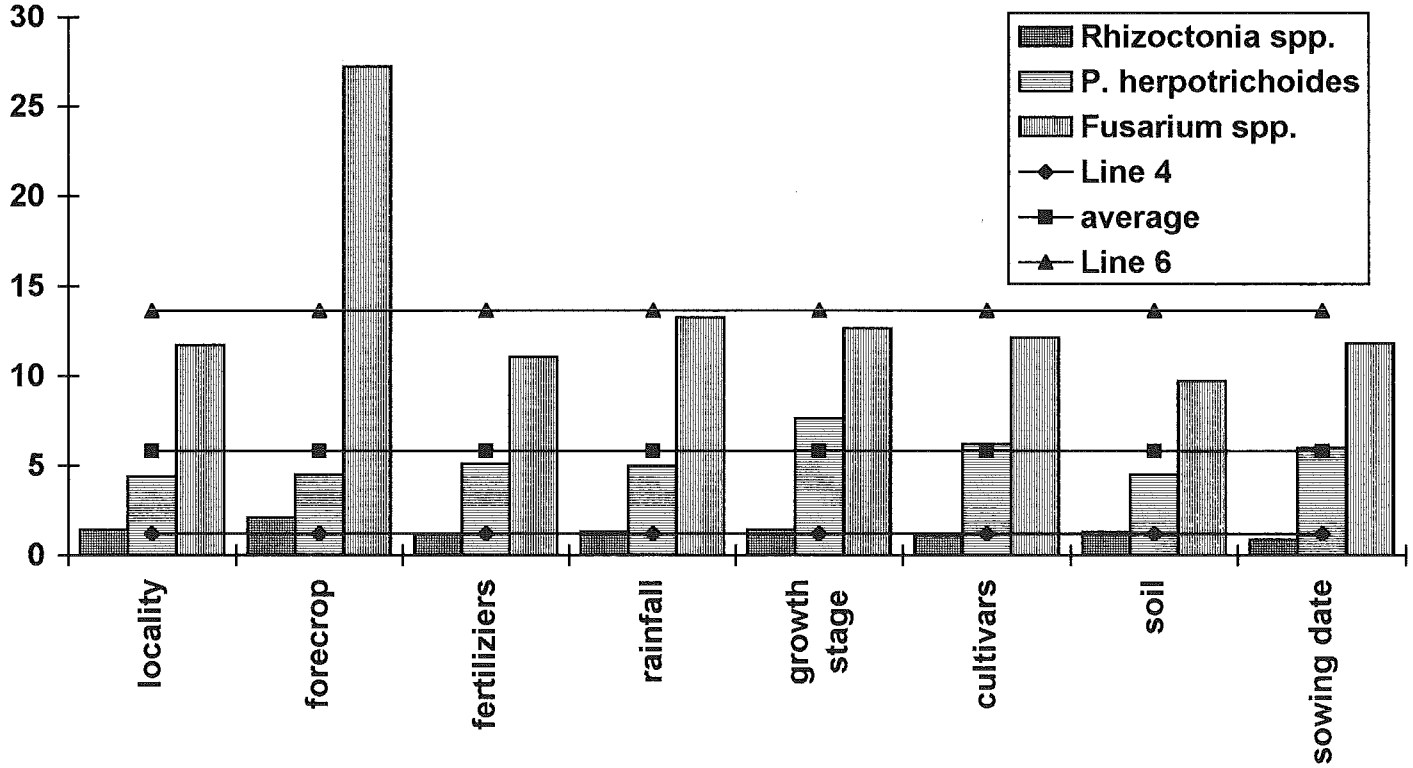
rainfall



growth stage



The influence of agricultural practice on stem base diseases



INVESTIGATION FOR RESISTANCE OF RYE TO LEAF RUST (*Puccinia recondita* f. sp. *secalis* Rob. ex Desm.)

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Introduction

Leaf rust caused by *Puccinia recondita* Rob. ex Desm. f. sp. *secalis* is one of the most important fungal diseases in rye worldwide because it leads to yield losses up to 40% (2). Use of host resistance remains the best approach to control this disease both in economical and ecological terms. To date, however, only a few genes for resistance have been identified in rye. In contrast, about 40 leaf rust resistance genes have been identified in wheat (4). Mains (3) was the first who found resistance to leaf rust and other fungal diseases to be controlled by one dominant gene, respectively. Musa et al. (5) reported leaf rust resistance in rye to be controlled by one or two genes in different inbred lines. Recently, Soloduchina (6) found a single resistance gene in each of 30 different backcrosses.
In the present project we try to detect new resistance genes and identify genetic markers using isozyme, RAPD and RFLP techniques.

Material and Methods

Genetic studies were conducted on three different self-fertile lines of rye and on the progeny of crosses between these lines. One of these lines (2527) was resistant to leaf rust, the remaining two lines (K 2630 and K 2635) were susceptible . Line 2527 was used in the crosses as the male parent. The F_2 and F_3 generations were obtained by selfing of F_1 and F_2 plants.

Crosses:	F_1	K 2630 x 2527
		K 2635 x 2527
	F_2	S (K 2630 x 2527) - 3291; 3291/1
		S (K 2635 x 2527) - 3293; 3293/1; 3293/2

In addition, we have backcrossed one of the F_1 generations with the lines 2527 and 2635. These backcrosses were also analyzed .

Backrosses: BC 1 F_1 (K2635 x 2527) x 2527
BC 2 F_1 (K2635 x 2527) x 2635

The plant material was inoculated with a population of leaf rust collected in Gross Lüsewitz. For the identification of resistant genotypes both *in vitro* tests and evaluations under field conditions (inbred lines) were performed.
In the *in vitro* tests, pieces of leaves were incubated on agar plates containing benzimidazol and inoculated twice with leaf rust spores. The spores were suspended in water and sprayed onto the plates. After both inoculations the plates were left for 24 h in the dark and at high humidity. Subsequently, the plates were incubated at 17°C under light. After 12-14 days, when the pustules were fully developed they were scored twice on a scale from 1-6 using a system of Frauenstein und Reichel (1).
Chi-square tests were used to compare the actual segregations with appropriate theoretical ratios.

Results

1. Inbred lines

Three lines were tested *in vitro* and under field conditions. Two of these lines (2630 and 2635) were susceptible and the line 2527 was resistant in both tests. We observed a good correspondence between field and *in vitro* tests. The results are represented in table 1.

Table 1: Reaction of inbred lines to leaf rust

Line	Number of plants	Tests	
		in vitro	field
		Number of plants (Type of infection)	
2527	12	12(1)	12(1)
	24	7(1); 16(2); 1(3)	
2630	22	22(5)	10(4); 12(5)
	20	4(3); 11(4); 5(5)	
2635	33	33(5)	13(4); 20(5)
	17	3(3); 10(4); 4(5)	

2. Crosses

The F_1 generation from cross 2635 x 2527 was uniformly resistant. Single plants from this F_1 were backcrossed with the lines 2635 and 2527. In the first BC we found a

segregation in resistant and susceptible phenotypes in a 3 : 1 ratio, in the second BC all plants were resistant (Table 2).
The F_2 plants from each crosses (Table 2) segregated in a 15 resistant : 1 susceptible ratio.

Table 2: Segregation for reaction to rye leaf rust in F_2 populations and in backcrosses

Combination		Number of plants	Segregation observed res.	sus.	theoretical	χ^2; p
3291	F_2	64	61	3	15 : 1	0,40; 0,5
3291/1	F_2	166	155	11	15 : 1	0,04; 0,9-0,95
3293	F_2	87	85	2	15 : 1	2,28; 0,1-0,3
		90	87	3	15 : 1	1,29; 0,1-0,3
3293/1	F_2	150	136	14	15 : 1	2,35; 0,1-0,3
3293/2	F_2	101	97	4	15 : 1	0,89; 0,3-0,5
		97	93	4	15 : 1	0,77; 0,3-0,5
F_1x 2635	BC	50	41	9	3 : 1	1,31; 0,1-0,3
		55	44	11	3 : 1	0,71; 0,3-0,5
F_1 x 2527	BC	42	42	0		
		40	40	0		

res. - resistant; sus. - susceptible, BC - Backcross

The results of the F_3 populations are documented in table 3. We have tested the progenies of single F_2 plants showing different types of infection. Some of these plants with the infection type 1 produced only resistant progenies whereas those with type 4, 5 and 6 gave rise to susceptible offspring without exception. In the progenies of the remaining plants we observed a segregation in resistant and susceptible plants.

Discussion

The results demonstrate that the tested F_1 generations were uniformly resistant, the F_2s displayed a segregation in resistant and susceptible phenotypes in a 15 : 1 ratio. In a backcross we observed a 3 : 1 ratio between resistant and susceptible plants. These results suggest a participation of two independent dominant genes in the control of leaf rust resistance in the present material. The data presented is in agreement with the findings of Mains et al. (3) and Musa et al. (5) who suggested the participation of 1 or 2 dominant genes in the control of leaf rust resistance.
The estimation of the F_3 populations demands additional tests. The size of progenies needs to be increased to allow for a more detailed statistic analysis of segregations.
Currently, additional progenies which are segregating in a qualitative manner for leaf rust resistance are under genetic analysis. The genetically characterized plant material may

serve as a valuable starting point for the mapping of major leaf rust resistance genes in rye and the search for tightly linked markers as a tool for marker-based selection.

Table 3: Segregation for reaction to leaf rust in F_3 population

Combination F_3	Number of plants	Class of reaction of F_2 parents	Segregation in the F_3 progeny res. : sus.	
6007/ 35	59	1;2	59	0
6007/160	15	1	15	0
6007/157	15	1 ; 2	8	7
6008/ 16	15	3 ; 4	2	13
6008/ 29	15	4 ; 5	0	15
6008/ 22	16	1 ; 2	11	5
6008/ 18	15	1	15	0
6008/109	52	1	52	0
6009/ 15	14	5 ; 6	0	14
6009/ 21	47	4 ; 5 ; 6	0	47
6009/ 27	15	2 ; 3	11	4
6009/ 28	15	1 ; 2	10	5
6009/ 36	17	1	17	0

res. - resistant; sus. - susceptible

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LACK OF ASSOCIATION BETWEEN *FUSARIUM* FOOT ROT AND HEAD BLIGHT RESISTANCE IN WINTER RYE

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Introduction

Fusarium culmorum (W.G. Sm.) Sacc. causes seedling blight, root rot, foot rot, and head blight in winter rye (*Secale cereale* L.) and other small grains, especially in temperate regions. In Middle Europe, the economically most important diseases are foot rot and head blight. Foot rot reduces water and nutrient transport and stem stability (lodging), both affecting yield components. Head blight causes severe yield losses (3, 5) and contamination of harvested grain with mycotoxins, predominantly deoxynivalenol and 3-acetyl-deoxynivalenol (7). Both diseases cannot be controlled effectively by fungicides (5, 6) and resistance selection should, therefore, be the best method for control. The present study was undertaken to analyze the relationship between foot rot and head blight resistance among winter rye inbred lines in the field.

Materials and Methods

Twenty inbred lines were grown in three-row (0.625 m^2) drilled microplots at Hohenheim (HOH) in 1992, 1993, and at Bad Schönborn (BSB) in 1992, 1993, 1994. In each environment (location-year combination) plants were arranged in three inoculation treatment blocks adjacent to each other: (1) foot rot, (2) head blight, and (3) no artificial inoculation. Genotypes were randomized according to a completely randomized block design in three replicates for each treatment block. For foot rot inoculation, colonized, crushed wheat grain material was spread onto the plants in November and March (4). For head blight inoculation, a suspension of 3 x 10^5 spores per ml was sprayed on each genotype at the respective flowering time at a rate of 90 ml per plot (3). Foot rot rating was performed at late milk ripening with 25 arbitrarily chosen stems per plot on a 1-9 scale (1 = no lesion visible, 9 = stem fully necrotized and softened, according to Miedaner et al. [4]). Head blight ratings were assessed plotwise three (BSB) and five (HOH) times, respectively, starting with the appearance of the first symptoms on a 1-9 scale (1 = no symptoms visible, 9 = 100% of spikelets and ears infected [5]). Arithmetic means across the rating dates were used for further analyses. Additionally, in three environments (HOH92, BSB92, BSB94) a *Fusarium*-specific ELISA (enzyme-linked immuno-

sorbent assay) described in detail by Beyer et al. (1) was used. The assay detects all *Fusarium* species with the same sensitivity, and shows no significant cross-reaction to *Pseudocercosporella herpotrichoides, Microdochium nivale, Ceratobasidium cereale, Drechslera sorokiniana* (1). To evaluate disease incidence of the individual foot rot fungi, necrotic lesions from 100 diseased stems per replicate and environment were incubated on agar (4). All statistical analyses were based on single plot data. The effects of genotypes, years, and replicates were assumed to be random variables.

Results

In five environments, *F. culmorum* was reisolated from necrotic stems at milk ripening in frequencies of 87 to 98%. Other *Fusarium* species and *Microdochium nivale* played a minor role only (9-29% and 0-19%, respectively). *Pseudocercosporella herpotrichoides* was found at two locations in frequencies of 2 to 7%. Disease severity was medium to high for both inoculated plant organs in all environments. Mean foot rot rating ranged between 3.5 and 6.2, mean head blight rating between 3.5 and 4.9 and mean ELISA absorbance between 0.33 and 0.83. All traits revealed significant ($P>0.05$) differences among the inbred lines in each of the environments.

Significant genotypic variation was also detected in the combined analysis across environments. Genotype x environment interaction variances were highly significant ($P=0.01$). Heritability estimates were of similiar size for foot rot and head blight rating (0.81 and 0.87, respectively), but considerably lower for ELISA absorbance ($h^2=0.48$).

Phenotypic and genotypic correlations between foot rot rating and head blight rating were low and nonsignificant (Fig. 1). A somewhat higher but still nonsignificant genotypic correlation occurred between ELISA absorbance and head blight rating ($r= 0.32$).

Discussion

Resistance to foot rot and head blight was tested in different treatment blocks to avoid any specific epidemiological interaction between fungal colonization of foot and head. No correlation between foot rot and head blight resistances existed despite similiar disease severities for foot rot and head blight ratings and significant genetic differences among the tested materials. *F. culmorum* was prevalently isolated from foot rot infected plants. Therefore, the missing correlation between foot rot and head blight resistances should not be caused by other foot-rot inducing pathogens. This conclusion is confirmed by the high absorbances found with the *Fusarium*-specific ELISA within inoculated stems. Different resistance mechanisms are, therefore, the most likely explanation for the lack of a correlation between foot rot and head blight rating.

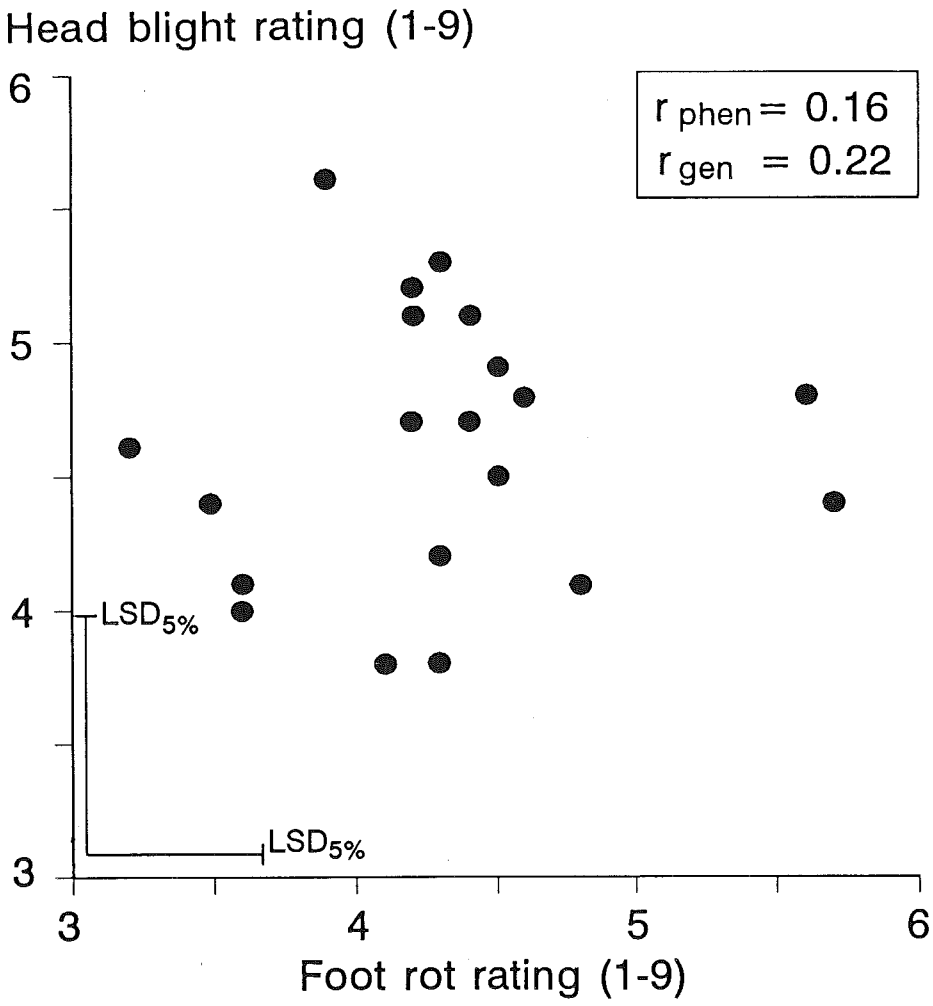

Fig. 1: Common distribution of foot rot and head blight rating (1-9 scale with 1 = healthy, 9 = 100% diseased) of 20 inbred lines after inoculation with *Fusarium culmorum* averaged across five environments (r_{phen}, r_{gen} = coefficient of phenotypic and genotypic correlation, resp. $LSD_{5\%}$ = Least significant difference at the 0.05 probability level)

In practical rye growing, resistance to both foot rot and head blight is needed, because *Fusarium* spores produced on diseased stems may enhance head blight infection (8) and *Fusarium* contaminated seeds lead to a higher seedling and foot infection (2). Separate breeding programs, however, are necessary for improving *Fusarium* resistance in the two plant organs. No correlated selection gain for foot rot resistance can be expected from improving head blight resistance, and *vice versa*. Resistance tests should be conducted in several environments, i.e. repeated over locations and/or years, to reduce the influence of the highly important genotype x environment interactions illustrated in this study and others (3, 4).

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THE STUDY OF POPULATION AREAS OF PUCCINIA RECONDITA F.SP.TRITICI AND PATHS OF SPORE MIGRATION IN CONNECTION WITH POSSIBILITIES OF EPIPHYTOTIA CONTROL.

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Introduction

The continental nature of wheat leaf rust puts on idea to propose regional deployment of race-specific genes of resistance. On our opinion, in the basis of this defense strategy lays knowledge of pathogen populations areas and possible paths of spore migration. Such kind of population characters have been studied for P. recondita f.sp.tritici in Canada (1,2), and U.S.A. (3), for P.graminis f.sp.tritici in U.S.A (4). The attepmts of detection of P.striiformis population areas inEurope were made by Stubbs (5). As for P.recondita populations areas in Europe and Asia we have very poor information though such kind of knowledge is very important for wheat defence. The objective of this study was to compare degree of similarity populations samples and to determine if significantly different populations of P.recondita existed within territory of wheat belt in the former U.S.S.R.(C.I.S). This investigation was performed during six years (1988-1993).

Material and methods

The fungus spore samples were collected from commercial winter and spring susceptible wheat cultivars in 24 localities of European part of U.S.S.R., in 5 ones of Caucasus part, and in 8 ones of Asian. In a whole 2479 single-pustule pathogen isolates were studied. The isolate virulence phenotypes were determined using our original set of differential including 9 genotypes: Thatcher lines with genes Lr1 and Lr2a, the cultivars Caucasus (lr26), SNW N5, Majestic (Australia), 47700 (Rodesia), Guan-fu-nai China), Jubiley (Bulgaria), Magnus (France), and the line of Magnus that has one from two genes of Magnus, it was named Mag (6). The Magnus has Lr26 and in addition gene Mag, the type reaction expressed by this gene is 1,2; (small pustule , surrounded by white paper type chlorosis). This gene can be detected when it is not being masked by Lr26. The virulence phenotypes (races) were named according to modified Habgood method (7).The methods of laboratory pathogen cultivation on detached seedling leaves based on application 0,040 ppm benzimidazole solution were used. These methods allowed to test virulence a sufficient number of fungus clones (8). The method of comparison of race frequency distribution in population samples was accepted to determine their belonging to some general population. The Zhivotovski index (9) $r = \sum \min (p_i , q_i)$, where p_i - frequency of ith phenotype in the first population and q_i - frequency of the ith phenotype in the second population, was used to measure the degree of similarity between populations. Identical populations will have an index 100%, and populations with no phenotypes in common will have an index 0% . The populations samples were grouped according to origin: West-European part (W.E.P.) , Volga region, Caucasus, Western-Asian part on base of the similarity comparison between them.

The data obtained in each year were united in order to escape mistakes connected with representativity of pathogen isolates.

Results

It was found that in each region there are the predominant races: They are races 322, 772 in Caucasus; races 430, 772 in W.E.P., races 474, 75 in Western-Asian part. In the Volga region each year we found both European and Asian races. In 1989 and 1990 years in Caucasus were detected "European" races 430, 772. In the same time in W.E.P. the typical "Caucasus" race 322 was detected. The similarity between Caucasus and other regions was low in 1988 (Table 1) ; it was low between Caucasus and Asian regions each year (0 - 5,4%). The similarity between Caucasus and W.E.P and Volga region was high during 1989-1993 years. Every year the resemblence between Western-European and Asian regions was low (1,2 - 13,2%). Neverthless, the similarity between Volga region and Western-Asian regions was rather high (13,2 - 61,1%). The similarity between Volga and Western regions was high (23,0 - 58,8%) also.

Table 1

The resemblence of phenotype composition of Puccinia recondita f.sp.tritici population samples from different geographical regions of CIS (%)

Year	Region	Western-European part (W.E.P.)	Volga region	Western-asian part
1988	Caucasus	0	5,4	0
	W.E.P.		23,0	8,2
	Volga region			61,1
1989	Caucasus	32,4	26,5	1,4
	W.E.P.		58,8	1,4
	Volga region			16,4
1990	Caucasus	39,3	28,8	5,4
	W.E.P.		49,5	13,2
	Volga region			52,5
1991	Caucasus	86,4	49,0	0
	W.E.P.		50,1	1,2
	Volga region			44,4
1992	Caucasus	93,8	27,6	2,3
	W.E.P.		32,2	6,2
	Volga region			20,6
1993	Caucasus	46,0	29,1	5,1
	W.E.P.		37,1	8,1
	Volga region			33,3

Discussion

We suppose on the territory of Russia and adjacent countries there are at least three populations of P.recondita f.sp.tritici: (i) European, (ii) Asian, (iii) Caucasian. We suppose coexistence European and Asian pathogen populations in the Volga region since the typical European and Asian races were detected there. Earlier in 1981-1987 years we have determined very low degree of similarity between Caucasian and European populations (10,11), the same phenomena we watched in 1988 year. By this reason we suppose that the high similarity between these two populations in 1989-1993 years was resulted from spore migration in direction from Caucasus to Europe, and opposite direction from Europe to Caucasus. The European and Asian populations during period 1988-1993 years were isolated from each other, we have not seen any significant spore migration between them. A possible strategy for epiphitoty control and prolonging the useful lifespan of wheat leaf rust resistance genes might be produced. We propose, it is neccessary to have independent sets of resistance genes for using in the breeding programms in European, Caucasian, Asian,and Volga regions according to detected pathogen populations areas and paths of spore migration.

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DETECTION OF *POLYMYXA BETAE* IN SUGARBEET ROOTS BY MOLECULAR TECHNIQUES

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Introduction

The soil-borne fungus *Polymyxa betae* Keskin (1) is an obligate parasite of sugarbeet roots. *P. betae* causes only little damage to sugarbeet (2) and under natural conditions it is necessary for the transmission of beet necrotic yellow vein virus (BNYVV) (3) and beet soil-borne virus (BSBV) (4). BNYVV is the causal agent of rhizomania which causes severe economical damage in sugarbeet. Since *P. betae* is involved in the incidence of rhizomania, a simple detection method for *P. betae* in sugarbeet roots would be useful for the examination of interactions between host, fungus and viruses.

Molecular techniques can provide a sensitive alternative to conventional detection of *P. betae* in sugarbeet roots by microscope as was already shown by Mutasa et al. (5). Here we describe the cloning of *P. betae*-specifc probes, the development of two PCR-based *P. betae* detection assays and their comparison with respect to sensitivity.

Material and Methods

Sugarbeet seedlings of the variety 'Hilma' were grown in BNYVV-infested or BNYVV-free soils. BNYVV content of the roots was determined by ELISA by G. Büttner, Institut für Zuckerrübenforschung (IFZ), Groß-Gerau and roots were kindly provided by G. Büttner for our investigations. DNA extractions were done according to Dellaporta et al. (6). RAPD-PCR reactions contained 5 units of AmpliTaq DNA Polymerase Stoffel Fragment (Perkin Elmer), 1x reaction buffer (10 mM Tris-HCl, 10 mM KCl, pH 8,3), 4 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of primer OPE-13 (5'-CCCGATTCGG-3', Operon Technologies) and 100 pg DNA template. The reaction mixture was heated to 94°C for 5 min, submitted to 45 cycles of 94°C for 1 min, 36°C for 1 min, 72°C for 2 min, and finally heated to 72°C for 10 min. PCR reactions with specific primers were carried out using 50 pmol of each primer, 1 ng DNA template and the same regards as above. The reaction mixture was heated to 94°C for 5 min, submitted to 35 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and finally heated to 72°C for 7 min. All incubations were done in a Perkin Elmer System 9600 thermal cycler. A 1.2 kb *P. betae*-specific fragment from RAPD-PCR amplification of *P. betae*-infected sugarbeet root DNA was isolated from the electrophoresis gel, purified and cloned in the plasmid pGEM-T (Promega). A Random genomic DNA plasmid library was prepared from EcoRI-fragmented DNA of *P. betae*-infected sugarbeet roots and screened for *P. betae* repetitive sequences according to Mutasa et al. (5). The positive clones obtained were labeled with digoxigenin and tested for their specificity to *P. betae* in Southern blot hybridization experiments as described by Boehringer Mannheim in their DIG System User's Guide for Filter Hybridization. Part of the sequencing was kindly done by A. Stegmann and S. König, Institute of Plant Genetics and Crop Research, Gatersleben.

Results

Samples of sideroots from sugarbeets were examined by microscope for the presence of *P. betae* resting spores (Tab. 1, column 5). Comparison of the results of *P. betae* and BNYVV determination showed that some sugarbeets were infected by virus-free and others by virus-carrying *P. betae* (Tab. 1, column 6). A *P. betae*-specific 1.2 kb DNA fragment was amplified from total genomic DNA of *P. betae*-infected sugarbeet roots by means of RAPD-PCR (Random Amplified Polymorphic DNA-Polymerase Chain Reaction) (Fig. 1).

Table 1: Comparison of the detection of *P. betae* in sugarbeet roots by RAPD-PCR and microscopic examination

	Number of sample	Description of sample by IFZ, Groß-Gerau	RAPD-PCR: 1) presence of 1.2 kb DNA fragment (Fig. 1)	Hybridization: signal on RAPD-PCR blot with 1.2 kb probe	Microscope: 2) presence of *P. betae* resting spores	ELISA: 3) presence of BNYVV E 405	virus
	1	12	(+)	+	+	0,617	+
	2	34	+++	+	++	0,041	(+)
	3	37	+	+	++	0,013	–
	4	43	++	+	+	1,680	++
	5	50	++	+	++	0,049	(+)
	6	53	++	+	++	1,255	++
	7	77	++	+	++	0,172	+
	8	81	+	+	+	0,012	–
	9	91	+	+	+	0,377	+
	10	98	+++	+	++	0,711	+
	11	101	+++	+	++	0,016	–
	12	129	++	+	++	0,844	+
→	13	145	+++	+	–	0,013	–
	14	148	+++	+	+++	0,956	+
	15	151, – control	–	–	–	0,012	–
	16	152, – control	–	–	–	0,011	–
→	17	153, – control	(+)	–	–	0,011	–
	18	154, + control	++	+	++	1,750	++
	19	155, + control	++	+	++	1,954	++
	20	156, + control	+++	+	++	1,830	++
→	21	172	(+)	–	–	0,011	–
→	22	173	(+)	–	–	0,011	–

1) Relative amount of 1.2 kb DNA fragment analyzed after electrophoresis in a 2% agarose gel (see Fig. 1) 2) Microscopic examination of the content of *P. betae* resting spores in at least five ca. 1 cm long root fragments from side roots. The symbols signify: – no resting spores, + some resting spores in at least one root fragment, ++ several resting spores in nearly every root fragment, +++ many resting spores in all root fragments tested. 3) ELISA detection of BNYVV with polyclonal antiserum. Measurement of the extinction at 405 nm. The symbol – signifies E405 values of 0 to 0,014, (+) of 0,015 to 0,099, + of 0,1 to 0,99, ++ of > 1. Arrows mark points of interest, mentioned in the text.

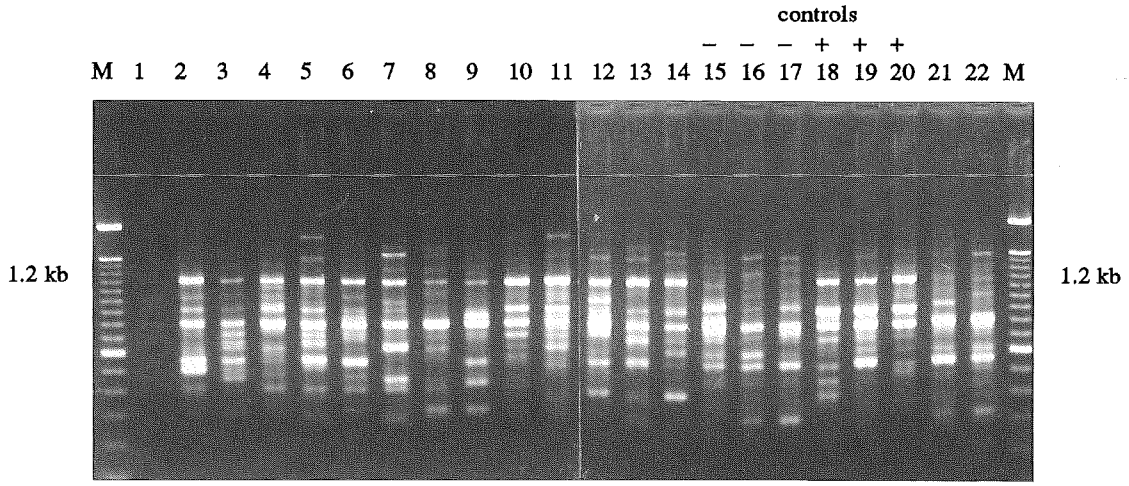

Figure 1: RAPD-PCR amplification of DNA extracted from *P. betae*-infected and healthy sugarbeet roots. Electrophoresis on 2% agarose gel stained with ethidium bromide. M = 100 bp marker, samples 15-17 = *P. betae*-negative controls, samples 18-20 = *P. betae*-positive controls.

As shown by comparison of RAPD-PCR patterns and microscopic examination of samples, there was a good correlation between the intensity of the 1.2 kb fragment in RAPD-PCR patterns and the amount of *P. betae* resting spores in sugarbeet roots (Tab. 1, columns 3 and 5) with the exception of one (Tab. 1, sample 13) out of altogether 42 samples tested.

RAPD-PCR amplification was sensitive enough to detect *P. betae* in 100 pg of DNA extracted from an approximately 1 cm fragment of weakly infected roots.

RAPD-PCR amplification of DNA from some sugarbeets resulted in patterns partly overlapping the fungus-specific DNA band as shown by weak 1.2 kb fragments in some samples (Fig. 1, lane 17, 21 and 22). Probing of Southern blots of RAPD-PCR gels with the cloned 1.2 kb *P. betae*-specific fragment revealed that these weak 1.2 kb bands are not related to *P. betae* (Tab.1, column 4).

When specific 20mer primers developed from the cloned 1.2 kb *P. betae*-specific fragment were employed, an exclusive amplification of DNA from *P. betae*-infected roots was obtained. The cloned 1.2 kb DNA fragment was also tested as a digoxigenin-labeled probe in Southern blot hybridizations, but turned out not to be sensitive enough to detect *P. betae* in 1 µg DNA from infected roots.

To obtain a more sensitive *P. betae*-specific hybridization probe a plasmid library with *Eco*RI-fragmented total genomic DNA from *P. betae*-infected sugarbeet roots was constructed and screened for *P. betae* multi-copy-sequences with digoxigenin-labeled DNA from purified *P. betae* resting spore preparations. Two *P. betae*-specific fragments (PbetaeBS1 approx. 1.55 kb and PbetaeBS2 approx. 1.8 kb) were found which detected *P. betae* in Southern blot hybridizations down to 100 ng and in dot blot hybridizations down to 1 ng of total genomic DNA from moderately infected sugarbeet roots.

Very weak cross-hybridization was detected using probe PbetaeBS1 with DNA from *P. graminis*-infected roots. *P. graminis* is a close relative of *P. betae* infecting roots of Gramineae (7).

Parts of the cloned *P. betae*-specific fragments were sequenced and specific 20 or 22mer primers were developed for the detection of *P. betae* by means of PCR amplification of DNA extracted from roots. Using PCR with different specific primer pairs, parts of the 1.8 kb and 1.55 kb DNA fragments were amplified from DNA of *P. betae*-infected sugarbeet roots (Fig. 2). In some cases an amplification signal was also obtained with DNA of *Polymyxa graminis*-infected barley roots (Fig. 2, lane 3). Under stringent PCR conditions no amplification products were obtained with these specific primer pairs when DNA from healthy sugarbeet roots was employed as template.

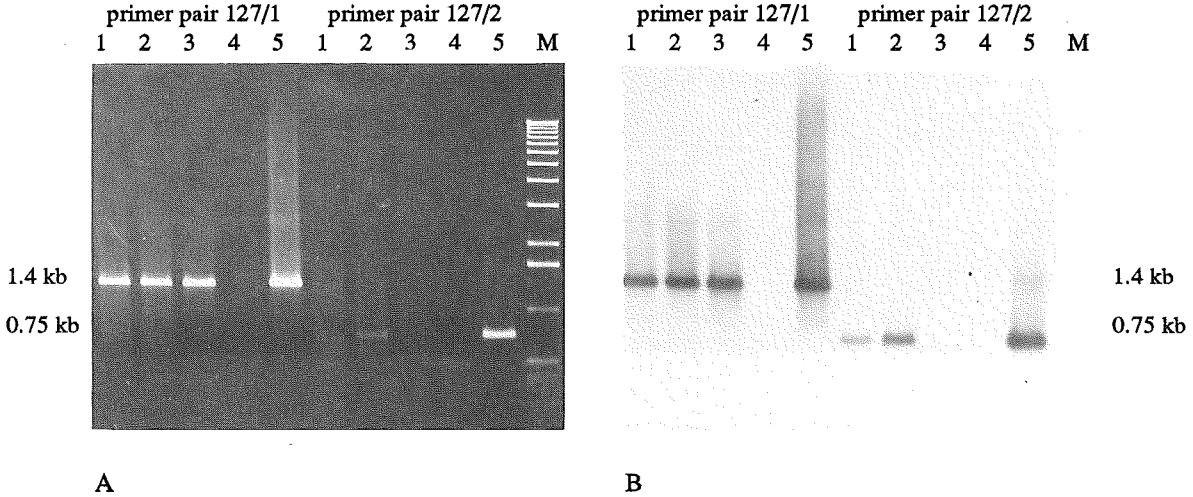

Figure 2: PCR amplification of DNA extracted from *P. betae*-infected and healthy sugarbeet roots and *P. graminis*-infected barley roots with specific primers for PbetaeBS1. A: 2% agarose gel stained with ethidium bromide. B: Southern blot of PCR gel probed with pPbetaeBS1. 1 = *P. betae*-infected sugarbeet, 2 = *P. betae*-infected sugarbeet, 3 = *P.graminis*-infected barley, 4 = healthy sugarbeet, 5 = plasmid pPbetaeBS1, M = 1 kb marker.

The efficiency of amplification varied very much among the primer pairs tested. Some PCR products were not or barely visible in the gel and Southern blot hybridization with cloned fragments was necessary to confirm their presence (Fig. 2, primer pair 127/2). PCR with specific primers was as sensitive as RAPD-PCR only with some of the tested primer pairs.

Discussion

RAPD-PCR amplification is a very sensitive method for *P. betae* detection. However, RAPD-PCR patterns of co-amplified DNA from sugarbeet or contaminating organisms may partly overlap the *P. betae*-specific 1.2 kb fragment rendering the interpretation difficult. The failure of the cloned 1.2 kb *P. betae*-specific RAPD-PCR amplification product to detect *P. betae* in Southern blot hybridization experiments might be due to its origin from a single- or low-copy-gene of the *P. betae* genome.

Two more sensitive *P. betae*-specific hybridization probes (1.55 and 1.8 kb) derived from multi-copy-sequences of the *P. betae* genome were obtained by cloning *Eco*RI-fragmented DNA from infected sugarbeet roots. The sensitivity of the two probes was comparable to the sensitivity of the 1.9 kb *P. betae*-specific probe described by Mutasa et al (5).

One of the two probes showed weak cross hybridization with DNA from *P. graminis*-infected roots. Amplification of *P. graminis* DNA was obtained with primers derived from both probes. The observed cross-reactivities are not critical for the detection of *P. betae* because of the different host range of both fungi. The genetic relationship of the two species is underlined by these results.

Specific primers developed from all three cloned *P. betae*-specific fragments differed greatly in amplification efficiency of DNA from *P. betae*-infected roots. This may be due to suboptimal annealing conditions or different DNA secondary structures in the neighbourhood of the priming sites. The sensivity of the best specific primer pairs under optimized amplification conditions was comparable with the sensitivity of RAPD-PCR amplification without the disadvantage of co-amplifying nonspecific fragments. Both methods are more sensitive and faster than microscopic examinations for estimation of the intensity of *P. betae* infection in roots.

We have started to use the *P. betae*-specific probes for *in-situ*-hybridization studies to investigate the role of *P. betae* in virus spread in sugarbeet roots. The *P. betae*-specific primers may also be employed for *in-situ*-PCR.

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CHARACTERIZATION OF THE RELATIONSHIP OF ISOLATES OF THE *PUCCINIA HORDEI OTTH* COLLECTION IN THE INSTITUTE OF EPIDEMIOLOGY AND RESISTANCE IN ASCHERSLEBEN

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Introduction:

Barley leaf rust (*Puccinia hordei Otth*) is present in Germany every year. The loss of yield is dependent on the time of infection and fluctuates between 15 to 40 percent.
The knowledge of the presence of virulence genes in leaf rust pathogen populations, of the development of populations in dependence on biotic and abiotic influences and also of possible combinations of the different virulence genes in the pathogen genotypes is a very important basis both for breeding of resistant cultivars and the judgement of the durability of resistance in actual cultivars. On the other hand these facts give important information for an effective plant protection.
In Aschersleben exists a pathogen collection with more than 300 isolates of *Puccinia hordei*.
For an effective test of material on resistance to *Puccinia hordei* it is not sufficient to know the virulence genes, it is also necessary to determine the relationships between the isolates. This knowledge provides an effective selection of characteristic isolates.
Therefore it is essential to determine the relationships of this isolates as a prerequisite for the evaluation of genetic resources and investigation of cultivars.

Methods :

1. estimate the virulence pattern of the isolates:

The Aschersleben pathogen collection of *Puccinia hordei* based on samples of attacked leaves collected with annually support from breeders and offices of plant protection and in cooperation with Dr. Felsenstein[1] of a mobile spore trap.
The determination of this collection was realised on the differential set by WALTHER (tab.2) using the key of measurement by LEVINE and CHEREWICK (tab.1).
The determination of virulence genes was carried out on the leaf segment test (length of leaves - 3 cm; benzimidazole agar 40 ppm - benzimidazole) with single pustules lines.
A different virulence pattern showed 96 isolates. These isolates were used to develop a numerical taxonomy to describe their relations.

tab 1 : key of measurement to estimate the virulence level by LEVINE and CHEREWICK, 1952 ; PARLEFLIET, 1976

i	=	no reaction, no infection
On,c,nc	=	highly resistant, no pustules, necrosis, chlorosis, chlorosis and necrosis
1	=	very small pustules in necrotic spots
2-	=	small pustules with low spore production in necrotic and chlorotic leaf area
2+	=	variably mesothetic, conglomeration of uredia of diverse sizes and types tending to integrate, both necrotic lesions and chlorosis usually present
3	=	moderatly susceptible, uredia of medium size usually slight chlorosis but no necrosis at infection centres
4	=	extremely susceptible, uredia predominantly large, necrotic lesions absent, chlorosis may be present

[1]Special thanks to Dr. Friedrich Felsenstein for collecting spores with the mobile spore trap

tab. 2 Differential set for barley leaf rust (*Puccinia hordei Otth*) and triple code nomenclature (race UN 8-1)

variety	resistance genes	reaction	factors	sum	
1 SUDAN	Pa 1	a	2^0	1 x 1 = 1	
2 PERUVIAN	Pa 2	r	2^1	2 x 0 = 0	
3 RIKA x F1	Pa 3+2r	r	2^2	4 x 0 = 0	**1**
4 GOLD	Pa 4	a		1 x 1 = 1	
5 CEBADA CAPA	Pa 7	r		2 x 0 = 0	
6 QUINN	Pa2+Pa5	r		4 x 0 = 0	**1**
7 BOLVIA	Pa2+Pa6	r		1 x 0 = 0	
8 EGYPT	Pa8	a		2 x 1 = 2	
9 HOR 500-1	(1d+1r)k	a		4 x 1 = 4	**6**
10 HOR 1132 sel.	2rk	r		1 x 0 = 0	
11 HOR 2596	Pa9	r		2 x 0 = 0	
12 HOR 679-3	Pa3	r		4 x 0 = 0	**0**
13 ESTATE	Pa3+1r	r		1 x 0 = 0	
14 ODERBRUCKER	Pa1	a		2 x 1 = 2	
15 REKA	Pa2+?	r		4 x 0 = 0	**2**
16 HOR 4280	1d+1r	r		1 x 0 = 0	
17 TRUMPF	(2d+1r)k	r		2 x 0 = 0	
18 LADA	(2r)k	r		4 x 0 = 0	**0**

The triple code for the race **UN 8-1** is **PH 116020**.

differential set by WALTHER and triple code calculation by FRAUENSTEIN et al

2. mathematical determination of the relations between the isolates :

Because the values ascertained in an ordinal scale, it is impossible to use these data for cluster analysis in a conventional way. Therefore the following algorithm was used to characterize the relations between the isolates :

1. transform the scale of measurement in a numerical code

notes	numerical code	
0	0	(highly resistant)
1	1	(resistant)
2-	1	(resistant)
2	2	(moderately resistant - moderat susceptible)
2+	3	(moderately susceptible)
3	4	(susceptible)
4	5	(highly susceptible)

2. determinate the polychoric correlation coeffizient by POSER (1974)

To estimate the correlation coefficient for these grouped data with an ordinal character the procedure by POSER was used. The objects are the 96 isolates and the characters are the virulence pattern on the 18 test varieties. Because the test variety 'Cebada Capa' (Pa7) is fully resistant to all isolates it shows no variance, these cultivar and the reaction were deleted.

$$r_{pol} = \frac{\sum_i \sum_j (f_{ij} * d_i * d_j)}{\sqrt{\sum (f_{i.} * d_i)^2 * \sum (f_{.j} * d_j)^2}}$$

k - number of categories of x i=1...k

m - number of categories of y j=1...m
-1< = rpol < +1

3. transport the correlation matrix in the factor analysis and estimate the factor loadings

The software 'STATISTIKA' was applied in order to estimate the factor loadings.
17 factors were extracted (number of factors = number of characters) with the method 'Principal components' by using the factor rotation 'VARIMAX, RAW'. The factor scorses could not estimated in this procedure, because the origin for the factor analysis was a correlation matrix.

4. estimate the factor scores

To calculate the factor scorses, each object in the origin data matrix was multiplied with each factor loading in the factor loading matrix. These results are metric parameters, suitable for clusteranalysis.

5. clusteranalysis

To classify the isolates the module 'Cluster Analysis' was applied contained in the software 'STATISTIKA' . The clustering method 'Joining Tree Clustering' with the amalgamation rule 'Ward's method' and the distance measure 'Euclidian Distances' was used.

6. results of clusteranalysis:

The result of the clusteranalysis is a dendrogram (graph1). This dendrogram shows the relations betwen the isolates. Members of each cluster are similar in their virulence pattern. The differences within the groups are small and without the groups high. The cluster algorithm 'Ward`s Method' produce approximate equally large groups with low variance within the clusters.

Results :

Five main groups and nine subgroups with different virulence patterns are estimated. To evaluate assortments of barley on resistance to *Puccinia hordei* it's not necessary to use all isolates. by means of the numerical taxonomy it is not difficult to select isolates for determining the basic of resistance on different samples. One isolate of each group is representative as a member of his cluster. This is an aconomical aspect of the determination of qualitative resistance in barley samples.

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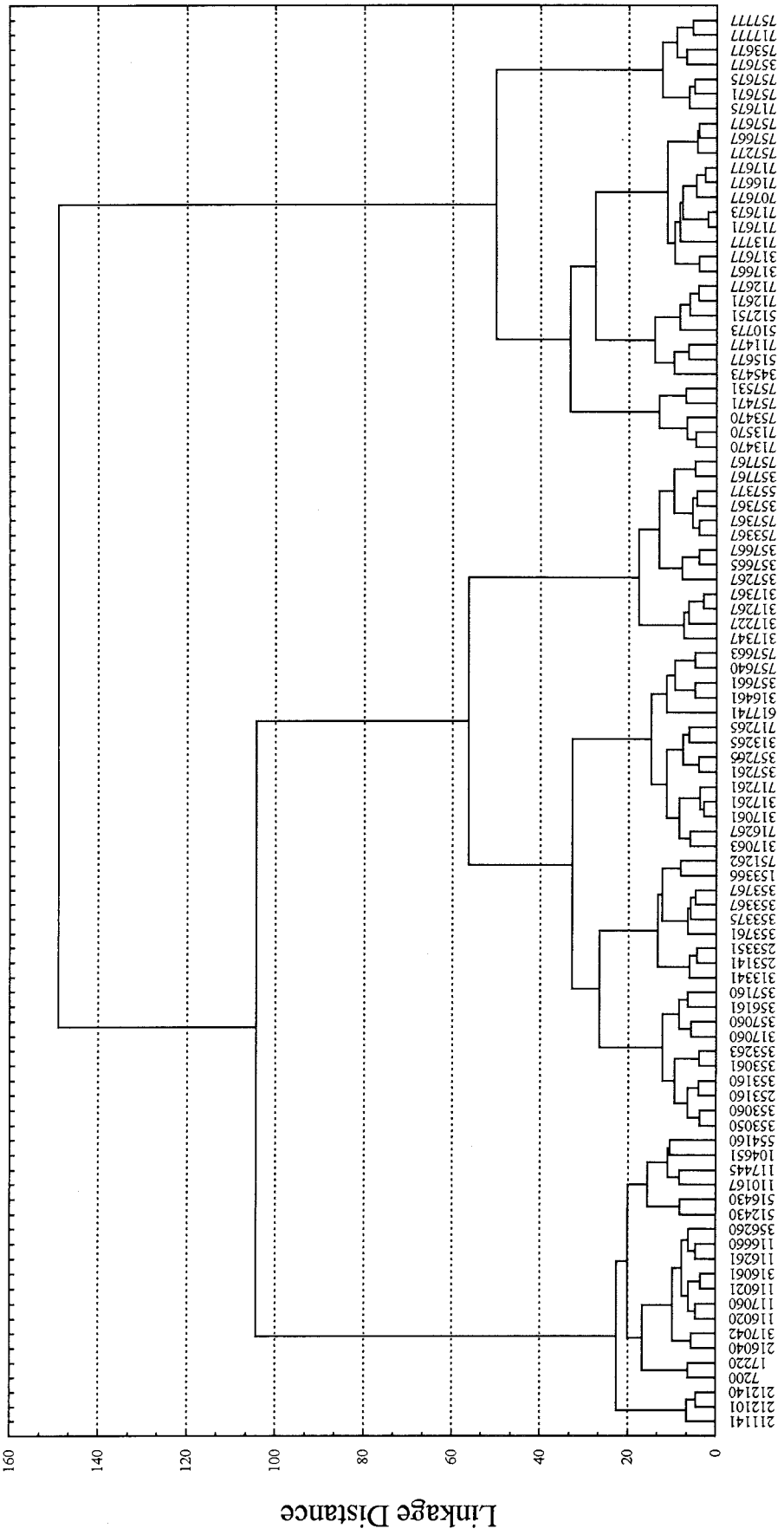
graph 1 : Tree Diagram for 96 Isolates

Amalgamation rule : Ward's method

Distance measure : Euclidean distances



PPOTEIN PATTERNS OF BARLEY LEAVES IN RESPONSE TO NET BLOTCH INFECTION AND TO SOME OTHER STRESSORS

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Introduction

Plants react to invading pathogens with the activation of a variety of defence responses. Obviously, in barley no evidence of phytoalexins exhibiting a defence function is available. But there are strong indications that besides the strengthening of the host cell wall, accumulation of pathogenesis-related (PL) proteins participate in the reactions confering resistance to the plants (1).

Material and Methods

Barley plants were inoculated in the seedling stage, 10 days after sawing. The inoculation with *Drechslera teres f. teres* was performed with a reisolate of the race 'Amelung' as a suspension of conidia in 0,01 % Tween 20. It was worked with the susceptible cultivar 'Karat', the cultivars 'Zenit' and 'Ming' were used as resistant counterparts.
For the extraction of the proteins, the primary leaves were powdered in liquid nitrogen, followed by a homogenization with an acidic extraction buffer (2). As extraction buffer for the isozyme analyses mostly was used 20% saccharose containing 20 mM DTT. Both the native and the sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) were carried out using 1 mm thick gradient gels (10-18% total concentration of acrylamide with 2% bisacrylamide as crosslinker). The sampling buffer according to Laemmli (3) was adjusted to a pH of 8.0. For isoelectric focusing (IEF) were used 0,3 mm thick polyacrylamide gels (5%) with a pH range of 3-10. Samples were applied to the gel surface on sample application pieces. The 2D-electrophoresis was performed first with a IEF, then for the second dimension ran a vertical SDS-pore gradient gel electrophoresis using the cutted strips from the IEF gels supported on sheets or the stripes with a immobilized pI (3-10) gradient (Immobiline DryStrip, Pharmacia).
Peroxidases in the gels were detected using the catechol/H_2O_2 system (4). Chitinase and ß-1,3-glucanase were detected by means of PAGE-overlay gels containing Remazol dyestuffs coupled to substrates (CM-Chitin-RBV resp. CM-Curdlan-RBB, Loewe Biochemica GmbH). The proteins were transferred to the overlay gels using capillary-forces with buffers as 0,1 M sodium phosphate/citrat pH 6.8 for chitinase and 0.1 M sodium acetat with a pH of 5.5 for ß-1,3-glucanase (5). Chitinase isozymes were detected as red signals on the blotting paper laying above the overlay gel. The ß-1,3-glucanase isoforms were visualized as cleared zones in the blue overlay gel.
For western blotting, the proteins were separated on polyacrylamide gels and electroblotted for 1 h at 15 V to PVFD membranes with 0.7 % acetic acid as a transfer buffer. After the immunoreaction (antisera were diluted 1:1500 with PBS-T) bands or spots were detected with a secondary antibody using alkaline phosphatase - conjugated goat anti-rabbit IgG diluted 1: 2000 in PBS-T and reacting the washed blots in NBT/BCIP reagents, finally with a detection of the enzyme alkaline phosphatase coupled on goat anti rabbit antibody.
For ELISA, the protein solutions from extractions were diluted 1:500 with coating buffer for their applications in the microtiter plates. Each IgG-solution was applied in a dilution of 1: 1000. The time-course studies were set up to sample the leaves every 12 or 24 hours after the inoculation.

Results and Discussion

In the primary leaves of barley plants infected with the net-blotch disease pathogen D*rechslera teres f. teres* were found the pathogenesis-related (PR) proteins by means of electrophoretic techniques. The accumulation of this PR-proteins occurred regardless of the used cultivar resistance against the fungal pathogen. Obviously, the amount of the induced proteins depends on the degree of the necrotization of the infected leaves. The PR-proteins were presented after isoelectric focusing and SDS - polyacrylamide gel electrophoresis by typical patterns of bands showing their extremely acidic or basic nature and a molecular weight range up to about 40 kDa, in generally.

Furthermore, the infection with the obligate pathogens causing brown rust or mildew results essentially in the same protein patterns as those we got by the perthotrophic pathogen *D. teres*. Also certain chemicals stimulated the barley plants to produce PR-proteins. So we proved atrazine, 2,6-dichloro-isonicotinic acid and a toxin fraction yielded from the culture filtrate of the fungus D. teres. We noticed small effects caused by 2,6-dichloroisonicotinic acid and atrazine (applied in subtoxic concentrations), which acting in some host-pathogen combinations as resistance inducers. But a strong effect we got with detached leaves treated with the toxin preparation. The toxin induced the same proteins like those known from the infection with the fungus. The strong accumulation of these infection-related proteins following the toxin treatment explain in our opinion why we got such a strong PR-protein induction in the case of the susceptible cv. 'Karat'. The toxin release seems to be responsible for it. In this connection remarkable are the symptoms of the toxin-treated leaves, which remember on the nets symptoms of the fungal infection. We have found them in the barley cultivars regardless of their cultivar-specific resistance against *D. teres*.

The isozyme analyses of the PR-proteins for peroxidase, chitinase, and ß-1,3-glucanase were performed after isoelectric focusing or after native pore-gradient electrophoresis by specific detection of each enzyme. The results of the IEF show a lot of bands for peroxidases in the neutral to basic area in the healthy plants , already, which were increased after infection. Chitinase-activities were expressed in the acidic as well as in the basic part of the IEF gel. The same was true for ß-1,3-glucanase: we found basic as well as acidic protein activities. After electrophoresis under nondenaturing conditions we got patterns for this three enzymes which have in common the occurence of many signals grouped in isoforms with long, middle and short running distances. After all we have to assume that the various isoforms we detected suggest some functional differentiation of these PR-proteins.

A first mapping of the PR-proteins was performed in the two-dimensional elpho-gel using five antisera against barley PR-proteins[1]. The used antigens for the immunization had been isolated from mildewed barley. The antisera reacted with the expected spots of the blotted 2D-gel from samples which we got from net blotch diseased barley as well as from leaves treated with the toxin from *D. teres*. So we assigned spots to the following PR-proteins: thaumatin-like protein (2 spots in the acidic part of the gel at about 16 kDa), chitinase (at 26 kDa in the acidic as well as in the basic), ß-1,3-glucanase (1 spot in the basic at 31 kDa) and PR-1a and PR-1b proteins (in the basic at about 15 kDa, not to differentiate because of cross reaction).

A mapping of some further proteins we have started now. These selected proteins are pathogenesis-related but was not considered until now. Starting with a 2D-elpho, we blotted the proteins onto PVDF membrane, cutted the Coomassie Blue - stained protein from the

[1] This work was supported by the generous gift of antisera from Dr. T. Bryngelsson

membrane for a micro-sequencing of the N-terminal end of the protein[2]. We investigated two groups. The first one include three proteins with a MW at about 23 kDa and pI-values in the slightly basic We attained a high percentage of alignment to the protein S and to the protein R (Fig. 1)., which are TL-proteins and were detected in the late developmental stage of barley seeds (6). The second group resolves into two spots in the acidic area of the gel with an apparent MW of about 21 kDa. In the short sequences we analyzed until now we didn't find any difference between the two proteins as well as between these proteins and the acidic TL-proteins described for isoforms with slightly different molecular masses (7, 8).

	5 10 15 20	identity to
protein 1	A T F T V I N K C Q Y T V **X** A	protein S, 93,3%
protein S	A T F T V I N K C Q Y T V W A A A V P A G G G	
protein 2	A T I T V V N R C S Y T V **X** P G A L P G	protein R, 95%
protein 3	A T I T V V N R **E** S Y T V **X** P	protein R, 86,6%
protein R	A T I T V V N R C S Y T V W P G A L P G G G V	

Fig. 1 Comparisons of the amino acid sequences of the N-terminal parts of the proteins 1 to 3 with the thaumatin-like proteins, the protein S and the protein R (6).

For PR1-a, PR-1b, TL-protein and for chitinase could be established an indirect ELISA (Fig. 2) which using the antisera mentioned before. This method was used to perform time-course studies. It could be made visible that the induction of the PR-proteins starts very soon whereas the necrotic symptoms are visible clearly later, at 3 dpi. We recorded essentially more or less a monophasic increase of PR-proteins with the time. The concentrations reached a certain saturation level at 3 to 3 ½ dpi.

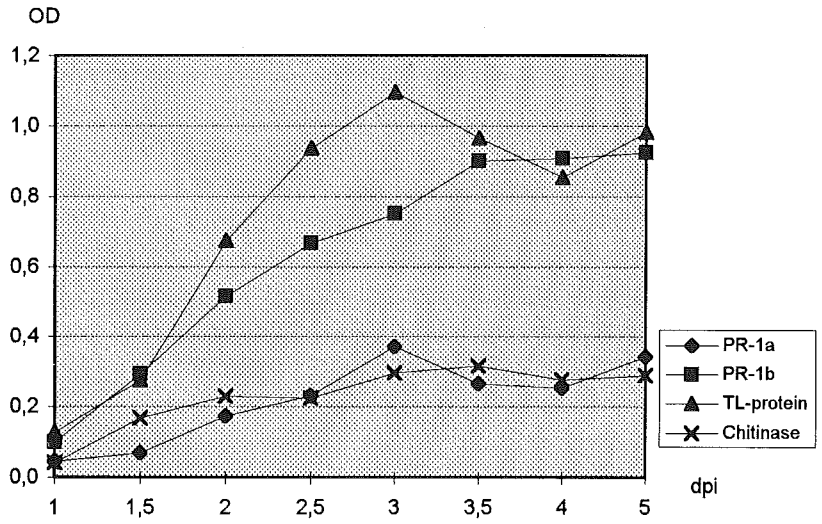

Fig. 2 Time-course of accumulation of PR-1a - and PR-1b - proteins, thaumatin-like protein, and chitinase after infection of barley seedlings (cv. Karat) with D. teres. Optical densities (OD) versus days past inoculation (dpi).

[2] The author wish to thank Dr. C. Horstmann for carrying out the sequencing and for the search in the data bank.

Finally it can be conclude that the great variety of PR-proteins, their functional assignment as well as their spatial and temporal distribution open a wide field for further work which will promote strongly the understanding of plant resistance.

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SOME ASPECTS CONCERNING THE EPIDEMIOLOGY OF POTATO LATE BLIGHT

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Introduction

Late blight is still the worst disease in the potato crop. Fortunately, nowadays it is possible to reduce crop losses to a tolerable extent by applying fungicides.

There are some prognosis models for the incidence of late blight, e.g. the negative prognosis and the prognosis systems of the former GDR. But all models are based on weather data and climatic conditions considering the plant growth development.

In a joint project of the Bayerisches Staatsministerium für Ernährung, Landwirtschaft und Forsten, the Bayerische Landesanstalt für Bodenkultur und Pflanzenbau, the Lehrstuhl für Phytopathologie and the Lehrstuhl für Pflanzenbau der T.U. München it is intended to develop a prognosis for potato late blight based on growth development and cultivar susceptibility, on weather conditions and on recording of inoculum levels. This project is intended to last five years.

In the following results from the first year of investigation are presented.

Material and methods

In 1994 samples of blighted potato plants were gathered and it was tried to isolate *Phytophthora infestans*. The isolates are indicated in table 1. They derived from different regions of Bavaria. It was tried to get to know wether these isolates had been in contact with systemic fungicides, e.g. Metalaxyl, because of possible resistance development to fungicides in the growing season. The isolates were kept on V8-Agar and crossed with known compatibility types to get information whether they belong to the A1 or A2 type of *Phytophthora infestans*.

Known sites of late blight appearance were provided with weather stations and spore traps. Weather data, especially rain events were fixed in a computer and combined with results from the spore trap. Sporangia in the spore trap were counted daily.

Results

As can be seen from table 1 the isolation of *Phytophthora infestans* in 1994 was rather late so that a contact with systemic fungicides has been rather probable.

Out of about 60 isolates (not all are listed in table 1) none belonged to the compatibility type A2.

In figure 1 the estimation of blight appearance, the sporangia in the trap per day have been combined with the weather conditions. It is sure that the sporangia release derives from the

rainfall of June 29th causing blighting of sprouts July 5th. This blighting gave birth to sporangia spreading. Due to optimal weather condition in July 5th and 6th, infection occurred and heavy blighting was observable on July 12th and 13th.

The narrow connection between rain events, temperature and sporangia release is shown in figure 2. There were rain events in August 10th, but they didn't cause sporangia release. The main cause for the lack of sporangia is a very dry period before for about two to three weeks. The plants have been really flaccid. The parasite evidently needed revitalization as well before being able to sporulate.

By aid of the spore trap the peaks of sporangia release during a day period can be observed. Figure 3 shows these events calculated over a time of about three weeks. The daily period of sporangia dispersal is very low from midnight up to about 8 a.m. Then the release reaches a peak at 10 a.m., decreasing in the following hours rather slowly to a very low level at midnight.

Discussion

The prediction of disease evidences, specially for potato leaf blight, needs an exact prognosis system which enables farmer to apply fungicides at exact terms in a correct dosis, depending on the resistance degree of the potato cultivar. Though some prognosis models exist, they are still insufficient especially for the farmers in the southern parts of Germany. The main cause for this situation is the lack of knowledge about the time of sporulation and evidence of spreading sporangia in the air.

Therefore it is an absolute necessity to know the time of sporangia appearance. Primary sporulation occurs only when the mycelium has grown from the tubers into the upper parts of the plant. Blight of sprouts and stems occurs occasionally in case of drought and dryness in the atmosphere. In that case the fungus dies in the infected leaves as well as leaves themselves. In the sprouts the fungus can still survive as the sprouts remain viably in case of drought. Sporulation occurs only in times of high humidity.

It is to point out that not every rain event will cause sporulation. Therefore not all favourable weather condition will result in new infections. Computer models based on weather conditions and growth development only indicate an infestation - in that case a fungicide application would be useless. For this reason it is necessary to look at the inoculum wether sporangia are spreading or not. Then a recommendation to the farmers is possible for applying fungicides against potato late blight.

In the last years the sexual cycle of *Phytophthora infestans* has been discussed, because oopsores had been found in some European countries. This would result in the possibility of the pathogen's overwintering as oospore. We don't know anything about this possibility. But it is our opinion that oospore overwintering is of minor importance if at all. Because in 1994 we were unable to find the A2 type of the parasite which is necessary for sexualism.

Tab. 1: Isolate samples 1994

isolate	origin	date of sampling	apply of fungicides	cultivar	mating type	leaf/tuber
Gr	Neuburg/Donau	Sep-93	?	Granola	A1	leaf
Ch	Immendorf/Augsburg	Sep-93	?	Christa	A1	l
In	Neuburg/Donau	Sep-93	?	Indira	A1	tuber
Qu	Nymphenburg/München	Apr-94	?	Quarta	A1	t
Pö	Pörnbach/Pfaffenhofen	Jun-94	?	?	A1	l
Sch8III	Scheyern,Vf,Erstbefall	30-Jun-94	keiner	Agria	A1	l
Bio	Scheyern,Bioanbau	1-Jul-94	keiner	Agria	A1	l
He	Herrngiersdorf/Abensbg.	Jul-94	18.6 u.27.6 Polyram Combi	Ponto	A1	l
Per	Perkam/Straubing	Jul-94	1xDit.Ultra,17.6.Ridomil MZ	Ute	A1	l
Is	Karlshof/Ismaning	Jul-94	21.6 Dit.Ultra,1.7 Manex,12.7 Sand	Aula	A1	l
Ny 2	Nymphenburg/München	Jul-94	gebeizt mit Acro.plus, sonst unbeh.	Agria	A1	l
Ny 3	"	Jul-94	gebeizt mit Rid. MZ sup. u. Tattoo,	Agria	A1	l
Ny 4	"	Jul-94	Ciluan, Acrobat plus	Desiree	A1	l
Ki	Kitzingen	Aug-94	?	?	A1	l
Vö	Vötting	Aug-94	?	?	A1	l
Pu	Pulling	Aug-94	?	?	A1	l
Ga	Gaden/Freising	Aug-94	?	?	A1	l
Mo	Moos/Ebersberg	Aug-94	?	?	A1	l
Em	Eckersmühlen/Roth	Sep-94	?	Pirola	A1	l
Br	Brunnau/Roth	Sep-94	?	?	A1	l
Ma	Manching/Ingolstadt	Sep-94	?	?	A1	l
Kg	Kinding/Altmühltal	Sep-94	?	?	A1	l
DüV	Dürnast	Sep-94	keiner	Indira	A1	l

Fig. 1: Experimental station Scheyern: Evaluation of disease, sporangia dispersal and weather conditions

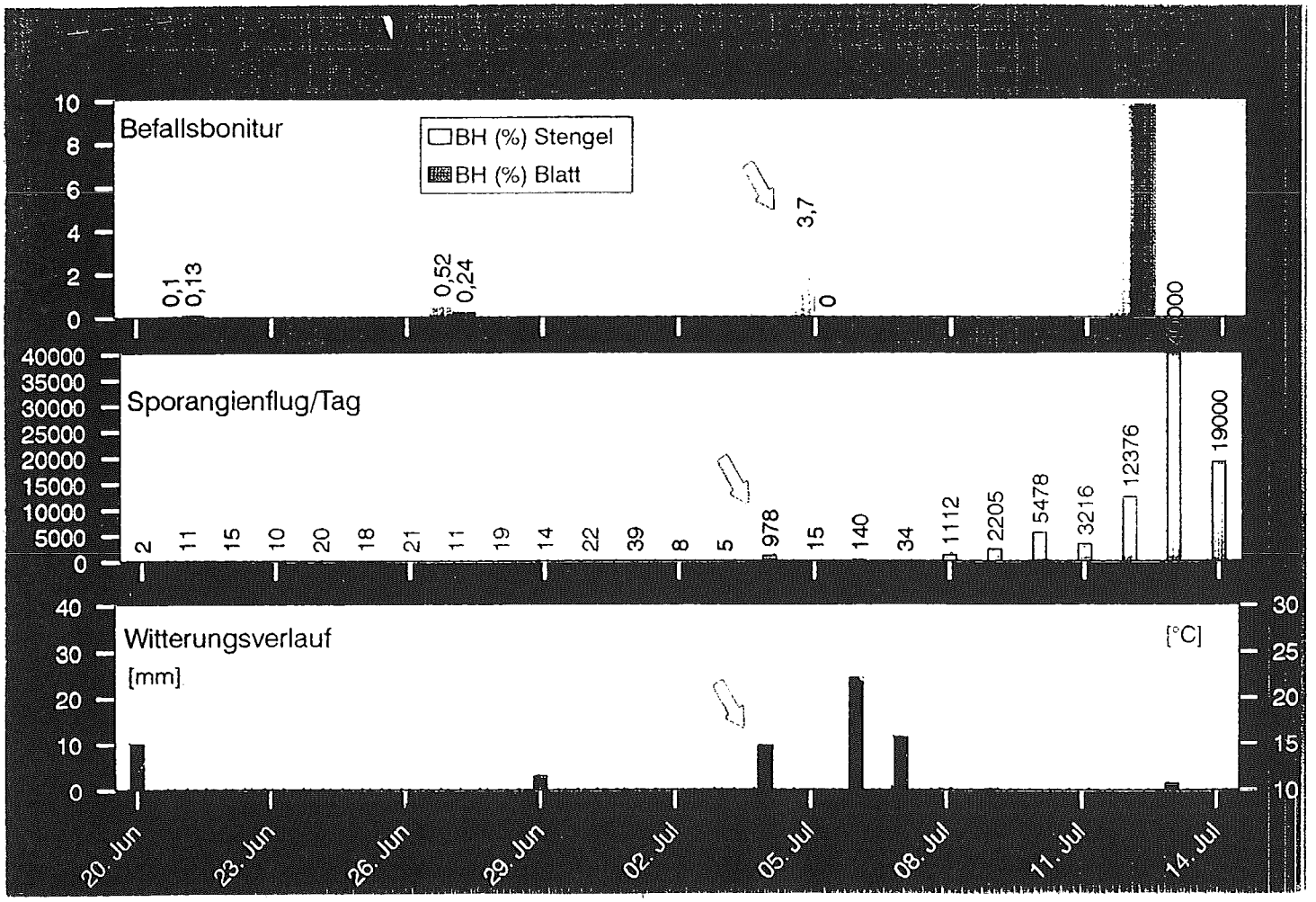


Fig. 2: Experimental station Scheyern: Sporangia dispersal and weather conditions; cv. Agria

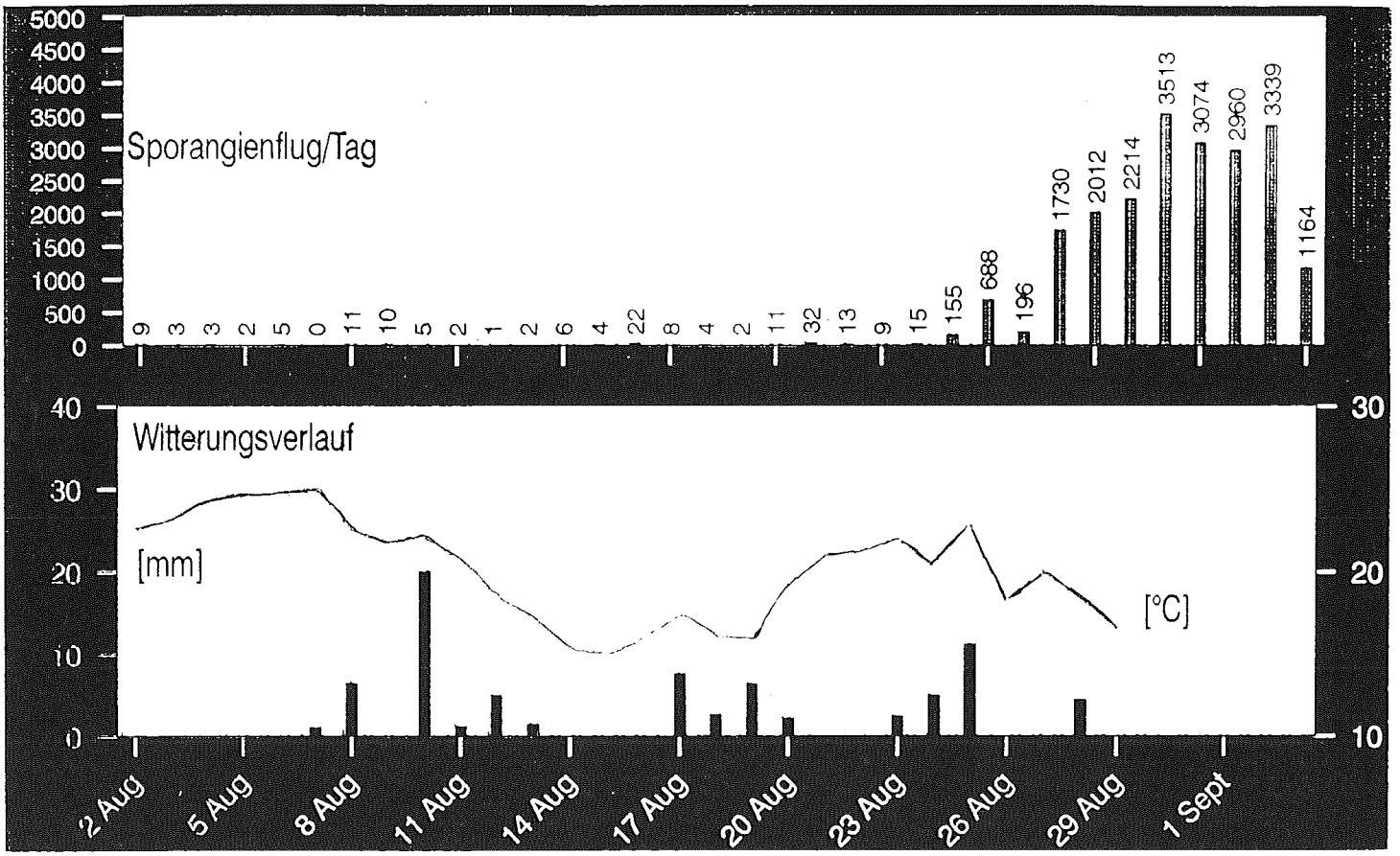
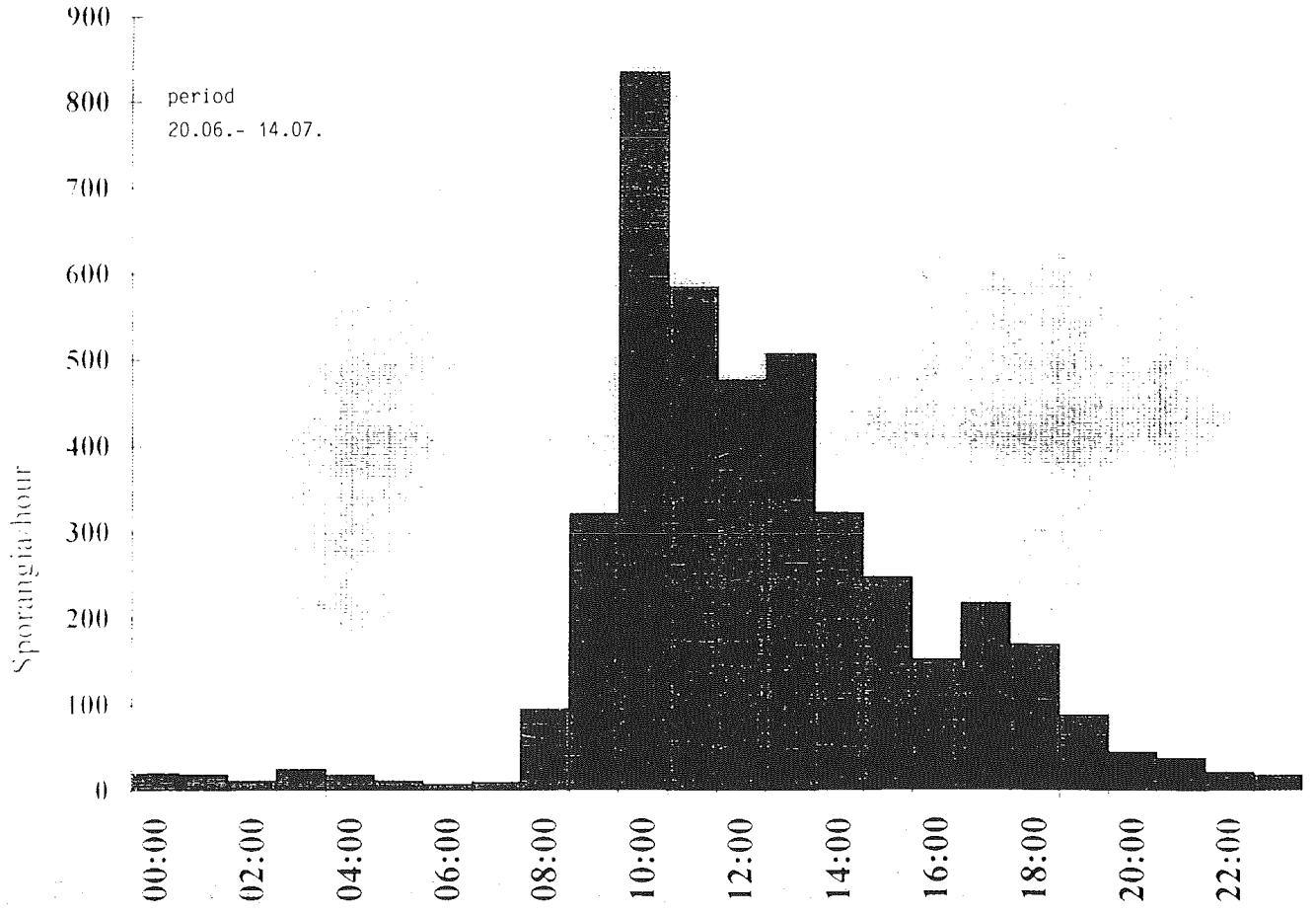


Fig. 3: Daily periodic sporangia dispersal



IDENTIFICATION OF MARKERS FOR LEAF RUST RESISTANCE IN RYE

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Introduction

Leaf rust (*Puccinia recondita* Rob. ex Desm. f. sp *secalis*) is one of the most important diseases of rye. For increasing the efficiency of resistance breeding, identification of individual major genes for leaf rust resistance as well as tightly linked markers is highly desirable. The goal of the present study is the characterization of resistant and susceptible inbred lines and their F_2s in respect to polymorphic isozyme and RAPD loci which could serve as markers for leaf rust resistance genes.

Material and Methods

Genotypes tested

Twentyone self-fertile inbred lines of rye with leaf rust resistance (selfing generation: $>S_3$) were chosen for marker studies. Four susceptible inbred lines, L 201-N and L 301-N (kindly supplied by H.H. Geiger, Hohenheim) and 2630 and 2635 were used as susceptible parents in the crosses with the resistant inbreds. The F_1plants were selfed to produce F_2-lines for marker studies.

Isozyme analysis

Fifteen enzyme systems comprising 44 isozyme loci were tested.

DNA techniques

Isolation of DNA was performed essentially as described by Saghai -Maroof et al.(1). RAPD reactions were performed as follows. Amplification reactions contained 10 mM Tris-HCl, ph 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0,001 % gelatine, 100 μM of each dNTP, 15 ng 10-mer primer (OPERON, Calif.), 40 ng target DNA, and 0,5 units Taq DNA polymerase (GIBCO BRL) in a volume of 18 μl, overlaid with one drop mineral oil (Sigma). Amplifications were performed in a Biometra thermal cycler (UNO-Thermo-block) programmed for 1 min. at 94 °C, 45 cycles of 1 min. at 94 °C , 1 min. at 36 °C and 2 min. at 72 °C. The amplification products were separated in 1,8 % agarose gels and visualized by ethidium bromide staining.

Results

Among the 21 inbred lines tested a minimum of six (L 04, L 05) and a maximum of twenty (L 09) isozyme polymorphisms was detected (table 1) Polymorphic loci were located mainly on chromosomes 4R, 5R, 6R and 7R. In addition to the twelve isozyme markers for chromosomes 1R - 6R , eight RAPD markers for the line L 40 were detected(fig. 1).
In lines L 22 and L 34 all the seven chromosomes carried polymorhic markers, whereas lines L 09, L 11 and L 40 comprised six chromosomes with suitable markers, respectively.

Table1: Identification of isozyme polymorphisms between leaf rust resistant (L 02-L 40) and susceptible inbred lines of rye (L 201-N; L 301-N; 2630; 2635)

Line	1R	2R	3R	4R	5R	6R	7R
L201-N x L04	*Pgi1*		*Aat4*	*Ep2* *Aat1*		*Ep1*	*Aat2*
L201-N x L05	*Pgi1*	*Sod2*		*Ep2* *Est10* *Aat1*			*Aat2*
L301-N x L09	*Mdh1*		*Aat4* *Mdh2*	*Ep2* *Aat1* *Lap2* *Est10*	*Adh2* *Aco2* *Est6,7,8*	*Aadh1* *Ep1* *Dia1* *Mdh2* *Aco1* *Lap1*	*Aat2* *Acph4*
L301-N x L11	*Mdh1*		*Aat4* *Mdh2*	*Ep2* *Dia2* *Est10* *Aat1*	*Est6* *Aco2*	*Ep1* *Dia1* *Aco1* *Lap1*	*Aat2*
L201-N x L13	*Pgi1*	*Sod2*		*Ep2* *Est10* *Aat1*		*Ep1*	*Aat2*
L301-N x L22	*Pgd3*	*Sod2*	*Mdh2*	*Ep2* *Lap2*	*Gpd*	*Aadh1*	*Acph2,3* *Acph4*
L301-N x L34	*Pgd3*	*ß-Glu*	*Mdh2*	*Dia2* *Est10* *Aat1* *Pgd1*	*Adh2* *Est6,7* *Aco2* *Gpd*	*Pgd2* *Est8*	*Acph2,3* *Acph4*
L301-N x L37	*Prx1* *Mdh1*	*ß-Glu* *Sod2*	*Aat4*	*Ep2* *Lap2* *Aat1*	*Est6*	*Aadh1* *Ep1* *Est8*	*Acph2,3*
L301-N x L40	*Pgd3*	*ß-Glu*	*Mdh2*	*Ep2* *Est10*	*Est6,7* *Gpd*	*Est8* *Lap1*	

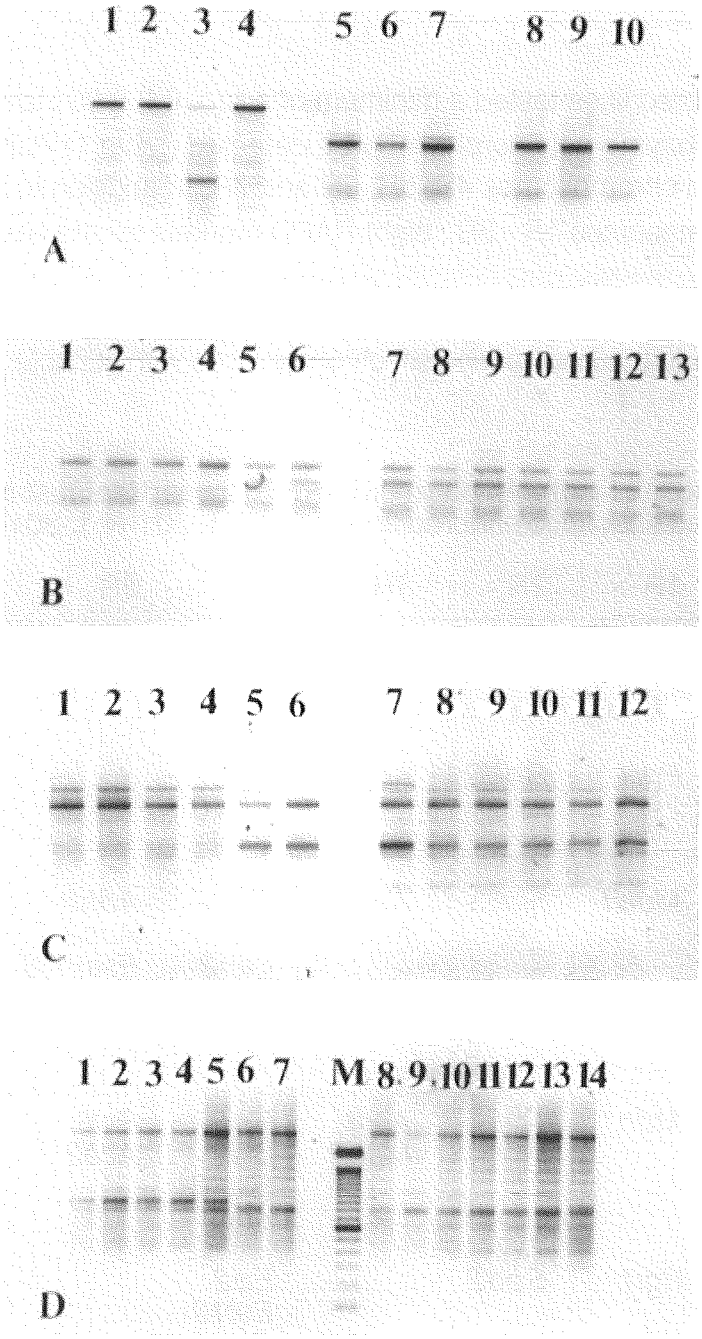

Figure1: RAPD patterns of resistant (L40; lanes 1-4) and susceptible lines 2630 and 2635 (lanes 5-14) using different OPERON primers A, OPO-02; B,OPO-07; C, OPO-03; D, OPO-04

Discussion

Presently, marker- facilitated selection for agronomically important genes has been reported in only few cases.For example, in the tomato breeding programmes the acid phosphatase locus *Aps*- 1, which is linked to the *Mi* gene for nematode resistance has been used (2). Wricke and Wehling (3) found a tight linkage between the *Prx* -7 isozyme locus and the self- incompatibility locus *S* in rye. Vahl et al. (4) detected endopeptidase as a biochemical isozyme marker for resistance to *Pseudocercosporella* in wheat. Linkage also exists between the *Prx* -7 isozyme locus and a putative major gene for pollen fertility restoration in rye (5). Very often the linkages are not close enough to be used efficiently in practical breeding. Thus, additional markers are needed to overcome the constraints of isozyme markers. Schachermayer et al. (6) found three RAPDs and one RFLP marker which allow the selection for leaf rust resistance in wheat.
In rye, we have started to analyze additional DNA markers (RAPDs, RFLPs, STS) for major leaf rust resistance genes. One of the polymorphic RAPD markers we have identified in our resistant material is OPA-07. This RAPD locus is located on the long arm of chromosome 6B in wheat and is absolutely linked to the leaf rust resistance gene *lr* 9 in this species. In respect to the homeology relationships of chromosomes and the synteny of genes among the grasses it will be interesting to examine whether this RAPD marker is also associated with leaf rust resistance in rye. Marker analysis in the respective F_2 progeny is currently performed.

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UTILIZATION OF GENETIC SOURCES FOR PRODUCING RESISTANT BASIC MATERIAL OF *BRASSICACEAE*

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Supplying varieties with biotic resistance to pathogens causing losses of quality and yield is an aim of first importance in plant breeding. At present, concerning cruciferous vegetables, there are only few chances for satisfactory solving this problem since crossing partners which possess efficient absolute or partial resistance and transmit it to the progeny in a durable manner are not yet available.
The Institute for Breeding of Vegetable, Medicinal and Spice Plants is assigned with the task of producing basic material which can be immediately used by the breeders. For this purpose economically important pathogens, clubroot (*Plasmodiophora brassicae*), leaf black spot (*Alternaria brassicicola, A. brassicae*), blackleg (*Phoma lingam*), and Turnip mosaic potyvirus (*TuMV*) are involved. The first step of the research programme is to find novel sources of resistance. For further treatment of the resistant material in order to amplify genetic variability and to transfer resistance into *Brassica oleracea* (white cabbage) conventional and advanced breeding methods are utilized. Particularly, to avoid cross incompatibility, intergeneric combinations will be conducted. Protoplast fusion, in vitro culture and gene transfer as well as molecular genetic (PCR-analysis) and flow cytophotometric methods will be used for producing and characterizing interspecific somatic hybrids. In a last step (pre-breeding) we have planned to establish lines possessing strong genes or additive gene effects to control one or more pathogens in a genotype basing on a genetically and epidemiologically stable level and to transfer these features into practically useful basic material.
In the last year, with a new conducted screening technique (13), more than 400 accessions of *B. oleracea* and *B. rapa* have been tested against isolates of *Alternaria brassicicola, A. brassicae, Phoma* and *Plasmodiophora,* respectively. All entries were moderately or highly susceptible to both *Alternaria* species, although the reaction of *A. brassicae* was significantly (α= 5%) lower. In tests with *Phoma* the proportion of moderately susceptible plants was higher than in the *Alternaria* tests, but since each of the genotype did not exhibit scoring lower than 1,0 (cut-off-point), all representatives

Tab. 1. Results of testing *Sinapis alba* accessions using leaves of intact young plants (int.) and detached young leaves (isol.) as target tissue for inoculation

Pathogen	*A. brassicicola*		*A. brassicae*		*Phoma*	
Target tissue	int.	isol.	int.	isol.	int.	isol.
Accessions tested	30	27	31	28	30	26
Resistance level (</=)	1,5	2,0	1,0	1,5	0,5	1,0
Disease index (DI; $\bar{x}$)	0,87	1,08	0,96	3,33	0	0,20
Range of reactions (DI)	0,09 3,88	0,17 2,42	0,26 2,27	1,47 4,88	0	0,04 0,67
Resistant accessions (%)	90,0	100,0	64,5	3,5	100,0	100,0

were classified as susceptible. Similar results have been recorded by (2), (3, 4), (8), and (5) concerning *Alternaria* and *Phoma,* respectively. Real chances of improving resistance properties against these pathogens are given in utilizing closely related species of *Brassica* or wild growing relatives, although there are , at present, practically no experiences about how this material should be introduced and utilized effectively. As stated by (2), (7), (10), (14) and (15) accessions of *B. juncea, B. carinata, B. souliei, Capsella bursa-pastoris, Camelina sativa* and, particularly, *Sinapis alba*, are possessing resistance features which are eventually transferable into *B. oleracea* and *B. rapa*. We tested 31 cultivars of *S. alba* and could show a large range of symptom expression from immune to highly susceptible reactions to *Alternaria* spec. and *Phoma* (Table 1). Some resistant lines are utilized as crossing partners in somatic hybridization.
Concerning *Plasmodiophora*, the majority of the *B. oleracea* and *B. rapa* (especially var. *pekinensis*) accessions have been shown as being highly susceptible. But with the exception of *B. oleracea* var. *costata*-lines, in all entries, including F_1-hybrid-varieties, resistant single plants have been found. Among 10 cultivated and wild relations of cruciferous species tested with clubroot (Table 2) particularly *Raphanus sativus* exhibited many accessions with immune or highly resistant response to clubroot. Similar results were recorded by (1), (9), (12) a. o., and there are some trials to introduce *Raphanus* resistance factors into cauliflower (6).

Tab. 2. Results of testing some cultivated and wild relatives of cruciferous species against *Plasmodiophora brassicae* using two races isolated from white cabbage (ECD 16/07/12) and Chinese cabbage (ECD 16/14/31), respectively

Species	*Brassica carinata*	*Brassica juncea*	*Brassica oleracea*	*Brassica rapa*	*Brassica tournefortii*	*Brassica napus*
Accessions tested	4	3	181	107	2	3
Single plants tested	62	48	2721	1714	17	47
Disease index (DI; $\overline{x}$)	9,0	7,8	6,0	6,2	7,4	2,9
Resistant plants (%)	0	2,1	9,6	13,8	0	68,1
cont.						
Species	*Camelina sativa*	*Capsella b.-pastoris*	*Crambe* spec.	*Raphanus sativus*	*Sinapis alba*	*Thlaspi arvense*
Accessions tested	6	6	12	112	4	1
Single plants tested	78	84	124	1619	64	13
Disease index (DI; $\overline{x}$)	8,5	2,5	7,0	1,7	8,7	0,9
Resistant plants (%)	3,9	36,9	9,7	69,0	0	92,3

For the comparison of the reaction of the different cruciferous vegetable to *TuMV* the relative virus concentration in the plants (DAS-ELISA, $A_{405\,nm}$) was used. As represented in Table 3 , in *B. oleracea* there was a high percentage of plants with immunity and/or resistance to isolate *TuMV1* in relation to isolate *TuMV2*.
In contrast, in *B. rapa*, *R. sativus* and *Armoracia rusticana*, only plants with immunity and/or resistance have been found. As recorded by other workers (11, 16) our data show that immunity and/or resistance is isolate specific. Furthermore the results suggest the importance of using as many *TuMV* isolates as available for resistance evaluations.
At present, more than 30 introductions of wild cruciferous relatives are tested in order to estimate ranges of resistance variability and to arrange a germplasm seed bank with putative partners for combination utilizable for protoplast fusions and gene transfers.

Tab. 3. Reaction of Cruciferae to *Turnip mosaic potyvirus*

Cruciferae	*TuMV* isolates	Genotypes total	Plants total	Plants ELISA negative (A 405 nm, 0-0,1)	
				absolute	%
Brassica oleracea ssp.	*TuMV 1*	108	1435	882	61,5
	TuMV 2	42	671	170	28,3
B. rapa ssp.	*TuMV 1*	26	367	0	0
	TuMV 2	7	84	23	27,4
Raphanus sativus	*TuMV 1*	2	20	20	100,0
	TuMV 2	1	30	24	80,0
Armoracia rusticana	*TuMV 2*	1	35	35	100,0

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SPREAD AND POPULATION STUDY OF CRYPHONECTRIA PARASITICA (MURR.) BARR IN HUNGARY.

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Introduction

Cryphonectria parasitica the causal agent of chestnut blight was first recorded in Europa in a forest near Genova (ITALY) in 1938. In Hungary the first occurence of the fungal disease was described by A. Körtvély in 1969. The Hungarian chestnut stands have about 200 thousend trees - mainly in coppices and less in orchards and forest (Figure 3.). The disease spreaded out rapidly in western Hungary. Chestnut blight occurred almost in every places of chestnut stands, and caused great decay.

The aim was to characterize the Hungarian populations of Cryphonectria parasitica and also to look for hypovirulence strains. Collected isolates were tested for vegetative compatibility groups and pathogenecity.

Materials and Methods

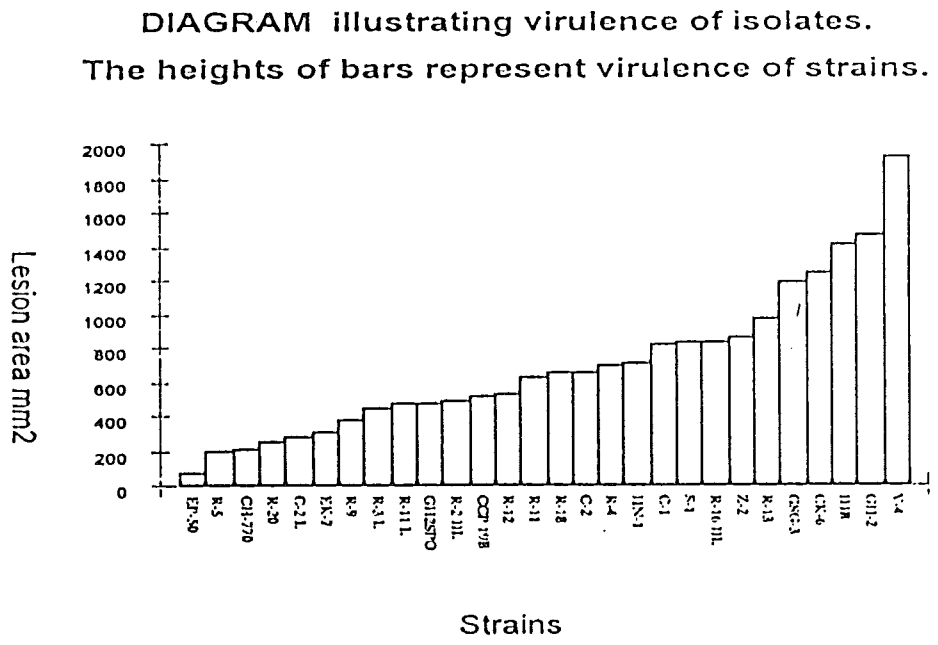
Fungal isolates

Samples were collected from western and southern Hungary where most of chestnut stands are. Bark pieces were taken from infected and probably healing trees by using knife and sterile Petri-dishes and placed on a PDA. After three or four days small agarpieces were transferred on media PDA containg metionin and biotine (PDA mb) (2,3,5).

Pathogenecity and virulence

were tested on dormant chestnut sticks. The sticks surfaces were desinfected by rinsing with NaOCl (approx. conc. 20%). Afterwards the ends were closed with paraffin. To gain a wound bark was removed with cork borer (5 mm) PDA - agarplugs carrying mycelium (25 mm^2) were put on the infection sites. The test was carried out at room temperature in dark (4.).
The sticks were stored in plastic containers having sections between each other. After fifteen days the areas of lesions were measured by planimeter.

Figure 1.



VEGETATIV COMPATIBILITY TEST

VC Group Code

VC group I.		VC group I.	VC group II.	VC group II.	
R-2 III.	Rezi	R-2 II.	SZ-1	SZ-1	Szombathely
R-4 III.	Rezi	R-4 III.	FS-1	FS-1	Fertőszentmiklós
R-11 I.	Rezi	R-11 I.	FS-4	FS-4	Fertőszentmiklós
R-11 II.	Rezi	R-11 II.	CK-3 I.	CK-3 I.	Cák
IHB-1p.	Iharosberény	IHB-1p	CK-3	CK-3	Cák
Z-1	Zengővárkony	Z-1	CSG-5	CSG-5	Csepreg
ZG-1	Zalaegerszeg	ZG-1	CS-2	CS-2	Csipkerek
ZG-4	Zalaegerszeg	ZG-4	V-1	V-1	Velem
S-1	Sand	S-1	V-3	V-3	Velem

Figure 2. VC-GROUPS found between western Hungarian strains with arrows indicating isolates for overlappings.

Figure 3. Chestnut stands in Hungary.

Vegetative compatibility test

The isolates were cultivated in four pieces on PDA mb. for 10 days, at 25 °C either barrage zones flanked by pycnidia or anastomoses were noted.
All confrontations were repeated four times (1.).

Results - Discussion

Virulence test

Most of Hungarian strains showed virulence of average extent. (Fig.1.). R-5, R-20, R-9 exhibited reduced virulence.

VC-groups

The test showed that exist two VC - Groups in western Hungary at least. Both of groups have overlapping areas of different extents. There were found compatibility between R-2 III. to V-3, R-4 II. to V-1, and S-1 to FS-1.

We have found reduced virulence strains in Hungary, but not sure hypovirulent ones. We would like to examine the strains with gel-electrophoresis to gain real results.

In western Hungary there are two Vegetativ Compatibility Groups of Cryphonectria parasitica at least. The Figure 2. showed the compatibility between the two groups.

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Pests and virus vectors

THE LADYBIRD FANTASY - PROSPECTS AND LIMITS TO THEIR USE IN THE BIOCONTROL OF APHIDS

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Introduction

Predators are generally considered to be less effective biocontrol agents than parasites. This is supported by many observations: out of 93 cases of "substantial" or "complete" biological control reported by van den Bosch & Messenger (1), only 10 cases of "substantial" and two cases of "complete" control involved only predators. The reductions in host density below the enemy free value (q value) by parasitoids are reported (2) to be an order of magnitude greater than by predators.

The famous exception is the control of the cottony-cushion scale, *Icerya purchasi*, by the ladybird beetle, *Rodolia cardinalis*. This outstanding success resulted in the widespread and haphazard introduction of natural enemies, which has been referred to as the period of the 'ladybird fantasy'(3). Many ladybirds were introduced during this period and apart from the above success this was possibly also due to the way these beetles have been perceived for centuries. The prevalence of holy attributes in their common names, in all European languages, might be seen to indicate divine intervention in pest aphid control.

Population Dynamics of Aphids and Ladybirds

Aphid populations characteristically show dramatic changes in abundance in time, with the species peaking in abundance at different times. That is, the prey of aphidophagous ladybirds occurs in patches that vary in quality both in space, and above all in time. Early studies revealed that the survival of the first instar larvae of ladybirds is dependent on an abundance of young aphids. This defines the aphid population density below which any eggs the ladybirds lay are unlikely to survive. The time from egg hatch to pupation in ladybirds spans more than one generation of aphids and is similar in duration to the period for which colonies or patches of aphids contain sufficient prey to sustain ladybird larvae. In addition to feeding on aphids ladybird larvae and adults will readily eat conspecific eggs and larvae. Both egg (5) and larval (6) cannibalism have frequently been observed in the field especially when prey is scarce. Field studies indicate that ladybirds tend to lay their eggs early in the development of an aphid colony and their larvae pupate just prior to the aphids in the patch becoming scarce.

Optimal Foraging by Ladybirds

A simulation model of the interaction between aphids and ladybirds, which takes the minimum aphid population density requirements of the first instar larvae of ladybirds and the risk of cannibalism into account, indicates that the best strategy is for the ladybirds to lay a few eggs at the beginning of the development of aphid colonies (7). If they lay their eggs later the larvae will not mature before the prey becomes scarce. In addition, if many eggs are laid, the larvae reduce the rate of increase of the aphid colony and cause an earlier decline in abundance. If this happens the larvae resort to cannibalism to survive. This results in the production of a few small adults, which are unlikely to overwinter successfully and have a low potential fecundity (8). That is, if ladybirds are to maximise their fitness they should lay a few eggs early in the development of an aphid colony (Fig. 1).

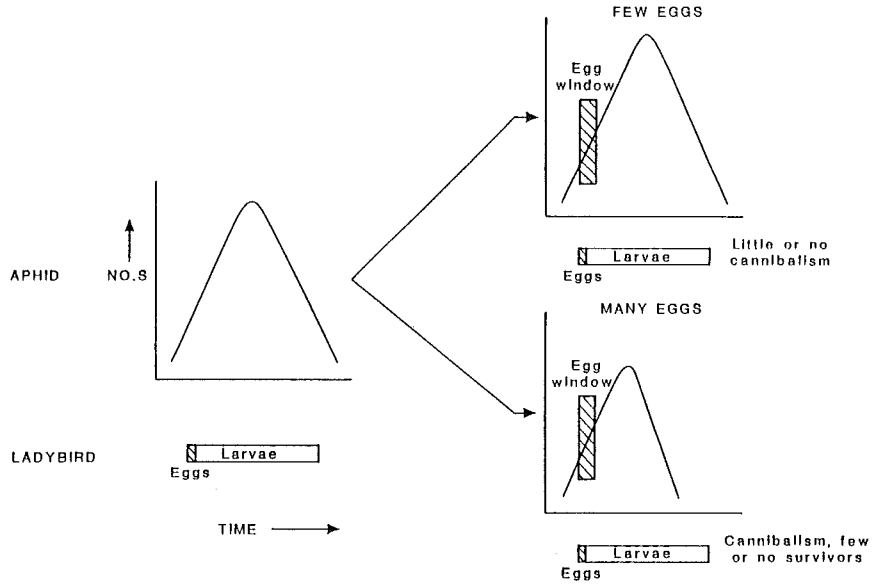

Fig. 1. Diagram illustrating when in the existence of a patch of aphids ladybirds should lay eggs, and how many they should lay in order to maximise their fitness.

What evidence is there that ladybirds forage optimally? *A. bipunctata* lays eggs on apple trees, nettles and wheat in this sequence as the aphid populations develop on these plants (9). In these different habitats most of the eggs are laid over a short period of time before each of these aphids peak in abundance (10). What causes the adults to cease laying eggs when the aphids are still increasing in abundance? In the laboratory, females of *A. bipunctata* kept on their own show a marked reproductive numerical response to increase in aphid abundance. In the presence of conspecific larvae, however, they are extremely reluctant to lay eggs and attempt to leave the area even though aphids are abundant. The females are responding to the chemical tracks left by the larvae, which appear to be acting as an 'oviposition inhibiting pheromone'. It is likely that the adaptive significance of this response is that it reduces the incidence of cannibalism. The consequence of this response is that fewer eggs are laid, which restricts their effectiveness as biocontrol agents.

Biological Control

The above we believe is characteristic of predators that are long lived relative to their prey. In the case of the ladybird, *Rodolia cardinalis*, an egg is laid under an adult female scale or its egg mass, and the lava completes its development by consuming the scale and its eggs (11). That is, the generation time of *Rodolia* and its prey are comparable. In the field, ladybirds in general are successful in controlling univoltine scales (1), but not polyvoltine aphids (12) and mites (13).

Thus instead of parasites vs predators there is a continuum of ratios of the generation times of the natural enemies to those of their prey (GTR), which is correlated with the q-values. In natural enemy/prey systems where the GTR is much larger than 1, the corresponding q-values are large. In parasite/host, predatory mite/herbivorous mite and *Rodolia cardinalis* /scale systems, the GTR is close to 1 and the corresponding q-values are small. Ladybirds successfully control their prey when the GTR is small and not so when the GTR is large as is the case for all aphidophagous ladybirds.

Conclusions

Thus ladybirds and other insect predators are unlikely to reduce greatly the rate of population increase in aphids. This leaves only one other means of reducing aphid rate of increase, which is to reduce the quality of the host plant for aphids. Most crops can be selected or genetically

engineered to be considerably more resistant to aphids. The prospect of using plant resistance to reduce pest aphid abundance is much more promising and largely unexploited.

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RESISTANCE OF SPRING TRITICALE TO GRAIN APHID

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Introduction

Importance of winter and spring triticale in Polish agriculture is increasing during the last decades. Similarly to other cereals, number of fungous diseases and pests decreasing quality and quantity of the triticale yield was arrived to that cereal crop. One of the most important group of insect pests on cereals are aphids (1). There are evidences that they occur on spring and winter triticale as well (2, 3).
A strong tendency to reduce even prophylactic application of synthetic pesticides led us to alternative plant protection methods. One of them is natural plant resistance against the herbivorous pests. There are good examples of moderately resistant wheat varieties to cereal aphids (4, 5, 6). On the other hand, both antixenotic and antibiotic resistance of the triticale to *Metopolophium dirhodum* (Walk.) and *Rhopalosiphum padi* (L.) was also reported (7, 8).
Present paper reports on the grain aphid (*Sitobion avenae* Fabr.) reaction to two Polish spring triticale cultivars.

Material and Methods

Aphids and plants
The grain aphid *S. avenae* came from the stock culture kept in an environmental cabin at the University in Siedlce. Seedlings of two spring triticale cultivars: Gabo and Jago were used in the experiments. The seedlings were grown in a medium nutrient compost in plastic pots 8.5 cm in diameter and 7.2 cm high. Plants were regularly watered and no extra fertiliser was added.
Entomological tests
Apterous adults were caged individually on four day old seedlings of the tested cultivars and allowed to deposit nymphs. After 24 hours, one nymph remained on a single plant; other offspring and the adult were removed. Development time, survival and reproduction of the individuals were observed daily until it's death. Tests were conducted in an environmental cabin at photoperiod 16h : 8h (light : dark) and temperature 20°C ± 2°C. Prereproductive period, daily fecundity, intrinsic rate of natural increase and percentage of survival were determined (6).
EPG recordings
Apterous adults were connected to 2 cm gold electrode, (20 μm wire in diameter), using conductive silver paint. Second electrode was inserted into the pot containing four day old triticale seedlings. The attached aphids were put on the central part of the seedlings and 24 h EPG recordings were performed by acquisition to a PC hard disk. The EPG recordings were conducted in Faraday's cage and analyses of EPG patterns were performed using STYLET 2.2 software on base of the waveform patterns

classified by Tjallingii (9). The considered aphid activities were: non-probing (NP), pathways (C), sieve element salivation (E1), sieve element ingestion (E2) and xylem ingestion (G). Number and time of the pattern duration for each model were estimated.

Results

The conducted experiments proved much higher level of the resistance to *S. avenae* in Gabo cultivar than Jago one at the seedlings stage. The aphids occurred on Gabo seedlings showed much longer time of the prereproductive period (time from the birth to maturity), much lower survival and daily fecundity and in addition lower value of intrinsic rate of natural increase (Tab. 1).

Table 1. Growth and development parameters of the grain aphid on spring triticale seedlings.

Growth and development parameters	Triticale cultivars	
	Gabo	Jago
Prereproductive period (days)	10.70 a	9.80 ab
Daily fecundity	0.47 b	1.45 a
Intrinsic rate of natural increase (r_m)	0.0267	0.1576
Survival (%)	15.20	75.00

Values in rows not followed by the same letter are significantly different at the 0.05% level (Duncan's test).

During the 24 hours EPG (electrical penetration graphs) recordings the grain aphid behaved quite different on those two spring triticales (Fig. 1).

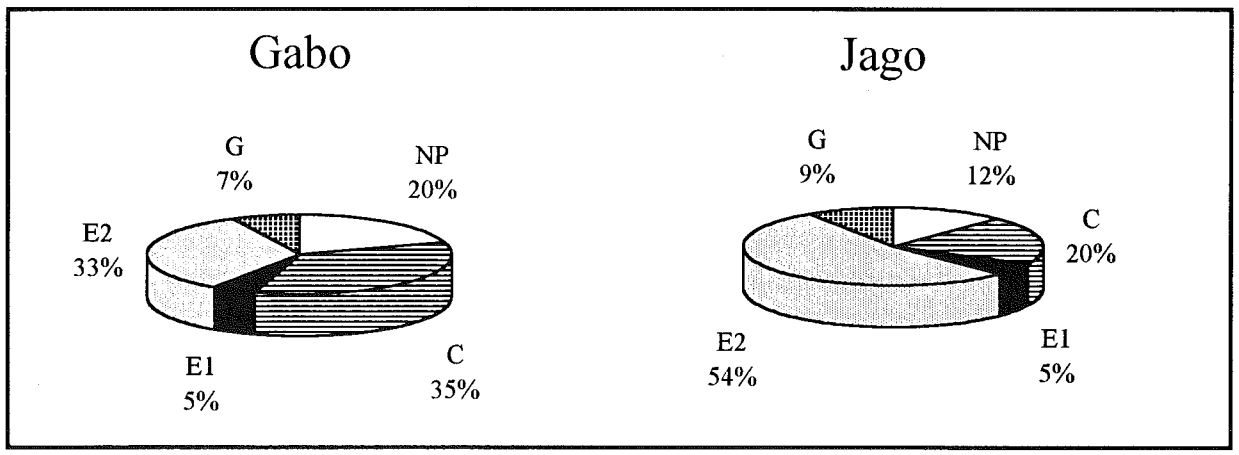

Fig.1. Percentage of the grain aphid activities on the spring triticale cultivars during 24h EPG recordings. NP - non-probing, C - pathways, E1 - sieve element salivation, E2 - sieve element ingestion, G - xylem ingestion.

There was much more aphid probes and clearly longer time of non-probing on the Gabo cvs, however average time of non-probing was shorter (Tab. 2). Similar results

were obtained for total time of pathway phase (penetration of epidermis and mesophyll tissues) and number of paths. Aphids fed on the moderately resistant cultivar showed much shorter phloem phase, this was clear in both total and average time of salivation into sieve elements and phloem ingestion. The phloem salivation and phloem ingestion were also twice more frequent and appeared much faster on the resistant seedlings. The xylem ingestion was slightly longer in aphids fed on Jago cultivar (Tab. 2).

Table 2. The grain aphid activities on the spring triticale seedlings during 24h of the EPG recordings.

EPG Parameters	Triticale cultivars	
	Gabo	Jago
Total time of non-probing	17397.5 a	10569.4 a
Number of non-probing	57.4 a	25.7 b
Average time of non-probing	303.1 a	411.3 a
Total time of pathway phase	29935.2 a	17306.4 b
Number of paths	79.8 a	39.6 b
Average time of path	375.1 a	437.0 a
Time before 1st probe	478.9 a	1050.3 a
Total time of sieve element salivation	4052.5 a	4573.5 a
Number of sieve element salivation	25.3 a	13.3 b
Average time of sieve element salivation	160.2 b	343.9 a
Time before 1st sieve element salivation	6635.5 a	9073.6 a
Total time of phloem ingestion	28733.3 a	45892.9 a
Number of phloem ingestion	11.0 a	5.0 b
Average time of phloem ingestion	2612.1 b	9178.6 a
Time before 1st phloem ingestion	7224.2 b	14808.3 a
Total time of xylem ingestion	6281.5 a	8057.8 a
Number of xylem ingestion	6.6 a	5.0 a
Average time of xylem ingestion	951.7 a	1611.6 a

Values in rows not followed by the same letter are significantly different at the 0.05% level (Duncan's test).

Discussion

Breeding of the resistant cultivars against the herbivorous insects requires a source of the resistant plant material and knowledge of the resistance mechanisms. Results presented here showed that Gabo cultivar is much more resistant than Jago at the seedlings stage. The resistance was clearly connected with different feeding behaviour, especially with quite dramatic reduction of the phloem phase on the resistant triticale. This resulted in slower development and growth reduction of the grain aphid on Gabo cultivar. So this cultivar was carrying some source of the aphid resistance at the seedlings stage. The level of the resistance might be pretty high since the susceptible here Jago, on the other hand was considered as less accepted spring triticale by the other cereal aphid species (10). Further work on the biochemical mechanism of the Gabo cultivar resistance that might be helpful for the breeders is in progress.

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EVALUATION OF CEREALS WITH RESISTANCE TO APHIDS

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Introduction

Aphids are the most important pests of cereals. In Europe, *Sitobion avenae* (FABR.), *Rhopalosiphum padi* (L.), *Metopolophium dirhodum* (WALK.) and *Rhopalosiphum maidis* (FITCH) are the most important cereal aphid species. The cultivated forms of cereals are more preferred by these aphids to the wild graminees and cultivated cereals are therefore more endangered. The aphids feed in the plant phloem and take nutrients away and thereby reduce the plant growth. Moreover, some transmit plant viruses, for example the cereal aphids transmit different strains of the barley yellow dwarf virus (BYDV). Especially in winter barley this virus is able to reduce yield considerably. To prevent these losses repeated applications of insecticides are necessary. In future the inbreeding of resistance genes in new cereal varieties may be helpful, to reduce or to eliminate these treatments. Therefore sources of resistance must be found and selected for plant breeders. A problem exists in finding resistance against the individual aphid species.

Resistance is defined as the property of a potential host plant to prevent or make more difficult the development of a pest on it. Therefore, it is necessary to study the development of the pest for judging resistance of a plant.

Depending on the effect of resistance factors on the pest we can differentiate between „nonpreference" (nonacceptance, antixenosis) and „antibiosis" (1).

In contrast, tolerance is defined as the property of a potential host plant to resist the development of a pest with low or without symptoms. To judge the tolerance of a plant therefore it is necessary to estimate the damaging effect of the pest or the intensity of plant symptoms.

Different phases can be distinguished in the relationship between aphid and its host plant (2).

Migrant aphids are attracted especially during the flight period by the colour of plants and/or in particular by odorous substances.

During the following conjunction period after there landing the aphids make probes to find a suitable host plant. Substances on the plant surface, especially waxes, are effective as attractants or repellents. In the same manner structures on the plant surfaces, for example hairs, can influence the reaction of the aphids. On plants accepted as suitable, the aphids settle or may leave the plant. The later case is described as „antixenosis".

During the exploitation, principal chemical components in the plant influence the settlement and development of the aphids. Less contents of essential amino acids or an unfavourable proportion between sugar and amino acids in the phloem sap are recognised as resistance factors. In case of antibiosis also the content of secondary plant substances, for example flavonoids such as DIMBOA or alkaloids such as gramine may be important factors.

Resistance breeding can have influence on such plant factors that have effects during attraction, conjunction and/or exploitation. Therefore it is necessary to obtain more knowledges about resistance mechanisms.

Resistance of plants against aphids in most cases is only quantitative. Therefore parameters are necessary to estimate the development of aphids and for resting the statistical significance of observed differences.

Different methods for the evaluation of plant resistance to aphids described in the literature include

- survival time of the starting population,
- fertility of the females
- duration of the different developmental stages of the larvae and their weight and
- mean developmental time and increase.

The registration of the probing behaviour by electrical penetration graph or estimation of produced honeydew may also give helpful informations about resistance properties of the plants.

In each of these cases, it is necessary to screen not only under controlled but also under field conditions. Here, more complex conditions affect the aphid population as well as the morphology and physiology of plant.

In genebanks there are many accessions collected from different geographic origins and there exist high degree of genetic variability in these genetic resources. But at the moment we have little or no knowledge about their resistance to pests and diseases. In co-operation with the Institute for Plant Genetic and Cultivated Plants in Gatersleben we have initiated an evaluation of some cereal forms of the genebank Gatersleben for resistance to aphids.

Material and Methods

The experiments were carried out using *Sitobion avenae*, *Rhopalosiphum padi* and *Metopolophium dirhodum*. Young plants were tested in a climatic chamber with constant conditions (22° C, 16 h light). Two larvae at the fourth stage were placed on ten plants of each accession when the plants have reached the second to third leaf stage. Fourteen days later the numbers of larvae, wingless and winged females were counted. The tests were replicated three times. Statistical evaluation was carried out by analysis of variance followed by Dunnet-Test. For comparing the test results we estimated the relations of the means of the tested accessions to the means of the standard (=1). We used as standard varieties „Alcedo" for wheat, „Erfa" for winter barley and „Haisa" for spring barley. These varieties were also used as host plants in the mass rearing of the aphids.

Results and Discussion

Up to now 203 accessions of barley and wheat were tested. The results showed that 29 accessions of the cereals supported a significant lower number of aphids compared to the standard varieties, therefore they were called as resistant (Tab. 1). But also we were able to detect accessions that were more susceptible than the standards.

Tab.1: Number of tested wheat- and barley accessions

Aphid species	No. tested	No. resistant
Rhopalosiphum padi	152	5
Sitobion avenae	125	16
Metopolophium dirhodum	109	12

Accessions with resistance to *M. dirhodum* were detected only in barley accessions. In the case of *Sitobion avenae* and *Rhopalosiphum padi*, the majority of resistant forms was found in wheat.

Only *Triticum baeoticum* BOISS. em. SCHIEM., *Hordeum jubatum* L. and *Hordeum bogdanii* WILENSKY showed resistance to more than one aphid species. The results of accessions tested under controlled conditions are as summarised in table 2.

Tab. 2: Index of resistance for the wheat and barley accessions with significant lower multiplication of aphids (index of resistance for the standard = 1)

Code	botanical group	*S. avenae*	*R. padi*	*M. dirhodum*
ATRI 1152	*Triticum aestivum* L. em. FIORI et PAOL. var. *milturum* (ALEF.) MANSF.	0,05		
ATRI 1822	*Triticum durum* DESF. var. *reichenbachii* KÖRN.		0,29	
ATRI 2737	*Triticum aestivum* L. em. FIORI et PAOL. var. *leucospermum* (KÖRN.) MANSF.	0,23		
ATRI 3419	*Triticum spelta* L. var. *coeruleum* ALEF.	0,17		
ATRI 4045	*Triticum turgidum* L. var. *coeleste* ALEF.	0,14		
ATRI 5416	*Triticum zhukovskyi* MEN. et ERIZ.	0,20		
ATRI 7131	*Triticum sphaerococcum* PERC. var. *globosum* PERC.		0,56	
ATRI 7301	*Triticum timopheevi* ZHUK. var. *nigrum* ERICZ.		0,39	
ATRI 768	*Triticum polonicum* L. var. *chrysospermum* KÖRN.		0,42	
ATRI 8541	*Triticum aestivum* L. em. FIORI et PAOL. var. *wernerianum* (KÖRN.) MANSF.	0,25		
ATRI 901	*Triticum dicoccon* SCHRANK var. *noricium* KÖRN.	0,12		
HTRI 1135	*Triticum aestivum* L. em. FIORI et PAOL. var. *milturum* (ALEF.) MANSF.	0,11		
HTRI 11557	*Triticum baeoticum* BOISS em. SCHIM var. *baeoticum*	0,11		0,06
HTRI 2401	*Triticum x* sp. (Kreuzung)	0,14		
HTRI 2692	*Triticum aestivum* L. em. FIORI et PAOL. var. *milturum* (ALEF.) MANSF.	0,13		
HTRI 6735	*Triticum urartu* THUM. *ex* GANDIL	0,12		
HTRI 7304	*Triticum x soveticum* ZHEBRAK.	0,19		
HTRI 7914	*Triticum aestivum* L. var. *lutescens* (ALEF.) MANSF.	0,16		
GRA 1000	*Hordeum chilense* ROEM et SCHULT.			0,18
GRA 605	*Hordeum bulbosum* L. *subsp. bulbosum*			0,47
GRA 615	*Hordeum violaceum* BOISS. *et* HU et			0,37
GRA 644	*Hordeum jubatum* L.	0,09	0,27	0,17
GRA 647	*Hordeum bogdanii* WILENSKY	0,01		0,16
GRA 876	*Hordeum turkestanicum* NEVSKY			0,24
HHOR 3097	*Hordeum vulgare* L. *con*var. *vulgare* var. *hybernum* VIB.			0,56
HHOR 4127	*Hordeum vulgare* L. *con*var. *vulgare* var. *hybernum* VIB.			0,57
HHOR 4157	*Hordeum vulgare* L. *con*var. *vulgare* var. *hybernum* VIB.			0,51
HHOR 4159	*Hordeum vulgare* L. *con*var. *vulgare* var. *hybernum* VIB.			0,52
HHOR 991	*Hordeum vulgare* L. *con*var. *vulgare* var. *hybernum* VIB.			0,66

The screening of these accessions under field conditions is in progress.

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THE IMPORTANCE OF TAXA BELOW THE SPECIES LEVEL FOR DEFINING THE VARIABILITY WITHIN SPECIES

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Introduction

Because they feed on phloem and are vectors of viruses aphids are important pests of crops. Up to now it has not been possible to secure the health of plants only by applying pesticides. Therefore, the resistence of plants to aphids and viruses has frequently been investigated. The aphids concerned are often very common species, which consist of subspecies and races whose biological performance can differ considerably. In the absence of an exact statement of the status of the aphids studied it is often very difficult to compare the results of different authors.

Material and methods

The biology of 4 strains of the pea aphid *Acyrthosiphon pisum* (1st. strain: *A. pisum destructor*, marking host plant: *Pisum sativum*, alatae males, green coloured; 2nd. strain: *A. pisum*, marking host plant: *Trifolium pratense & Medicago sativa*, apterous males, red coloured; 3rd. strain: *A. pisum*, marking host plant: *Trifolium pratense*, apterous males, red coloured; 4th. strain: *A. pisum*, marking host plant: *Lotus uliginosus*, apterous males, yellow coloured) were studied. These strains have been cultivated parthenogenetically for several years and are isolated from one another in secure cages.
The biological performance of the aphids was determined on specific plants at constant temperatures of 15, 20 and 25 °C and a photoperiod of 16 h . The following parameters were determined:
1. Developmental time (D),
2. Adult weight,
3. Mean relative growth rate (MRGR), and
4. Fecundity (numbers of embryos).

To reduce the effects of previous host plant all strains of *A. pisum* were reared on bean.

Results

In all strains the developmental time decreased with increase in temperature. Both red strains (strain 2 & 3) produced the largest adults at 20 °C. The true green pea aphid (strain 1) produced similar sized aphids at 15 °C and 20 °C but significantly smaller individuals at 25 °C (Fig. 1). The yellow strain (strain 4) produced similar sized adults at all temperatures. The mean relative growth rates of the strains did not differ at 15 °C but at the higher temperatures the red strains performed best (Fig.2). At 20 °C the yellow strain did worst, and at 25 °C the performance of the green strain declined significantly.
The numbers of the embryos varied similarly. The red strains had more well developed embryos at the higher temperatures. At 20 °C the yellow strain had the least well developed embryos and at 25 °C the green strain had significantly fewer embryos with pigmented eye spots (Fig. 3).

Discussion

Aphids of the *A. pisum* complex are a pest in Germany as they infest pea and *Medicago* crops. However, different strains infest these two crops. Strains 1, 3 and 4 do not hibernate on *Medicago*. In choice experiments using pea, clover, *Medicago* and different species of vetch (*Vicia villosa*, *V. cracca* and *V. hirsuta*) the sexuparae of strain 1 preferred vetch. Although under laboratory conditions the fundatrices of strain 1 can develop successfully on clover, under natural conditions they do so only

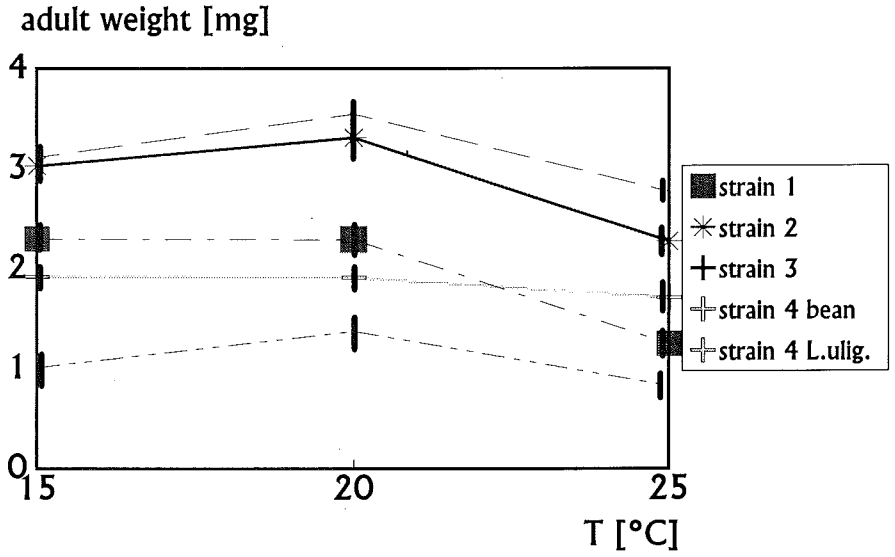

Fig. 1. The relationships between the adult weight of apterous individuals of 4 strains of *A. pisum,* reared on *Vicia faba* or *Lotus uliginosus,* and temperature.

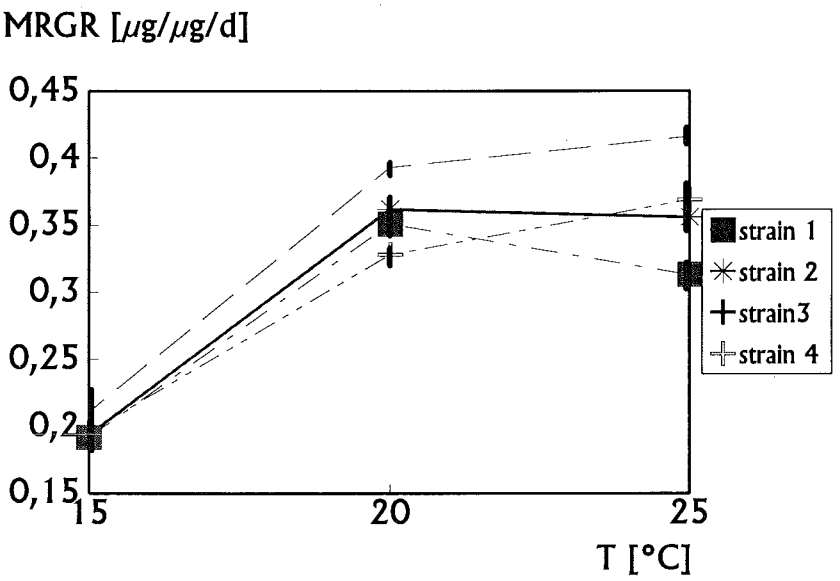

Fig. 2. The relationships between the MRGR of apterous individuals of 4 strains of *A. pisum,* reared on *Vicia faba,* and temperature.

on species of vetch. The strains of *A. pisum* differ in -colour, -hostplants, -showing host alternation, -temporal and quantitative production of alatae, -having alatae or apterous males.
In terms of growth and reproduction at high temperatures strains 2 and 3 perform best.
In other areas different combinations of characters can be expected. *A. pisum* was introduced approximately 120 years ago into North America and in 1982 into Australia (1). From the originally small number of clones that colonized these continents a multitude of new clones have developed, with

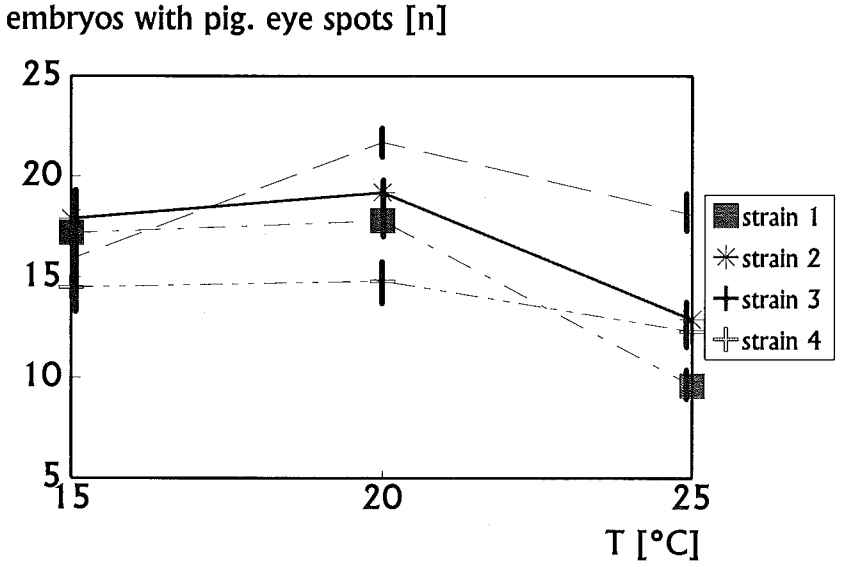

Fig. 3. The relationships between the number of embryos with pigmented eye spots in apterous individuals of 4 strains of *A. pisum,* reared on *Vicia faba,* and temperature.

different characters to those observed in Europe. For example the red and green clones that infest *Medicago* have males, which are either all alate, 50 % alate or all apterous (2). In Germany the green strain 1 (*A. pisum destructor*, which colonise pea) only produces alate males (3).
In other aphid species host races are also being described, for example, in *A. gossypii* of English origin (4). Strains originally collected from chrysanthemum can not be transfered to cucumber and *vice versa*. Dutch material of *A. gossypii* also includes genetically distinct host races that infest cucumber and chrysanthemum (5). In cereal aphids, *Sitobion avenae* collected from different host species (6) and the different colour strains of *S. avenae* collected from winter wheat show differences in performance (7).

Studies of persistent viruses, especially, should consider the variability of the vector - virus relationship as viruses show a high degree of specificity to both plant and aphid. Virus transmission experiments using pea aphid are frequently reported. However, the results are contradictory as different virus - vector - host plant - complexes were used. For example, Erhardt and Schmutterer (8) used *Vicia faba*, others used *Lathyrus odoratus* (9) or *Pisum sativum* (10). The studies of Hinz (11) clearly show differences in the ability of pea aphid strains to transmit virus. Transmission experiments with hybrids of *A. pisum* strains have shown, that the transmission character of a virus is genetically fixed and the ability to transmit it inherited dominantly (12).
This variability in the biology of the subspecies and host races of pest aphids is of fundamental importance to studies on resistance and virus transmission. The identification of species, and *especially taxa below the species level* is difficult, and can only be done with the help of a specialist.

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