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Integrated Control of Pome Fruit Diseases

editor:

L. Parisi

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Organisation Internationale de Lutte Biologique et Intégrée contre les Animaux et les Plantes Nuisibles
West Palaearctic Regional Section / Section Régionale Ouest Paléarctique

IOBC / WPRS

Working group "Integrated Plant Protection in Orchards"

Subgroup "Integrated Control of Pome Fruit Diseases"

PROCEEDINGS OF 5th WORKSHOP ON INTEGRATED CONTROL OF POME FRUIT DISEASES

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Opening of the 5th Workshop on Integrated Control of Pome Fruit Diseases

Tuesday 24 August 1999

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Président de la Commission "Recherche, Développement Technologique, Affaires Internationales et Européennes et Enseignement Supérieur"

Une particularité française : le découpage administratif et les différents échelons de décisions

Depuis le début des années 80', l'une des particularités du système politico-administratif français est l'existence de différents entités (l'Etat, les régions, les départements et les communes, sans oublier l'Union Européenne) dotées de compétences spécifiques gérées de manière autonome. Chaque collectivité peut donc définir les normes de ses actions et choisir les modalités de ses propres interventions.

Le Conseil Régional des Pays de la Loire, que je représente ici, a donc compétence pour intervenir dans la promotion du développement économique, dans les domaines scientifique, notamment celui de la Recherche, culturel, de la formation et dans l'aménagement du territoire régional.

The specificity of the french political and administrative system is the existence of different local entities. Each local authority has its own competences and a managerial autonomy. It is the case for Regional Research policies.

L'intérêt d'une politique de recherche régionale

En France, les politiques conduites au niveau national en matière de recherche sont axées, d'une part, sur la reconnaissance et l'excellence internationale à travers une forte production de publications. Et, d'autre part, sur une répartition territoriale des structures de recherche dont l'objectif est d'éviter la dispersion des hommes et des moyens dans des domaines proches. On assiste ainsi à la constitution de pôles de compétences regroupées sur un site.

Une région française, prend en considération d'autres paramètres pour développer et mener une politique indépendante dans ce domaine : elle privilégie le lien entre la recherche et le développement économique de son « territoire », à travers ses filières d'excellence.

Ce lien permet aux interrogations « scientifiques » des entreprises régionales de trouver leurs réponses dans les structures de Recherche soutenues par la région. En traitant les problèmes « sur le terrain » grâce à la recherche appliquée, elle se diversifie au sein d'une filière et progresse plus en amont ou en aval du problème soulevé.

Compared with French national policy, the Region of Pays de la Loire fosters the link between research and economic development of its area, through its leading industries. Applied research can comply with different levels of industrial and scientific needs.

D'autre part, la notoriété d'une région au niveau international en matière de recherche encourage l'implantation de nouvelles entreprises du secteur concerné. C'est pourquoi, la Région s'applique à maintenir et à développer sur son territoire des équipes de recherche performantes dans ses filières d'excellence.

The reputation of a region in terms of research facilities is a key factor of new firms establishement. That is why the Region of Pays de la Loire focus on the developement of successful searcher teams in its leading industrial sectors.

La Recherche dans les Pays de la Loire : bilan et évolution

Les indicateurs économiques placent la Région des Pays de la Loire à la 4ème place des Régions françaises, mais les indicateurs de la Recherche et du Développement Technologique la placent en 10ème position. Si l'effectif des chercheurs a doublé depuis 1990, la Région des Pays de la Loire ne représente encore que 2,5% de la Recherche française.

Les explications de cette situation sont nombreuses. Les universités sont jeunes, hormis l'Université Catholique de l'Ouest, les écoles d'ingénieurs également. Le tissu économique de PME, axé surtout sur des activités de main d'œuvre, ne se prête pas toujours à un développement de la matière grise. Les grands groupes présents n'ont souvent dans la Région que des unités de production. Le domaine où existe un continuum est celui de la navale (civile et plaisance).

Nowadays, for different reasons, the Region of Pays de la Loire only represents 2.5% of the French research, but indicators show a positive evolution characterized by an important dynamics of all actors : searchers, laboratories, firms.

Les indicateurs montrent cependant une évolution positive de la recherche dans les Pays de la Loire. Elle est caractérisée par une forte dynamique de tous ses acteurs. Plusieurs équipes de chercheurs ont atteint un rayonnement national et international. Certains pôles d'excellence ont acquis un éventail de compétences leur permettant d'accéder à la compétition scientifique et technologique : l'agroalimentaire et le végétal (le Maine-et-loire est le premier département producteur de semences potagères et florales, le 2ème producteur de pommes avec 10% de la production nationale), les matériaux et la mécanique, la santé, les sciences et technologies marines. Des projets phares se développent dans le secteur de la physique nucléaire, le génie génétique, l'acoustique, le génie naval. Et les PME-PMI disposent d'un réseau de diffusion technologique performant lié à l'Agence Régionale de Développement Technologique, une force pour les Pays de la Loire. Par ailleurs, la Région a fait un effort sans précédent au profit de la recherche avec un budget passant de 14,7 millions de francs en 1992 à 48,8 millions en 1998.

La Recherche en Pays de la Loire : les grandes orientations futures

A la veille des négociations avec l'Etat et l'Europe, la Région des Pays de la Loire a élaboré sa vision à long terme de l'Aménagement du territoire régional. La stratégie adoptée s'appuie sur l'idée d'une institution régionale forte qui dans un contexte de mondialisation des échanges et en même temps de recherche d'une solidarité territoriale, se donne la capacité d'accroître son influence et se trouve la mieux placée pour fédérer les acteurs.

Pour les années à venir, le plan stratégique régional a fait de la Recherche, de l'Innovation et des Transferts de technologie une priorité.

For the next years, first priority will be given to research, innovation and technological transfers.

La Région souhaite doubler, en moins de 10 ans, le potentiel de recherche régionale en confortant ses filières et les disciplines à haute spécificité. En équipant les laboratoires au meilleur niveau afin d'attirer des chercheurs reconnus ou de jeunes thésards, pour s'implanter dans notre région. En soutenant les programmes de recherche performant qui émergeront du programme « Pays de la Loire Recherche 2006 ». Enfin, en fédérant les chercheurs de la région avec ceux des régions voisines, notamment la Bretagne, afin d'obtenir la taille critique pour accéder aux programmes de recherche internationaux.

The objective is to reach a twice more human and material research potential than today in the next 10 years in our region.

La Région souhaite également encourager les partenariats entre laboratoires, chercheurs et entreprises pour que tout projet de recherche trouve une déclinaison en entreprise. La contrepartie de l'aide régionale apportée à la recherche consiste à établir une relation privilégiée avec une ou plusieurs PME-PMI ligériennes au travers de la mise à disposition d'un chercheur, d'un thésard ou d'un étudiant. Les grands laboratoires de la région doivent donner l'exemple et sont prêts à le faire. S'agissant des dossiers en négociation, signalons notamment la mise en œuvre d'un vaste programme de recherche sur le végétal, l'horticulture, les semences et les terroirs viticoles qui associe l'INRA, l'Université et les professionnels, et conforte la reconnaissance internationale du pôle végétal angevin. L'objectif est d'établir 1000 projets de cette nature sur dix ans. L'Agence Pays de la Loire Innovation, seule structure régionale agissant dans le domaine du développement technologique, en assurant le suivi.

Les changements profonds de la compétitivité économique des entreprises les engagent à se mobiliser plus fortement en direction de l'innovation : elles doivent réaliser un effort plus grand dans leur propre recherche, un partenariat plus marqué avec la recherche publique, des démarches d'alliances nationales ou transnationales. Dans ce but, le Région souhaite renforcer les moyens d'ingénierie de projets et veille stratégique notamment au sein de l'Agence Pays de la Loire Innovation, et coordonner les moyens humains au service du développement technologique : création d'entreprises innovantes, partenariat chercheurs-entreprises, partenariats interrégionaux notamment avec la Bretagne. Le Pôle Agronomique de l'Ouest constitue un exemple réussi de ce type d'association interrégionale d'entreprises et de chercheurs dans le domaine de l'agro-alimentaire. Par ailleurs, grâce aux technopoles, de jeunes entrepreneurs ou chercheurs ont pu développer des entreprises. Dans ce but, une «fédération régionale» des technopoles et autres acteurs ligériens, sous l'égide du Conseil Régional, apparaît comme une piste de travail intéressante à mener en cohérence avec l'ANVAR.

Partnerships between laboratories, searchers and firms will be fostered so that each research project can find an implentation in a firm. The Region also wants to encourage firm creations and interregional partnerships with Brittany.

Enfin, la région veut renforcer l'utilisation des technologies de l'information et de la communication (TIC). Le réseau interrégional Ouest Recherche, mis en place dès 1992, a permis la transmission de données et de produits multi-média entre les universités, centres de recherche et IUT. Un nouveau réseau interrégional à haut débit entre la Bretagne et les Pays de la Loire doit être opérationnel dès le début de l'an 2000 et se développera sur les dix prochaines années. Il concerne en priorité la recherche et l'enseignement supérieur, mais ses

applications pourront servir d'autres domaines, comme la santé, la formation continue, la culture, le tourisme. Il devrait constituer une force d'attraction pour l'implantation d'entreprises et le développement de nouvelles activités.

The Region will reinforce the use of information and communication technologies. A new network related to research and higher education will be operational next year. This project should be attractive for setting up new firms and developing new industrial activities.

Integrated Control of Pome Fruit Diseases
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50 years of research on biological control

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Abstract : Ever since apples are grown on a commercial basis, apple scab management has always been a major component of production. Scientists have looked at biological control as an alternative strategy for more than 50 years. Over the years, several collections of microorganisms, mostly apple leaf inhabitants, were studied. In the fifties, scientists were encouraged by the great successes obtained with antibiotics for the control of animal diseases and tried the same approach for apple scab. Unfortunately, most of the antibiotics discovered (culture filtrates) were fungistatic, not fungicidal. During the seventies, with the advances in microbial ecology, researchers investigated the interactions between the apple leaf microflora and *V. inaequalis* development. From these studies, it was concluded that several bacteria and fungi interfere with the development of pseudothecia but not enough to provide substantial reduction in inoculum potential. During the eighties, *A. bombacina* was identified as a potential biological control agent because it has the potential to completely eliminate ascospore production. Large field trials were never conducted with *A. bombacina*, so its real potential is still to be determined. In 1997, an another fungal antagonist, *Microsphaeropsis* sp., was identified. This fungal antagonist inhibited up to 98% of the ascospore production when tests were conducted under controlled conditions. However, when tested in the field the real potential is approximately 75 to 80%. The requirements for the commercialization of a biofungicide are difficult to meet, but in a near future it will be possible to improve known antagonists and hopefully discoverer new candidates. Nevertheless, biological control can potentially have a role in an integrated scab management especially for organic growers, small orchards or orchards located near urban areas.

Key words : antibiosis, disease management, mycoparasitism, *Venturia inaequalis*

Introduction

Apple scab, caused by *Venturia inaequalis* (Cooke) G. Wint., is a limitation to apple production wherever apples are grown. Despite years of research and development, it is still the most economically important disease of apple worldwide. Losses due to scab vary from one location to another and depend on disease pressure and prevailing weather conditions. Cost of fungicide also varies as it depends on control programs and products used. As an example, in New York State, the average annual costs for chemical sprays are 250$U.S. per Ha (Burr *et al.*, 1996). During years with high disease pressure, apple scab may make apple production unprofitable.

Apple scab control relies, in most part, on the use of chemical fungicides. There is a slow adoption of resistant cultivars as they generally do not possess the required agronomic characteristics such as fruit quality, yield and fruit storability (MacHardy, 1996). Over the last 25 years, several forecasting systems were developed and proposed to apple growers (Jones *et al.*, 1980 ; Gadoury and MacHardy, 1982). These systems aimed to a better timing of fungicide applications and most of them are based on predicting the severity of infection periods and/or estimating the level of maturity of pseudothecia. In general, these predicting systems are useful only as a general indication or under low disease pressure. In eastern Canada, there is a tendency among the apple growers to return to a calendar spray program

because it is simple and it usually ensures a minimum of scab control.

Because of the problems associated with the intensive use of chemical fungicides among which, cost, risk of resistance development in the *V. inaequalis* populations and sustainability of a control program based only on pesticides, scientists have explored alternative strategies. These strategies have included pruning to increase air circulation and consequently reducing leaf wetness duration, leaf litter burning, use of earthworms to increase leaf decomposition and biological control. This review paper focuses mainly on biological control based on either the introduction of a microbial control agent or on the manipulation of naturally occurring microorganism populations.

Biological control of apple scab

Biological control is often seen as recently emerging from microbial biotechnology, but in fact research on biological control of apple scab has been conducted for more than 50 years. Because of the nature of the *V. inaequalis* life cycle, studies on biological control have focused on either interrupting the overwintering of the perfect stage or on controlling leaf infection during the spring and summer (Figure 1).

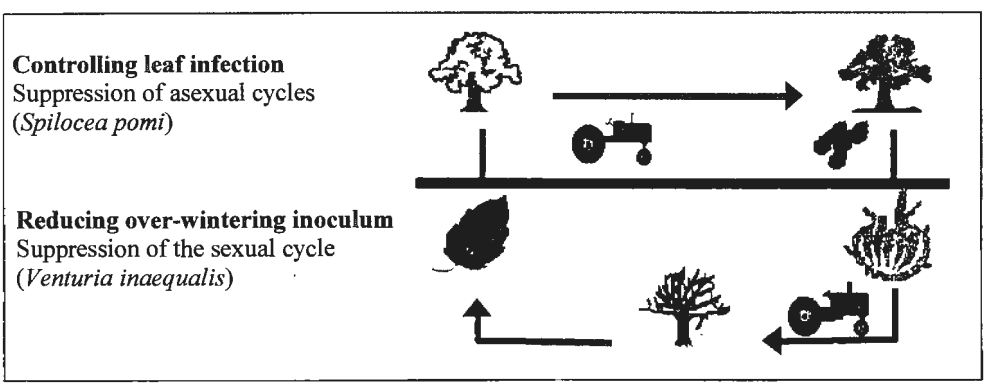

Figure 1. Strategies for biological control

The pioneers

Cinq-Mars in 1949, was a pioneer in the field of biological control of apple scab. He was the first scientist to isolate microorganisms from apple leaves. His collection contained more than 25 different organisms among which, were fungi, bacteria and yeast. Using these organisms, he looked at the interaction of *V. inaequalis* and sterile culture filtrates of the isolated microorganisms. He showed that some of these organisms, mostly *Penicillium* species produced antibiotics that inhibited mycelial growth of *V. inaequalis*. He also demonstrated that some the fungi were able to enhance leaf decomposition and consequently may interfere with pseudothecia development. In 1953, Ross pursued the work initiated by Cinq-Mars and extended the collection of microorganisms from apple leaves collected in Quebec and Nova Scotia. Ross was the first to conduct an *in* vitro experiment to show the effects of leaf decomposition on pseudothecia development. Ross was one of the first scientists to evoke the possibility of using the antagonist itself instead of the antibiotics produced by the organism. A few years later, inspired by the remarkable success obtained with antibiotics for the control of animal diseases, Simard and collaborators (1957) made a new

collection of microorganisms with the hope of finding antibiotic producers. Out of their new collection, they observed that 34 fungi produced antibiotics that significantly inhibited mycelial growth of *V. inaequalis*. Unfortunately, in further tests they observed that the effects of the substances produced by apple leaf inhabitants was mainly fungistactic, not fungicidal.

In 1962, Hirst and Stedman, reported the results and conclusion of several years of research on the supply and liberation of ascospores of *V. inaequalis*. They suggested that low ascospore concentrations observed in some orchards may be due to the presence of naturally occurring saprophytes. However, the first real ecological study of apple leaf microorganisms in relation to *V. inaequalis* was conducted by Crosse *et al.*, in 1968. They reported that fall application of urea interfered with the overwintering of pseudothecia of *V. inaequalis* and favored bacterial population development. They observed that the diversity of apple leaf microflora was modified following urea application. The bacterial populations shifted from one predominantly 'gram positive' to a population dominated by 'gram negative' bacteria. Fluorescent pseudomonads became more important and many were found to supress the development of *V. inaequalis*. Consequently they hypothesized that a fall application of urea may help reducing initial inoculum of *V. inaequalis* by enhancing leaf decomposition, disturbing pseudothecia development, and favoring populations of microbes antagonistic to *V. inaequalis*.

In 1969, Hislop and Cox complemented this work by investigating the effects of fungicides on the size and diversity of populations of microorganisms living on apple leaves. Hislop and Cox's work represented a turning point in the history of scab biological control, as it was the first study to look at a possible integration of microbial and chemical control.

In 1970, Burchill and Cook conducted the first field study on the effects of a fall application of urea on apple leaf fungal flora. They observed that dipping leaves in urea stimulated the leaf colonization by *Cladosporium* sp., *Epicoccum* sp., and *Pistillaria* sp.. Spraying apple leaves with urea had a similar effect on *Cladosporium* sp., but also stimulated *Alternaria* sp. and *Fusarium* sp.. When *Fusarium sporotrichioides* and *F. avenaceum* were inoculated on to leaf disks the development of pseudothecia of *V. inaequalis* was suppressed. From this study they also concluded that the bacterial populations could both stimulate or inhibit pseudothecia development.

The eighties

After a period of lull, Andrews *et al.*, in 1983, reinitiated the research on apple scab biological control. First, they made another collection of apple leaf inhabitants and evaluated the effects of these leaf colonizers on *V. inaequalis* vegetative growth and conidial germination. Fifty microorganisms were evaluated and the eight most antagonistic fungi were *Aureobasidium pullulans*, *Trichoderma viridae*, *Chaetomiun globosum*, *Microsphaeropsis olivacea*, and two unidentified actinomycetes. From a series of experiments, they selected the antagonist *Chaetomiun globosum* based on its efficacy and consistency. From their observations, they suggested that the antagonistic activity was due to both nutrient competition and antibiosis. In parallel to these experiments, Heye (1982), and Heye and Andrews (1983) looked at the possibility to screen fungal antagonists on the basis of their ability to inhibit pseudothecia development rather than vegetative growth. From their fungal collection, they screened 57 apple leaf saprophytes, and selected *Athelia bombacina* because of its ability to completely inhibit *V. inaequalis* pseudothecia development.

From the lab to the orchard

In 1984, Cullen *et al.*, evaluated the potential of *Chaetomiun globosum* as biofungicide against apple scab. A spore suspension of *C. globosum* applied every 1 to 2 weeks to apple trees in an orchard reduced scab severity by 20% as compared to the non treated control. In

further trials, Boudreau and Andrews (1987) demonstrated that *C. globosum* did not colonize apple leaves even when biological control activity was observed. They observed that dead cells of *C. globosum* were as effective as alive cells in preventing leaf infection by *V. inaequalis*. Unfortunately, they also noted that the antibiotics produced by *C. globosum* are short-lived and loose their activity when exposed to common physical regimes such as prolonged light.

A. bombacia was tested under field conditions for its efficacy to reduce ascospore inoculum. In a first field trial, Young and Andrews (1990) showed, using immunocytochemical detection that *A. bombacina*, when applied to naturally infected apple leaves, inhibits both growth of hyphae and initiation of pseudothecia by *V. inaequalis*. From these studies the fungus *Athelia bombacina* Pers. was identified as a potential biological control agent. However, complete pseudothecia inhibition was obtained in field trials only when very high antagonist inoculum doses were used (Heye and Andrews, 1983). When a lower rate was used, pseudothecial inhibition was reduced to only 60 to 70% (Miedtke and Kennel, 1990). More recently, *A. bombacina* was used as a positive control in a field evaluation of potential biocotrol agents (Carisse *et al.*, 2000). In this experiment, with relatively low amounts of inoculum, the ascospore inhibition by *A. bombacia* was 84.2%, although *Trichoderma* sp. and *Microsphaeropsis* sp. were as good as *A. bombacina* in reducing ascospore production.

As the research on the development of biofungicides evolve, the required characteristics of the microbial agents are becoming more clearly defined. To be commercialized, a microbial control agent must be effective at an economic dose defined by the mode of industrial production, be easy to implement by growers and provide observable benefits for the growers. In this context, *A. bombacina* will be difficult to commercialize because of the amount of inoculum required and because large-scale production of *Basidiomycetes* is, in general, costly.

Recently, in 1996, Burr *et al.*, screened 931 strains of bacteria and yeast isolated from apple leaves. They looked at the effects of these microorganisms on mycelial growth, conidial germination, and scab development on seedlings, 92 isolates significantly inhibited *V. inaequalis* mycelial growth, 32 inhibited conidial germination and 104 of them significantly reduced scab severity on apple seedlings. Unfortunately, they did not observe a correlation between *in vitro* and *in vivo* tests and the promising bacteria were not tested under field conditions. In this study, Burr *et al.* (1996), aimed at developing a microbial control agent to be used against the leaf infection, but this approach is hazardous because it is much more difficult to attack *V. inaequalis* in its active phase (parasitic phase) than when it is in a saprophyte phase. Futhermore, it will be difficult for a biofungicide to compete with available chemical fungicides on the basis of cost and efficiency. Several researchers (Palmiter, 1946, Burchill and Hutton, 1965, Palmiter, 1946) demonstrated that reducing the ascospore inoculum results in smaller disease pressure the following spring and consequently make secure scab management, possibly with less fungicides sprays. But it is only in 1993 that MacHardy *et al.* demonstrated that it is possible to delay the first fungicide applications up to pink stage when the inoculum potential is very low. This strategy of biological control was investigated again by Philion *et al.* in 1997. From another collection of apple leaf fungal inhabitants they screened the isolates for their ability to inhibit ascospores production in *in vitro* tests. The candidates were also selected on the basis of their ability to colonize apple leaves under the relatively cold conditions generally encountered in the fall, their potential for industrial production and multiple modes of action. The candidates *Diplodia* sp., *Trichoderma* sp, *Ophiostoma* sp., *Microsphaeropsis* sp. and *M. arundinis*, were further tested in the field with detached scabbed apple leaves. The fungal antagonist *Microsphaeropsis* sp. (strain P130A) was selected (Benyagoub *et al.*, 1998, Bernier *et al.*, 1996, Philion *et al.*, 1997) and tested in large plots. The efficacy of this isolated to inhibit ascospore production varied from

98% in *in vitro* tests to 75% in apple orchards.

Future of biological control

Despite the tremendous amount of research and publications on biological control of plant pathogens, there are only few biofungicides registered in the world, most of them being commercialized for specific niches such as high value crops for which there is a demand for pesticide free products. To be commercialized, bifungicides must be effective (at least as compared to available chemical fungicides), consistent in it effectiveness, adaptable to IPM, thus compatible with chemicals and other biological treatments, must be compatible with common agricultural practices, environmentally friendly and not more expensive than available chemicals. Furthermore, biofungicides are often made of a single strain of an antagonist that was selected on the basis of its specificity, in practice this limit the use of the biofungicide against other diseases. In the near future, research on biological control will probably focus on enhancing expression of active gene in biocontrol agents such as degrading enzymes. Research on biological control and interactions between plant pathogens and antagonists may help discovering new molecules such as the Strobilurins produced by *Strobilurus tenacellus*.

Most probably biofungicides will have to be used in conjunction with other products in an integrated control program. As mentioned previously, this implies that the biocontrol agent is compatible with chemical fungicides and agronomic practice commonly used. This may result in a more complex management program including some changes in some grower's practices. On the other hand it may provide a more sustainable scab management program because it is based on more than one single strategy. Biological control of apple scab will provide an alternative for organic apple producers, growers located in or near urban areas and home gardeners.

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Protection of apple against fire blight induced by avirulent mutants of *Erwinia amylovora*

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Abstract : Protective effect of two types of *hrp Erwinia amylovora* mutants against fire blight was investigated using two experimental systems: inoculation of apple flowers or apple seedlings in the glasshouse. Our results showed that a *hrp* regulatory mutant exhibited high level of protection when inoculated simultaneously with the virulent strain. In contrast, the *hrp* secretory mutant induced a poor control in the same experimental conditions. Dynamics of bacterial populations in these interactions were studied in the same material by sampling at different intervals after inoculations and plating on selective media. The protective effect observed with the regulatory mutant was consistently associated with a decrease in the population level of the coinoculated virulent strain. This suggested an activation of a host defense response triggered by the regulatory mutant. Indeed, the increase of activity of PAL and POD, two enzymes involved in plant defense response induced by the protective mutant, supports this hypothesis. Conversely the secretory mutant was unable to induce such activities. It is therefore likely that plant defense mechanisms triggered by specific mutant play an active role in the observed protection. Nevertheless other mechanisms may be involved. They are currently under investigation.

Keywords : *hrp* mutants, plant defense response, apple, *Erwinia amylovora*

Introduction

Fire blight caused by *Erwinia amylovora* is the most serious bacterial disease affecting apple and pear trees. Genetic studies of this necrogenic bacteria led to the identification of a gene cluster named *hrp,* essential to the hypersensitive response on non host plants and to the pathogenicity on host plants (Barny *et al.,* 1990). Three functions have been assigned to this cluster: gene regulation, protein secretion, and production of elicitors and pathogenicity factors (Lindgren, 1997).

Fire blight can be only partially controlled through the use of resistant plant genotypes or application of antibacterial compounds such as copper or streptomycin. However, copper is often phytotoxic, and streptomycin is banned in many countries in Europe. Alternative control methods have been investigated such as biological control using microbial antagonists like *Pantoea agglomerans* or *Pseudomonas fluorescens* (Lindow *et al.,* 1996).

In order to explore other methods of biological control we studied the protective ability of *hrp* mutants through a bioassay that utilized a wound inoculation procedure (Tharaud *et al,* 1997). The aim of this paper was i) to evaluate the protective effect on non-wound inoculated apple seedlings and flowers of two types of *hrp* mutants: a *hrp* secretory mutant and a *hrp* regulatory mutant and ii) to investigate some mechanisms involved in the observed protection.

Material and Methods

Bacterial strains and culture media

We used a virulent strain of *E. amylovora*, CFBP1430, and two of its transposon mutants : PMV6023, an avirulent *hrp* secretory mutant (Barny *et al.* 1990) and PMV6046, an avirulent mutant altered in the regulatory functions of the *hrp* cluster (Tharaud *et al.* 1997). For population dynamics studies a spontaneous mutant of CFBP1430 resistant to spectinomycin (100 mg/ml) was used. Bacteria were grown routinely for 24 h at 27°C on King's medium B (KB), supplemented with the appropriate antibiotic.

Plant material

Young apple seedlings (5-9 leaves) from open-pollinated cv. Golden Delicious were grown in the greenhouse. Branches of apple tree of the cv. Golden Delicious were collected in the orchard in early spring then placed in the greenhouse to allow blooming.

Inoculation methods and assessment of results

Bacterial suspensions (avirulent mutants 3. 10^9 c.f.u. ml^{-1}, virulent CFBP1430 3. 10^8 c.f.u. ml^{-1}) were prepared in sterile water. Apple seedlings were sprayed with distilled water, inoculated by spraying to run-off with the bacterial suspensions and covered for 24 h with a transparent plastic bag to maintain high relative humidity. Apple flowers were inoculated with a 30 ml droplet of bacterial suspension inserted onto the hypanthium (Faize *et al.*, 1999).

For seedlings and blossom bioassays two inoculation procedures were used (Tharaud *et al.* 1997) : i) sequential inoculation, in which the avirulent mutant was inoculated 24 hours before the virulent strain and ii) co-inoculation in which the two strains were mixed and immediately inoculated.

A seedling or a flower was considered protected when no necrosis could be detected on the stem or on the fruitlet, respectively. Percentages of infected seedlings or fruitlets were compared to water controls.

Population dynamics

Five seedlings or flowers were separately ground in phosphate buffer 0.2 M pH 7, then suitable dilutions were plated onto KB supplemented with the appropriate antibiotic.

Enzymatic analyses

Leaves of apple seedlings were vacuum infiltrated with bacterial suspensions (10^9 c.f.u. ml^{-1}) as described by Faize *et al.*, 1999. For the phenylalanine ammonia lyase (PAL) extraction leaf tissues were ground to powder in liquid nitrogen. The powder was mixed into the extraction solution (50 mM Tris-HCl pH 8 supplemented with 1 mM of PMSF and 10% (w/v) of PVP25). After 30 min of centrifugation at 16000 x g the supernatant was assayed for PAL activity (Whetten *et al.* 1992). For determination of the guaiacol peroxidase (POD) activity leaf tissues were ground in 50 mM phosphate buffer pH 7.5, containing 0.01% of triton, 1mM of 2-mercaptoethanol, 1 mM of PMSF and 8% (w/v) of PVPP. The homogenate was centrifuged at 10000 x g for 10 min and the supernatant was assayed for POD activity (Moerschbacher *et al.* 1986).

Results and discussion

Protective ability of the mutants

We compared the protective ability of the two *hrp* mutants through two bioassays: inoculation of apple seedlings and inoculation of apple flowers. Our results (Table 1) showed that in the

two bioassays the protective ability of the regulatory mutant (PMV6046) was significantly higher than that of the secretory mutant (PMV6023) when co-inoculated with the virulent strain. With sequential inoculation procedure no significant protective effect was observed. However, the differential efficacy of the regulatory mutant could be noticed if an avirulent/virulent ratio of 50 : 1 was applied , only through apple flowers bioassay (data not shown).

Population dynamics

Dynamics of the bacterial growth was studied on apple seedlings (Fig. 1) and apple flowers (Fig. 2). Under single inoculation procedure, the virulent CFBP1430 multiplied in the seedlings or flowers for the whole duration of the experiment whereas populations of the two avirulent mutants decreased. When co-inoculated with the regulatory mutant PMV6046, the population of the virulent strain decreased over time ; when co-inoculated with the secretory mutant PMV6023 the population of the virulent strain remained stable (seedlings bioassay) or increased (flowers bioassay). These data indicated that the protective effect of the regulatory mutant was closely related to the inability of the virulent strain to multiply. These results suggested an activation of host defense responses triggered by the regulatory mutant.

Table 1. Comparison of the protective ability of the secretory mutant PMV6023 and the regulatory mutant PMV6046 on apple seedlings and apple flowers

	Seedlings		Flowers	
	Col[1]	Sl[2]	Col	Sl
Control	90[3]	90	79[4]	79
PMV6023	68	78	56	53
PMV6046	**20**[5]	72	**8**	46

[1]Col = Co-inoculation): seedlings or flowers were inoculated with the avirulent mutant or water (control) at the same time as the virulent strain CFBP1430.

[2]Sl (Sequential Inoculation): seedlings or flowers were inoculated with the avirulent mutant 24 h before applying the virulent strain CFBP1430.

[3]Percentage of infected seedlings assessed three weeks after inoculation. Twenty plants were inoculated. Results are means from three experiments.

[4]Percentage of infected fruitlets assessed two weeks after inoculation. Results are means from six replicate branches bearing 30 flowers excised from apple trees in 1997. The experiment was repeated in 1998 and similar results were obtained.

[5]Results in bold type are significantly different from the water control at $P < 0.05$.

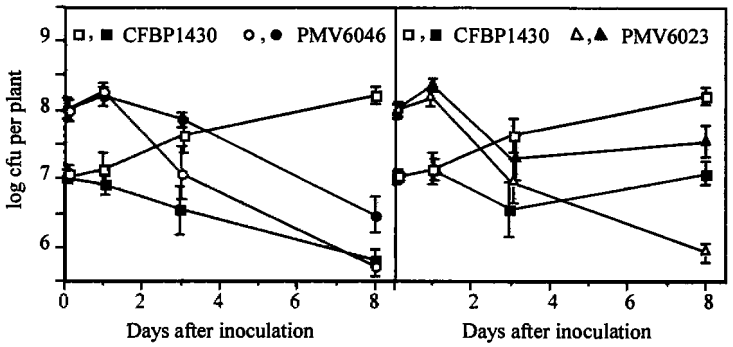

Figure 1. Bacterial growth on apple seedlings. Bacteria were inoculated alone (open symbols) or co-inoculated (closed symbols). Means of 5 replicates with standard error (from Faize *et al.*, 1999).

Enzymatic activities

In order to investigate the above hypothesis we analyzed the activity of two enzymes involved in the plant defense response (PAL and POD). Results (Fig. 3) showed an activation of these two enzymes with the regulatory mutant whereas no activation was observed with the secretory mutant or in the control.

Other mechanisms

We looked for a possible direct antagonism between an avirulent mutant and the virulent strain. Preliminary results showed that another regulatory mutant at least inhibited *in vitro* growth of the virulent strain, when the secretory mutant did not.

Understanding mechanisms involved in this protection may provide useful basis for new biological control approaches.

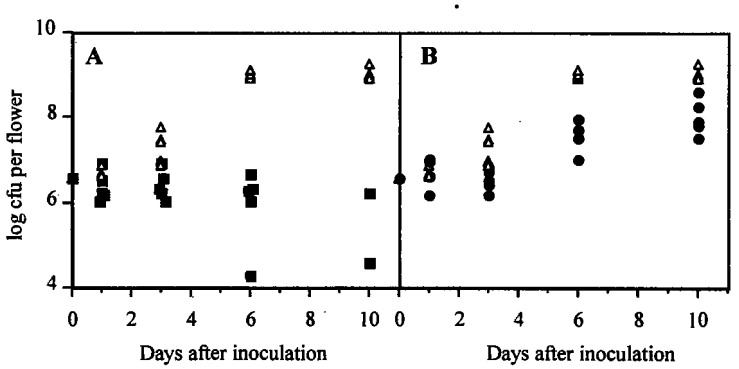

Figure 2. CFBP1430 growth on apple flowers inoculated alone (open symbols) or co-inoculated (closed symbols) with PMV6046 (A) or PMV6023 (B).

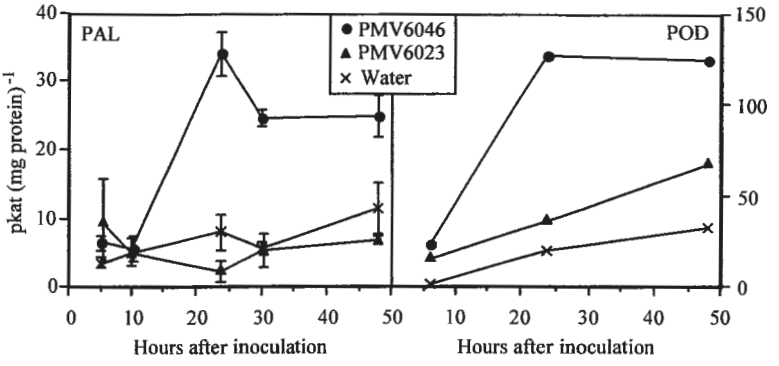

Figure 3. Time-course of PAL and POD following infiltration of leaves with avirulent mutants PMV6023 and PMV6046. For PAL activity results are means of four replicates with standard error. For POD activity, data of a typical experiment are presented (from Faize *et al.*, 1999).

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Antagonistic effect of *Bacillus subtilis* BS 2924 upon fire blight bacteria

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Abstract : The *Bacillus subtilis BS 2924* showed antagonism to *Erwinia amylovora in vitro* and in some cases on fruits of 'Conference' pear. Antagonist failed to survive in field spray, however, it influenced the movement of *E. amylovora* in plant and on chromatography paper in the laboratory. Bacilli produced an antibiotic, which is able to prevent fire blight symptoms on flowers in the laboratory and to change pattern of ring formation by chemotactic cells of the pathogen.

Key words : *Erwinia amylovora*, pear, field survival

Introduction

Non pathogenic strains of *Erwinia herbicola, Pseudomonas fluorescens* and *P. syringae* are the most often cited antagonists of *Erwinia amylovora*, the fire blight agent (Sobiczewski *et al.* 1997). Zeller and Wolf (1996) studied antagonistic bacteria from the *Erwinia, Pseudomonas* and *Bacillus* genera isolated from various plants. Authors estimated *E. herbicola* as most effective in suppression of *E. amylovora* and comparable by its effect with streptomycin treatment. Earlier, Abo-El-Dahab and El-Goorani (1964) showed *in vitro* antagonistic effect of a *B. subtilis* strain upon fire blight agent also comparable to the antibiotic action.

However, no commercial products were developed using these bacteria. It seems possible that antagonistic potential of *Bacillus* spp. isolates is not studied enough. As *Bacillus* are spore forming microbes, it will be easy to produce and store the biocontrol formulations based on them. The aim of this work was to study selected stain of *B. subtilis* as an antagonist of *E. amylovora.*

Material and methods

The *B. subtilis* BS 2924 was isolated in Ukraine and antagonistic activity of this strain was demonstrated earlier against *Botrytis fabae* and *Botrytis cinerea* on *Faba* bean leaves (Sharga 1997). The origin of *E. amylovora* strains is indicated in Table 1.

The antagonistic effect of *B. subtilis* BS 2924 against *E. amylovora* strains was evaluated in dual cultivation *in vitro* on 5% sucrose agar plates. For antibiotic production antagonist was cultivated onto liquid medium used by Sharga and Lyon (1998). The dialysis tubing from Medical International Ltd (London, UK) and benzoilated dialysis tubing from SIGMA, which retain compounds with an M_r value greater than 12 400 and 2000 D, respectively, were used as molecular meshes for evaluation of molecular weight of the antibiotic.

To study the influence of *Bacillus* and its antibiotic onto movement of *E. amylovora* within plant vessels, autoclaved wooden pear shoots were dipped by bottom end into 2 ml of *B. subtilis* BS 2924 culture (grown over night onto 5% sucrose potato broth) for 3 days and

then into suspension of *E. amylovora* Ea 394 at 10^9 cfu/ml for the same period of time. Control shoots in a sterile atmosphere were inoculated with *E. amylovora* Ea 394 suspension only. The shoots were dried, cut and stamped against 5% sucrose agar plates with (for suppression of *B. subtilis* BS 2924) or without (for control of *B. subtilis* BS 2924 movement) 0.15% lincomycin hydrochloride. The *E. amylovora* Ea 394 is resistant to lincomycin hydrochloride. The penetration ability test was designed also with use of crenate strips cut from Whatman (Whatman International Ltd. Maidstone, U.K.) 3MM Chr paper and from DIN A4 No 2043 b and No 2045 b paper muster/samples from Schleicher & Schuell (Dassel, Germany). They were dipped into night broth of *B. subtilis* BS 2924 culture or its 20 times concentrated (by freeze-drying) culture filtrate (CFC) in a same way as in experiment with the shoots. However, later strips were in contact with *E. amylovora* Ea 394 suspension only for night.

The ability of antibiotic from bacilli to influence the chemotaxis was evaluated onto medium of Adler (1966) by placing Whatman paper disks impregnated with CFC in the center of agar plate in front of the moving bound of the cells of particular *E. amylovora* strain inoculated at the plate periphery. Plates in control were seeded with suspensions of *E. amylovora* only.

Plates inoculated with different combinations of antagonist suspension at 10^2-10^{10} cfu/ml and *E. amylovora* Ea 394 at 10^9 cfu/ml with antagonist or with its CFC in advance of fire blight agent onto restricted sites of fruit damaged with a cork-borer and a knife were incubated at 30°C.

The pear flowers were first dipped into CFC, than placed onto support to be in contact by their bottom ends the same concentrate into Petri dish. The *E. amylovora* Ea 394 at 10^6-10^9 cfu/ml was inoculated onto tops of flowers treated or untreated with the concentrate and kept under 16 h photoperiod at 30°C till symptoms of fire blight developed (usually 4 days).

The field experiments were carried out to look on protective effect of the bacilli applied as spray of suspension at concentration 10^{10} cfu/ml onto 'Conference' pear flowers just at petals fall. As fire blight has not been introduced in Ukraine yet, the inoculation of very young fruits and flowers with *E. amylovora* Ea 394 suspensions (0.02 ml drop/pistil) were done in the laboratory *in vitro* and kept in the same conditions for 4 days.

The antagonism in rich medium was examined in combined cultivation onto 5% sucrose potato broth. The Erlenmeyer flasks (0.5 L) filled with 50 ml of sterile medium were inoculated with 0.1 ml of *B. subtilis* BS 2924 and *E. amylovora* Ea 394 at levels of 6×10^9 and 4×10^8 cfu/ml, respectively. The antagonist and fire blight agent were also inoculated separately from the same suspensions and cultivated as single organism cultures in control. The amount of viable cells in the cultures (cfu/ml) was estimated at 7-hours-intervals by plating out samples onto 5% sucrose agar with or without 0.15% lincomycin hydrochloride.

Results and Discussion

As it shown in Table 1, *B. subtilis* BS 2924 antibiotic was active against *E. amylovora* strains isolated from different places. This result supports the idea of a great similarity between the *E. amylovora* isolates and of a broad spectrum of action of *B. subtilis* BS 2924 antibiotic.

The bacilli CFC showed protective effect onto pear flowers *in vitro*. This demonstrated good diffusion of antimicrobic substance(s) into plant tissues. However, when *E. amylovora* Ea 394 was inoculated as 0.02 ml drops at concentration 10^8 or 10^9 cfu/ml about 5% and 7% of flowers developed fire blight infection (blackening of all flower parts and ooze from ovary and pistil). All flowers showed symptoms in control inoculations.

During petals fall pollen as source of nutrients for bacilli was limited. The conditions onto aerial surface of pear were harsh (UV irradiation, low temperatures, shortage of water

and leaching sites). Because of these circumstances, the *B. subtilis* BS 2924, applied as water suspension, failed to establish properly onto very young fruit surfaces of pear (flowers just after petals fall) in field conditions. The *B. subtilis* BS 2924 concentration onto very young fruit surface remained almost unchanged at laboratory and declined about 10 times during 24h period into field. No protective effect was observed for field spray.

Very high concentrations of bacilli (not less than 10^4 cfu/mm^2) are needed to decrease or prevent the development of fire blight symptoms onto damaged sites of developed pear fruits. Possibly, the production of antibiotic by bacilli was limited into wound site and high number of bacterial cells was necessary for pathogen niche exclusion.

Table 1. Sensitivity of *E. amylovora* strains to *B. subtilis* BS 2924 antibiotic in agar plate test

E. amylovora strains	Host plant	Sensitivity to bacilli antibiotic
Ea- 4 #	apple	+
Ea- 95 #	apple	++
659 *	apple	++
691 *	apple	++
Ea- 311 #	quince	+
610 *	hawthorn	++
651 *	hawthorn	++
684 *	hawthorn	++
Ea-103 #	medlar	+
Ea 394 *	pear	+
NCPPB 2024	pear	++
NCPPB 8705	pear	++
661 *	*Sorbus* spp.	++

Note : # strain isolated by Dr. Veljko Gavrilovic in Ugoslavia
* strain isolated by Dr. Piotr Sobiczewski in Poland
+ sensitive isolate (1-15 mm in radius zone of inhibition)
++ extremely sensitive isolate (15 mm or more in radius zone of inhibition).

The antibiotic was able to diffuse through either of dialysis tubing. According to this test, the molecular weight of antibiotic is not greater than 2000 D. However M_r value should be estimated also by other methods, because inhibition zone after diffusion of antibiotic through benzoilated tubing was somewhat smaller in size. Larger than 12 400 and 2000 D molecules can diffuse through the tubings in case of thin linear molecular structure of the antibiotic.

The colonization of autoclaved wooden shoots within 3 days by *B. subtilis* BS 2924 decreased the subsequent movement of *E. amylovora* Ea 394 through the shoot vessels. The colonization of capillary tubes in crenate paper strips with the antagonist also decreased the penetration of *E. amylovora* Ea 394. Possibly, this was observed because of pathogen niche exclusion and antibiotic production by antagonist (as broth culture was used for colonization). The CFC significantly decreased the movement of fire blight agent up along the strips or shoots as concentration of antibiotic into plant vessels increased. In comparison with control,

bacteria moved about 9 and 3 times less in CFC treated strips or shoots respectively (Table 2). The *B. subtilis* BS 2924 should be studied in more details for endophytic properties, as such properties were reported to exist in *B. subtilis* colonizing tissues of woody plants (Mundt & Hinkle 1976 ; Gardner *et al.* 1982; Misaghi & Donndelinger 1990). It is quite possible that endophytic bacilli may be discovered in pear and apple tree also.

The antibiotic substance diffusing into the medium from the Whatman paper disk treated with CFC caused curving of chemotactic band of *E. amylovora* Ea 394, 610, 691 or NCPPB 8705 cells within 4 days. The moving bacteria tended to not approach the disk containing CFC antibiotic. This resulted in deformations of ring patterns produced by chemotactic cells of fire blight agent.

Table 2. Movement of *E. amylovora* Ea 394 in material colonized by *B. subtilis* BS 2924 or pretreated with its culture filtrate concentrate.

Material	Penetration ability of bacteria, mm*	
	B. subtilis BS 2924	*E. amylovora* Ea 394
Pear shoots untreated	90 ± 5	68 ± 13
Pear shoots colonized by *B. subtilis* BS 2924		45 ± 5
Pear shoots treated by CFC		24 ± 3
3MM Chr untreated	160 ± 10	190 ± 5
3MM Chr colonized by *B. subtilis* BS 2924		115 ± 3
3MM Chr paper treated by CFC		20 ± 4
No 2043 b untreated	135 ± 5	162 ± 6
No 2043 b colonized by *B. subtilis* BS 2924		125 ± 3
No 2043 b treated by CFC		22 ± 2
No 2045 b untreated	160 ± 5	160 ± 4
No 2045 b colonized by *B. subtilis* BS 2924		135 ± 8
No 2045 b treated by CFC		18 ± 3

Note: *data are the means ± standard error from 5 replicates.

It is important to take into account that for given antagonist more than one mechanism may operate to suppress a pathogen, and the significance of a particular mechanism may vary with the conditions onto aerial plant surface. That is why an experiment was needed to confirm the antagonistic effect of *B. subtilis* BS 2924 in conditions when starting nutritive resources for arriving antagonist and pathogen were of rich value.

As showed in Figure1, growth of the fire blight agent was suppressed by the presence of antagonist which is possibly due to the production of antibiotic by bacilli. The multiplication of bacilli was almost the same as in control up to 21 hr of mixed cultivation. However, after 21 hr, the viable cells number of BS 2924 decreased to lower level in mixed culture than in single culture. Presumably, this results from shortage of nutrients due to their consumption by populations of antagonist and pathogen. Thus, competition for nutrients was more acute after populations of cells of combined microorganisms passed their maximum levels. However, it remains to be determined in further studies, if *E. amylovora* Ea 394 is able to produce in dual culture any substance toxic to *B. subtilis* BS 2924 cells.

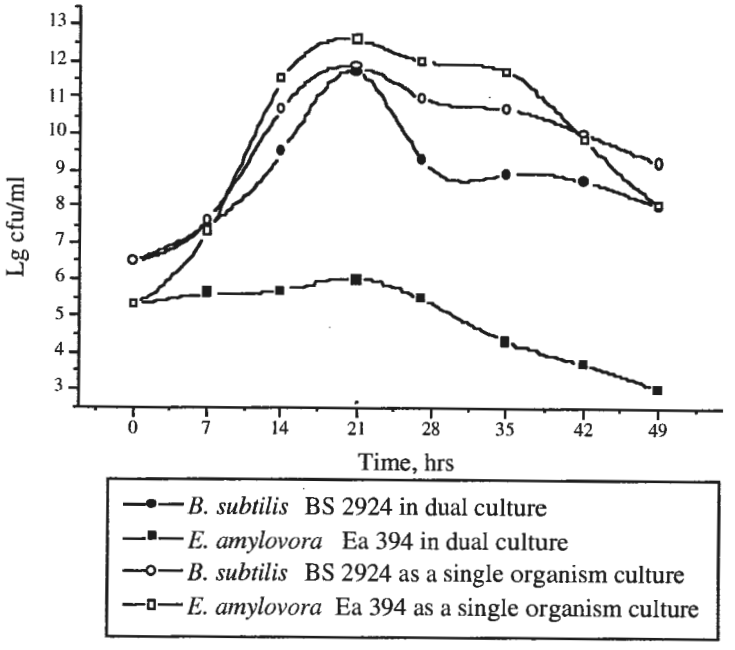

Figure 1. Growth of antagonist and fire blight agent in dual and single organism culture

Recent studies in our laboratory are aimed at answering the question of the resistance of *Erwinia amylovora* to *B. subtilis* BS 2924 antibiotic and how long and in which formulation the antibiotic remains active onto plants in comparison with streptomycin sulfate.

The *Bacillus subtilis* was already reported as effective biological control agent for postharvest fungal diseases caused by *Botrytis* and *Penicillium* on apple under controlled conditions (Sholberg *et al.* 1995).

The formulation should be developed for better survival of the *B. subtilis* BS 2924 on apple or/and pear above ground plant surface in the field. Experiments should be done to look if the use of this formulation may be compatible with other treatments. More detailed studies of *B. subtilis* BS 2924 antibiotic are needed to verify if it can be used as streptomycin, with no danger for man and nature.

Acknowledgements

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Integrated biological control of apple scab

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Abstract : Apple scab, caused by *Venturia inaequalis* (Cooke) Wint., is a serious disease of apple and causes damage to fruit wherever the spring is cool and wet. Apple growers rely heavily on the use of fungicides to control the disease. In orchards where scab was properly controlled the previous year, apple growers are often able to reduce their number of fungicide sprays by delaying the first sprays and/or ending spraying earlier. On the other hand, when the ascospore potential is high, reduced spray programs are commercially unacceptable to growers. Several inoculum reduction strategies, including mechanical removal or destruction of fallen leaves, fall applications of chemicals and urea have been investigated over the last 50 years. Most of these chemicals have showed good efficacy but their use pose several problems such as possible detrimental effects on the tree and may promote the spread of resistance in *V. inaequalis* populations. Recently, the possibility of using a fungal antagonist to reduce ascospore production has been investigated *in vitro* and under natural conditions (Carisse *et al.*, 1999). In large-scale experiments, fall applications of *Microsphaeropsis* sp. (strain P130A) resulted in a reduction in the total amount of airborne ascospores caught the following spring by 80%. The objective of the present study was to evaluate the potential of this biological control agent combined with spore monitoring for the control of apple scab. The experiment was conducted during two apple scab seasons on one and three sites for the first and second year, respectively. One third of each orchard was treated with the biocontrol agent applied in the fall. The following spring, volumetric spore traps were installed in each of the three orchard sections. Two sections (one treated with the biological control agent and the other one not treated) were sprayed based on an ascospore threshold of 1.5 ascospores per cubic meter of air. The third section was sprayed according to the local recommendations regardless of the ascospore concentration. Scab was evaluated on cluster leaves and vegetative shoots. Overall, the application of the biological control agent resulted in a reduction of fungicide sprays of 20 to 40% depending on the site.

Key words : ascospore inhibition, fugal antagonists, inoculum reduction, *Venturia inaequalis*

Introduction

Apple scab is the most important apple disease in eastern Canada. The relatively cool and humid weather in the spring is particularly favourable to scab development which may endanger the entire crop if appropriate control measures are not applied. Fungicides are presently the only method of control and these sprays represent an important input of costs to growers. Most research on apple scab has been focused on the control of primary infections, and it has resulted in spray schedules, which are expensive, environmentally unsound and not necessarily effective every year. The objective of our research program on apple scab management was to develop an Integrated biological control strategy. In general, high level IPM strategies result in a reduction in the number of fungicide applications. However, it is only reliable in orchards with low ascospore potential (PAD) and over the years, it is expected that inoculum will build up and eventually making IPM strategies unreliable.

In eastern Canada, ascospores are the main source of primary inoculum and it is assumed

that overwintering ascospores are solely responsible for scab development in the early spring. The strategy we developed consists in applying a biological control agent to apple leaves in the fall to inhibit sexual stage development and consequently reduce the ascospore potential. The following spring, the ascospore density is monitored with spore traps and the decision of applying a fungicide is made on the basis of inoculum potential reduction (MacHardy *et al.*, 1993) and number of ascospores present in the air (Philion *et al.*, 1997). This approach to apple scab management was tested for two years in Frelighsburg, Québec and has proven to be efficient in reducing ascospore production and number of fungicides required.

The objective of the present study was to evaluate the potential of the biofungicide alone and mixed with urea in an integrated apple scab management program.

Materials and methods

Microsphaeropsis sp (strain P130A) was isolated from dead apple leaves collected in the fall of 1994 (Bernier *et al.*, 1996). It was tested in *in vitro* (Philion *et al.*, 1997) and in the field (Carisse *et al.*, 2000) for its ability to inhibit ascospore production. *Microsphaeropsis* sp (strain P130A) reduced ascospore production by 85 to 98% and by 75 to 85 % under controlled and field conditions, respectively. Recently, Benyagoub *et al.* in 1997 studied the interaction between *Microsphaeropsis* sp (strain P130A) and *V. inaequalis* showing that the antagonist was able to penetrate directly the *V. inaequalis* cell wall inducing reduced growth or cell death. The biofungicide consisted of frozen spores of strain P130A applied at a rate of 1×10^{11}spores/ha. The biofungicide alone or mixed with urea 5% (46% N), was applied at a rate of 1125 L/ha (about 1.2-1.8L/tree) using a gasoline powered Solo backpack portable sprayer or spraying equipment available on the farms. The biofungicide applications were done on October 8th at Vineland; on October 16th at Frelighsburg; and on October 22th at Kentville. Prior to treatment application, fall scab was evaluated by counting the number of scabbed leaves and the number of lesions per scabbed leaf on at least 50 branches (100 at Frelighsburg) per experimental sub-plot. The treatments are described in Table 1.

Table 1. Description of the treatments

1.	Fall application of the biofungicide on the trees after harvest but before leaf fall. Spring fungicides applied based on ascospore concentration.
2.	No application of biofungicide. Spring fungicides applied at the same time as treatment 1
3.	Fall application of the biofungicide mixed with 5% urea on the trees after harvest but before leaf fall. Spring fungicides applied based on ascospore concentration.
4.	No application of biofungicide or urea. Spring fungicides applied at the same time as treatment 3
5.	Fall application of 5% urea on the trees after harvest but before leaf fall. Spring fungicides applied based on ascospore concentration.
6.	No application of biofungicide or urea. Spring fungicides applied according to grower's practices (or government recommendations).

At each site, the experimental design was a non-replicated trial and the same experiment was conducted at 3 sites (Frelighsburg, Vineland and Kentville) so that the design of the entire experiment was a completely randomized design replicated over space. Before the first ascospore ejection, one spore sampler was installed in each of the sub-plot, and ran for four to six hours following the beginning of each rain. The sampling heads were set at 40 cm above the ground and shielded from the rain with a plastic cover installed at 10 cm above the heads.

For each rain conducive to infection, the number of ascospores on the entire surface of the exposed area of the rods was counted and ascospore concentration was expressed as the number of ascospores per cubic meter of air sampled. Fungicides were applied when at least 15 ascospores per rod, which corresponded to approximately 1 to 2 ascospores per cubic meter of air, were sampled. Scab severity (number of lesions per scabbed leaves) and incidence (number of scabbed leaves) on 50 branches per sub-plot were estimated at the end of the primary scab season.

Results and discussion

Only the results from the trials in Kentville and Frelighsburg are presented because the level of scab was too low in Vineland to test the biofungicide (no scab in any of the sub-plots). The fall incidence of scabbed leaves varied from 2.31 to 5.00 and 2.86 to 11.37 at Ketville and Frelighsburg, respectively. The scab severity varied from 0.37 to 0.95 lesions per leaf and 0.35 to 3.33 lesions per leaf at Kentville and Frelighsburg, respectively. Overall, the level of scab was much higher in Frelighsburg than in Ketville.

Table 2. Fall scab evaluation in Kentville and Frelighsburg

Treatment	Kentville Incidence	Severity	Frelighsburg Incidence	Severity
1	5.00	0.95	4.47	1.72
2	3.85	0.68	4.10	1.95
3	2.31	0.37	5.56	1.43
4	3.20	0.49	8.04	1.85
5	4.37	0.61	11.37	3.33
6	3.42	0.85	2.85	0.35

Incidence was expressed as % scabbed leaves and severity as the average number of lesions per scabbed leaves.

During the primary scab season, in Kentville, nine fungicides were applied in the untreated plots (Table 3) resulting in 4% leaf scab (Figure 1). All the other treatments resulted in complete control of scab on both leaves and fruits, however the best reduction in ascospore concentration was observed in the plot treated with the biofungicide mixed with urea. Scab control in the treated plots was achieved with only five fungicides, which represent a significant reduction in the amount of fungicide required (45% reduction). In Frelighsburg, we observed a much higher reduction in the ascospore concentration following the fall treatments, more than 75% in the plots treated with the biofungicide alone or mixed with urea (Figure 1). In the untreated plot and treated plots fungicides were sprayed six times and five times, respectively (Table 3). Scab severity on leaves was 12.07, 7.12, 2.21, and 1.18% in the untreated plot, plots treated with urea, the biofungicide, and the biofungicide mixed with urea, respectively (Figure 1).

This trial was conducted in orchards with different levels of inoculum. At Kentville, the potential of inoculum was low and the fall application of the biofungicide alone or mixed with urea resulted in a substantial reduction in the number of fungicides required (five as compared to nine in the untreated plot). At Frelighsburg, where the inoculum potential was very high, the fall application of the biofungicide alone or mixed with urea resulted in a small reduction

in the number of fungicides required (five as compared to six in the untreated plot). However, the reduction in the level of scab was substantial (2.21 and 1.18 as compared to 12% in the untreated plot).

Table 3. Description of the fungicides applied

	Treatments				
Date	1 and 2	3 and 4	6	5	Products
Kentville					
May 05	-	-	-	spray	Maestro
May 10	-	-	-	spray	Maestro
May 13	spray	spray	spray	spray	Maestro, Nova, Zinctrac
May 17	spray	spray	spray	spray	Maestro, Nova, Zinctrac
May 21	spray	spray	spray	spray	Equal
May 27	spray	spray	spray	spray	Maestro, Nova
June 03	-	-	-	spray	Maestro
June 11	spray	spray	spray	spray	Equal
June 16	-	-	-	spray	Maestro
Frelighsburg					
May 05	spray	spray	spray	spray	Polyram
May 11	spray	spray	spray	spray	Polyram, Nova
May 21	spray	spray	spray	spray	Nova, Captan
May 27	spray	spray	spray	spray	Nova
June 01	spray	spray	spray	spray	Equal
June 11	-	-	-	spray	Nova, Captan

The fungicides were applied at the following rate : Maestro 4 Kg/ha ; Nova 340g/ha ; Zinctrac 1L/ha ; Equal 22.5kg/ha ; Polyram 80DF 3kg/ha ; Captan 80WP 3.75Kg/ha.

The results are in accordance with commercial situations where growers with good scab control will be interested in using the biofungicide to save on fungicide sprays and those who have problems with scab control will be interested in additional tools to maintain their scab level under the economic threshold.

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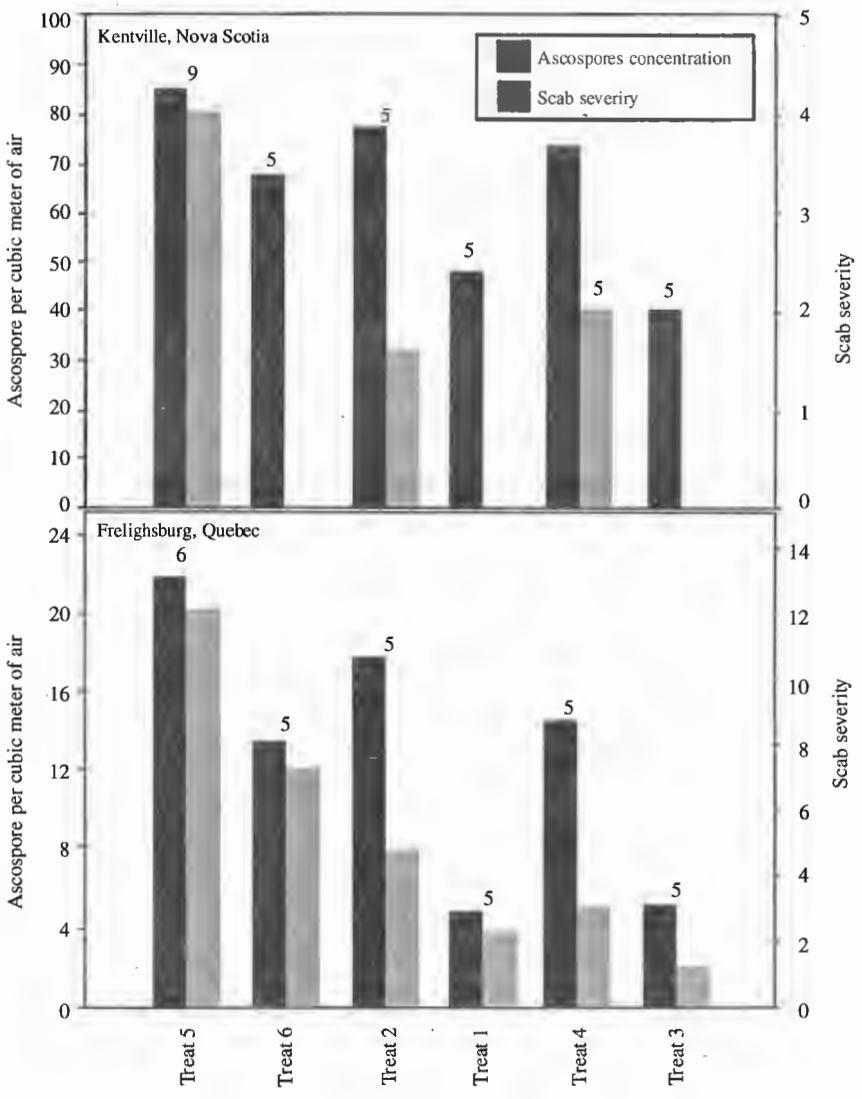

Figure 1. Ascospore concentration and scab severity in untreated and treated orchard plots. Treatments were as followed: 1 : fall application of the biofungicide; 2 : no fall treatment, sprayed as in treatment 1; 3 : fall application of the biofungicide mixed with 5% urea; 4: no fall treatment, sprayed as in treatment 3; 5: no fall treatment; 6: fall application of 5% urea. The numbers represent the number of fungicide sprays.

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Covering Apple Fruits with Multi-layer Fruit Bags Reduces Defects

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Abstract : Apple fruit quality is reduced by fungal diseases, physiological disorders, and insect pests. To improve quality for specialized markets in the Orient, apple growers in Japan and in the United States (for export) sometimes place multi-layer bags on fruit. The object of our research was to determine whether or not covering apples with bags reduces the occurrence of fruit defects under Kentucky conditions. We also used bag application as an experimental tool to determine when during the growing season apple defects occur. Eight experiments were conducted 1995-1998 in four different commercial or experiment station apple orchards using 'Red Delicious,' 'York,' and 'Golden Delicious' cultivars. Each experimental treatment consisted of applying multi-layer fruit bags (Kobayashi Bag Mfg. Co., Ltd., Japan) to apple fruits in May or early June, 18-29 days after petal fall when the fruits were 2.0-2.5 cm diameter. Fruits without bags were left as controls. Disease pressure varied from year to year and fruits without bags ranged from 18-100% defective. Over the eight experiments, 77-99 percent of bagged and 0-82 percent of control fruit were defect-free. Causes of fruit defects listed by order of frequency of occurrence were flyspeck, sooty blotch, cork spot, codling moth, stink bug and San Jose scale. Sooty blotch and flyspeck occurred in all experiments and were significantly (P=0.05) reduced by bag application in each case. Cork spot occurred in six of eight experiments and was significantly decreased with bags each time. Codling moth was found in four experiments, and in two cases use of bags caused significant damage reduction. Measurable stink bug and San Jose scale damage was found in three and two experiments, respectively, but incidences were low and decreases due to bagging were not significant. In bag application timing experiments, sooty blotch and flyspeck were decreased the most when bags remained on the fruits the full season, but disease could be significantly curtailed if bags were left for as little as 55-70 days, especially during July and August. Cork spot was significantly reduced when bags were applied as little as 40-45 days before harvest and fruit coverage from mid July through August was most effective at decreasing cork spot.

Key words : multi-layer fruit bags, sooty blotch, flyspeck, cork spot, apple fruit defects

Introduction

High quality apple markets require blemish-free fruit. For specialized markets in the Orient, apple growers in Japan and in the United States (for export) sometimes place multi-layer bags on fruit to improve quality. In addition to improving quality, placing bags on fruit as a substitute for pesticide application could benefit "organic" apple producers. Although too labor-intensive for most commercial growers, fruit grown in the home garden may be responsive to such treatments.

In Kentucky, fruit quality is reduced by fungal diseases, physiological disorders, and insect pests. Sooty blotch, (*Peltaster fructicola, Geastrumia polystigmatis, Leptodontium elatius* and other fungi) and flyspeck (*Zygophiala jamaicensis)* are common in temperate regions with warm, humid growing seasons. Cork spot (calcium and/or boron deficiency) markedly reduces fruit quality. Insect pests such as codling moth (*Cydia pomonella*), stink bug (*Euschistus servus*), and San Jose scale (*Quadraspidiotus periciosus*), also cause fruit defects. Work done in the USA in Kentucky (Hartman, 1996a, 1996b ; Smigell & Hartman,

1997a, 1997b, 1997c, 1997d), North Carolina (Brown & Sutton, 1995), and New York (Rosenberger, 1997), showed that empirical models could be made relating accumulated hours of leaf wetness during the growing season to occurrence of sooty blotch and flyspeck. For example, initiating fungicide applications for sooty blotch and flyspeck control could be adjusted by knowing how many leaf wetness hours had accumulated (Hartman & Smigell, 1997d).

The object of our research was to determine whether or not covering apples with bags reduces the occurrence of fruit defects. We also used bag application as an experimental tool to determine when during the growing season certain apple defects occur, and if fruit bagging would affect the relationship between leaf wetness accumulations and occurrence of sooty blotch and flyspeck.

Material and methods

Eight experiments were conducted over four years in four different commercial or experiment station apple orchards using 'Red Delicious,' 'York,' and 'Golden Delicious' cultivars. Each experimental treatment consisted of applying multi-layer fruit bags (Kobayashi Bag Mfg. Co., Ltd., Japan) to numerous fruits selected at random over several trees. Treatments were randomized and replicated for statistical purposes. Bags were applied according to manufacturers instructions in May or early June, 18-29 days after petal fall when the fruits were 2.0-2.5 cm diameter. Fruits without bags were left as controls. Prior to bag application, a standard chemical spray program was used to manage early-season diseases and insects. After bag application, pesticides were no longer used. Depending on cultivar and location, bags were removed 96-124 days later either at harvest or two weeks prior to harvest (to promote fruit coloration). Treatments were evaluated at harvest by visually estimating the percentage of fruit with each of several defects. Results were analyzed using a means comparison test.

In four experiments to determine the best timing for bag application for sooty blotch and flyspeck management, leaf wetness was measured in the orchard using self-contained weather stations (Envirocaster, Neogen Corp., Lansing, MI, USA; METOS Gottfried Pessl, Weiz, Austria). Leaf wetness hours were summed from the time of first cover (10 days after petal fall) until harvest. Bags were applied when fruits were 2.0-2.5 cm diameter or at selected intervals thereafter. In some experiments, bags were removed at selected intervals following application, thus providing a tool for determining when sooty blotch and flyspeck were active, or when cork spot could best be mitigated. Timing of bag applications are presented in the results tables.

Results and discussion

Effects of bags on all fruit defects

Disease pressure varied from year to year and fruits without bags ranged from 18-100% defective. Over the eight experiments, 77-99 % of bagged and 0-82 % of control fruit were defect-free. Causes of fruit defects listed by order of frequency of occurrence were flyspeck, sooty blotch, cork spot, codling moth, stink bug and San Jose scale (Table 1). Sooty blotch and flyspeck occurred in all experiments and were significantly (P=0.05) reduced by bag application in each case. Cork spot occurred in six of eight experiments and was significantly decreased with bags each time. Codling moth was found in four experiments, and in two cases use of bags caused significant damage reduction. Measurable stink bug and San Jose scale damage was found in three and two experiments, respectively, but incidence was low and decreases due to bagging were not significant.

Table 1. Effect of fruit bag application on apple fruit defects 1995-1998

Treatment	Average percent fruits affected by insects, diseases and disorders						Percent defect-free fruit
	Flyspeck	Sooty blotch *	Cork spot	Codling moth	San Jose scale **	Stink bug *	
Bag	3.9	1.7	6.0	5.6	5.8	2.4	77.6
No bag	87.5	75.3	39.8	18.0	17.0	7.7	5.8

Treatment differences were statistically significant (P=0.05) each year for sooty blotch, flyspeck, cork spot, and defect-free fruit; for codling moth, 2 of 4 years; and not significant for stink bug and San Jose scale.
* Observed in three of four experiments.
** Observed in two of four experiments.

Effects of bags on sooty blotch and flyspeck

In bag application timing experiments, sooty blotch and flyspeck were decreased the most when bags remained on the fruits the full season (Table 2), but as long as bags were placed on the trees before 175 hours of leaf wetness had accumulated, disease was significantly reduced.

Table 2. Effect of fruit bag application timing and accumulated leaf wetness hours on sooty blotch and flyspeck incidence, 1995 ('Golden Delicious')

Date of fruit bagging	Accumulated hours of leaf wetness at first treatment	Disease rating 9 Sept
17 May	56	0.3 a
31 May	114	1.4 b
14 June	176	2.9 c
29 June	224	3.4 cd
13 July	259	3.5 de
27 July	298	4.0 e
Control		3.7 de

Rating : 0 = no disease, 1 = trace - 5%, 2 = 6 - 25%,
3 = 25 - 50%, 4 = > 50%
Numbers in a column followed by the same letter are not significantly different (P = 0.05)

By varying the time of bag application and removal, it was found that disease could be significantly curtailed on fruit with bags on them, especially during July and August (Table 3). The same effect was observed when this experiment was done two more times, at a different location, and on 'Red Delicious' (results not shown). Indeed, disease could be significantly reduced with as little as 4-5 weeks of bag cover at the appropriate time (Table 4).

Table 3. Effect of timing of fruit bag application and removal on sooty blotch and flyspeck incidence, 1996 ('York').

Bag application and removal dates	Number of days apples in bags	Total hours of leaf wetness without bags	Disease rating 19 Sept
Varying the time of bag removal			
No-bag control	0	313	4.6 h
4 June - 19 June	15	230	3.8 g
4 June - 4 July	32	205	3.2 f
4 June - 17 July	45	169	2.5 e
4 June - 1 Aug	60	107	1.5 d
4 June - 17 Aug	76	92	1.0 bc
4 June - 8 Sept	98	77	0.2 a
Varying time of bag application			
4 June - 8 Sept	98	77	0.2 a
19 June - 8 Sept	83	160	0.9 b
4 July - 8 Sept	66	185	1.0 bc
17 July - 8 Sept	53	221	1.4 cd
1 Aug - 8 Sept	38	283	2.9 ef
18 Aug - 8 Sept	22	298	3.3 f
No-bag control	0	313	4.6 h

Rating : 0 = no disease, 1 = trace - 5%, 2 = 6 - 25%, 3 = 25 - 50%, 4 = 50 - 75%, 5 = > 75%.
Numbers in a column followed by the same letter are not significantly different (P = 0.05)

Table 4. Effect of varying the timing of 4-5 week fruit bag coverage periods on sooty blotch and flyspeck incidence, 1997 ('York')

Bag application and removal dates	Number of days apples in bags	Total hours of leaf wetness without bags	Disease rating 14 Oct
No-bag control	0	341	1.4 cd
10 June - 7 July	27	217	1.1 bc
7 July - 6 Aug	30	319	0.9 b
22 July - 24 Aug	33	261	0.9 b
6 Aug - 10 Sept	35	244	0.9 b
24 Aug - 22 Sept	29	309	1.0 bc
10 Sept - 8 Oct	28	341	1.4 cd
10 June - 8 Oct	120	97	0.0 a

Rating : 0 = no disease, 1 = trace - 5%, 2 = 6 - 25%, 3 = 25 - 50%, 4 = > 50%.
Numbers in a column followed by the same letter are not significantly different (P = 0.05)

Effects of bags on cork spot

Cork spot was significantly reduced at harvest when bags were applied as little as 40-45 days before harvest (Table 5). This experiment was repeated in another orchard on another cultivar and the same effects were observed (data not shown). Fruit coverage with bags from mid-July through August was most effective at decreasing cork spot (Table 6).

Table 5. Effect of timing of fruit bag application and removal on cork spot incidence, 1996 ('Red Delicious').

Varying time of bag removal			Varying time of bag application		
Bag application and removal dates	Number of days apples in bags	Number of cork spots per apple	Bag application and removal dates	Number of days apples in bags	Number of cork spots per apple
No-bag control	0	1.1 c	20 May - 27 Aug	100	0.2 a
20 May - 3 June	14	0.9 bc	3 June - 27 Aug	86	0.2 a
20 May - 18 June	29	0.9 bc	18 June - 27 Aug	71	0.1 a
20 May - 3 July	44	0.8 bc	3 July - 27 Aug	55	0.1 a
20 May - 18 July	59	0.4 bc	18 July - 27 Aug	40	0.2 a
20 May - 30 July	71	0.6 bc	30 July - 27 Aug	28	0.5 ab
20 May - 13 Aug	84	0.2 a	13 Aug - 27 Aug	14	0.9 bc
20 May - 27 Aug	100	0.2 a	No-bag control	0	1.1 c

Numbers in a column followed by the same letter are not significantly different (P = 0.05)

Table 6. Effect of varying the timing of 4-5 week fruit bag covering periods on cork spot incidence, 1997 ('York')

Bag application and removal dates	Days apples in bags	Cork spots per apple
No-bag control	0	1.5 c
10 June - 7 July	27	0.8 ab
7 July - 6 Aug	30	1.1 bc
22 July - 24 Aug	33	0.3 a
6 Aug - 10 Sept	35	0.3 a
24 Aug - 22 Sept	29	0.8 ab
10 Sept - 8 Oct	28	0.8 ab
10 June - 8 Oct	120	0.2 a

Numbers in a column followed by the same letter are not significantly different (P = 0.05)

Defects caused by fungi and insects may be reduced by the physical barrier to pests and inoculum imposed by bags. In the case of sooty blotch and flyspeck, a reduction in fruit surface wetness may account for reduced disease. Neither of these explanations would apply to cork spot, so some other mechanism must account for cork spot reduction. Fruit bagging improves fruit quality and could reduce pesticide inputs. Although impractical for all but specialty apple growers, use of fruit bags might be employed by researchers to study the biology of apple diseases, physiological disorders and insect pests.

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Bion® induces systemic defense responses in apple and protects against fire blight infections

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Abstract : Bion® [benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester], a Novartis product also known as Actigard®, has been reported to activate systemic acquired resistance (SAR) in several crops (mainly annual plants). We investigated the potential of Bion® to induce resistance to bacterial fire blight of *Maloideae* (*Erwinia amylovora*). Apple seedlings and trees of cv. Golden Delicious were sprayed with Bion® and inoculated with *E. amylovora* according to several protocols (various delays between treatment and inoculation, single or repeated sprays, diverse inoculation procedures). In each case, results of infection showed a significant level of protection against fire blight after Bion® applications when compared to a water control. Leaves of seedlings were also assayed for two defense-related enzymes : peroxidases and ß-1,3-glucanases. Both enzymes were progressively activated in plants after Bion® applications, locally (in treated leaves) as well as systemically (in untreated upper leaves). The protection, associated with the accumulation of defense-related proteins, suggests that Bion® promotes induced systemic resistance in a perennial crop such as apple. Although promising for a new approach of control of fire blight, this compound needs further investigations before its practical use.

Key words : fire blight, *Erwinia amylovora*, apple, Bion®, induced resistance

Introduction

Chemical control of fire blight, a disease of *Maloideae* caused by the bacterium *Erwinia amylovora*, relies essentially upon the use of bactericides, antibiotics and copper compounds, which provide a protective barrier between the plant and the pathogen. The chemical activation of natural plant defenses is an emerging strategy in plant protection (Lucas, 1999) and we investigated this strategy on apple against fire blight using the Novartis product Bion®.

Bion® (benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester) has been described as a systemic acquired resistance (SAR - for review see Sticher *et al.*, 1997) inducer and its protective effect against several fungal and bacterial diseases has been demonstrated on various annual crops (including tobacco [Friedrich *et al.*, 1996], wheat [Görlach *et al.* 1996], bean [Siegrist *et al.*, 1997], maize [Morris *et al.*, 1998], cucumber [Narusaka *et al.*, 1999]) and one perennial crop (Japanese pear [Ishii *et al.*, 1999]).

In this paper we show that Bion® has a protective effect against fire blight on apple in greenhouse and in orchard. We also establish a relationship between this protection and the activation of two well known families of defense-related enzymes, peroxidases (involved in cell wall reinforcement) and ß-1,3-glucanases (PR-proteins with antifungal activities).

Material and methods

Biological material
Experiments were performed on apple seedlings from open-pollinated cv. Golden Delicious and on potted Golden Delicious trees in the greenhouse, and on adult Golden Delicious trees in an experimental orchard. The virulent French standard strain CFBP1430 of *E. amylovora* was used for inoculation.

Plant protection experiments
Seedlings were sprayed once with Bion® (200 mg/l active ingredient) at intervals from 10 to 2 days prior to inoculation. The day of inoculation, the youngest expanded leaf of each plant was wounded (two cuts across the midrib) and bacterial suspension (10^8 cfu/ml) was sprayed 4 hrs later. Bion® was compared to a water control and to streptomycin (100 mg/l - the standard against fire blight) both sprayed immediately after wounding. Percentage of infected seedlings was assessed within 3 weeks after inoculation. Trees in the greenhouse received two repeated sprays of Bion® (100 mg/l) or water 7 and 2 days before shoot inoculation with a syringe (10^7 cfu/ml). Percentage of infected shoots was assessed within 3 weeks after inoculation.Trees in the orchard received four repeated sprays of Bion® (100 mg/l) or water around the blooming period, 7 and 2 days before inoculation and 7 and 14 days after inoculation. Inoculation was performed by spraying the bacterial suspension (10^8 cfu/ml) on clusters at the full bloom stage. Percentages of infected clusters was assessed 4 weeks after inoculation.

Assay for enzymatic activities
In a first set of experiments, young leaves were sampled from apple seedlings undergoing the same schedule of treatments as described above. In a second set of experiments, only one young fully expanded leaf per seedling was carefully sprayed with Bion® (200 mg/l) or water and the treated leaves and the untreated upper leaves were separately sampled the following days. After sampling, leaves were immediately homogenized in a suitable buffer and supernatants were spectrophotometrically assayed for enzyme activities. Determination of peroxidase activity was based upon the oxidation of guaiacol in the presence of hydrogen peroxide, as described by Chance and Maehly (1955). ß-1,3-glucanase activity was determined according to the method of Wirth and Wolf (1992) with some modifications.

Results and discussion

Bion® provided a significant control of fire blight on apple seedlings whatever the delay between treatment and inoculation (Table 1). This protection was as effective as the protection obtained with streptomycin. On trees in greenhouse and in orchard, a significant reduction of infection was also obtained after treatment with Bion® when compared with the water control (Table 2).

Concerning defense mechanisms, apple seedlings treated with Bion® showed an activation of both enzymes, peroxidases and ß-1,3-glucanases, which increased with the delay between treatment and sampling for enzyme extraction (Table 1). Streptomycin did not induce any activation when compared with the water control. A clear relationship could be established between the induction of both biochemical markers and the obtained protection.

Defense-related enzymes were not only progressively activated in treated leaves but also in untreated upper leaves (Table 3). The delay for a significant activation was longer in untreated leaves (10 days) than in treated leaves (3-5 days). These results demonstrates that application of Bion® induces not only locally but also systemically apple defense mechanisms, and that this elicitation lasted for at least 17 days in our conditions.

Table 1. Protection against fire blight and activation of peroxidases and ß-1,3-glucanases induced by Bion® on apple seedlings

Treatments	Times before inoc./sampling	Percentages of infected plants	Enzyme activities*	
			peroxidases	glucanases
Bion®	10 days	13	909	2,27
	8 days	10	797	1,72
	6 days	17	746	1,56
	4 days	20	687	2,02
	2 days	25	419	0,83
Water	4 hrs	84	128	0,10
Streptomycin	4 hrs	27	173	0,12

*Enzyme activities in leaves equivalent to those inoculated. Peroxidase activities expressed in nmole tetraguaiacol/mg protein/min ; ß-1,3-glucanases expressed in absorbance/mg protein/min.

Table 2. Efficacy of Bion® against fire blight on Golden Delicious trees in greenhouse and in orchard

Treatments	Percentages of infection*	
	in greenhouse (shoots)	in orchard (clusters)
Bion	30	14
Water	90	30

*20 shoots inoculated per treatment ; 500 clusters inoculated per treatment

Table 3. Time-course of peroxidase and ß-1,3-glucanase activities locally (treated leaves) and systemically (untreated upper leaves) in apple seedlings after application of Bion®

Days after treatment	Activities* in treated leaves		Activities in untreated leaves	
	peroxidases	glucanases	peroxidases	glucanases
3	24	0,68	9	0,06
5	138	0,78	6	0,28
7	383	1,66	39	0,05
10	491	1,72	138	0,38
12	422	1,53	82	0,62
14	631	2,73	143	1,66
17	579	1,30	185	0,61

*Data presented as the numerical differences of enzyme activities between treated and control plants. Peroxidase activities expressed in nmole tetraguaiacol/mg protein/min ; ß-1,3-glucanases expressed in absorbance/mg protein/min.

This study gives the first evidence that Bion® induces SAR in apple. The overall level of protection of apple obtained with this chemical as well as the observed stability of its effect in protection and in enzyme activation several days after sprays suggest that Bion® could provide a new approach of control of fire blight. However there is a need for further investigations to solve several unanswered questions before the practical use of such a plant activator in the field (dose responses, spray schedule, etc). Besides, enlargements of the use of Bion® to other host plant of fire blight (especially pear) and to other pathogens of apple (especially *Venturia inaequalis*) remain opened questions. Finally one can also wonder if the prolonged elicitation of defense mechanisms will have consequences on the tree growth and on the quality and quantity of fruit crop.

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Recent research on ascospore discharge in *Venturia inaequalis*

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Abstract : The present paper presents a summary of the most recent work on climatological conditions affecting ascospore discharge in *Venturia inaequalis*, the apple scab fungus, carried out at our two institutes. Experiments were performed under laboratory conditions by means of a wind tunnel and in the field using volumetric spore traps, either with artificial irrigation or under natural rain. One purpose of the studies was to reveal artificial laboratory conditions which could affect suppression of ascospore release normally observed under orchard conditions. Both air containing low relative humidity passing over leaf samples during simulated rain and extended maturation of the pseudothecial populations increased the number of spores released during darkness. These factors can thus explain some of the discrepancy between previously reported lab and field results. Field experiments with artificial irrigation showed that the possibility of high ascospore release during darkness increased once 80% or more of the season's ascosporic inoculum had matured. The earlier observation of the stimulating effect red light has on ascospore release was confirmed. Under lab conditions, light intensities similar to what was recorded in the field around sunrise on rainy days, stimulated ascospore release. The rate of discharge increased with increasing light intensities up to a level normally occurring 2-3 hours after sunrise. When leaf samples were exposed to light and simulated rain in the lab, the rate of release increased over time. Thus, the delay in reaching the peak rate of ascospore release after sunrise may be due to the combined impact of increasing light intensity after sunrise and the intrinsic increase in rate of release over time. Ascospore release was monitored in the wind tunnel at temperatures of 1-8°C in daylight-balanced light. Low temperatures resulted in a lag phase in the cumulative distribution of ascospore release, where few or no ascospores were detected. The time until first detection of a given quantity of inoculum was inversely proportional to temperature. There was a reduction in the rate of release from 8 to 1°C, and consequently there was a reduction in the number of ascospores released at any given time. Where temperatures below 10°C coincided with continious rain and leaf wetness during night and day in field studies in Norway, few ascospores were released until 4-5 hours after sunrise. High quantities of ascospores were recorded during nights with dew in two Norwegian orchards. Episodes where more than 1% of the season's inoculum was released during dew occurred around bloom of apple, and followed more than 2 days of fair weather (clear, warm days and cool, humid nights). Field studies showed that protracted dry periods with no or little rain not only delayed ascospore release, but also maturation, and consequently extended the season for ascospore release. Recommendations for management of the apple scab fungus is discussed.

Key words : aerobiology, apple scab, dew, epidemiology, light intensity, light quality, spore discharge, temperature, *Venturia inaequalis*

Introduction

Apple scab is caused by the ascomycete *Venturia inaequalis* (Cooke) G. Wint. The apple scab fungus can survive winter in young wood and fruit buds in the anamorph stage (*Spilocaea pomi* Fr.). However, in most apple growing areas of the world, the principal way to overwinter is as fruiting bodies in leaf litter on the ground. During rain in spring and early summer,

ascospores are released from pseudothecia in leaf litter, and causes primary infections in leaf and fruit tissue.

There have been many contributions to the understanding of apple scab epidemiology to secure a better timing and reduce the number of fungicide sprays. However, in many regions of the world growers still spray regularly on a protective schedule or combined with postinfection sprays according to infection tables (Mills 1944, MacHardy and Gadoury 1989). Many growers and grower advisors have been reluctant to adapt new information into their management programs on how e.g. climatic factors, tree growth, and size of inoculum affects the possibilities for infection and disease development of the apple scab fungus. Due to either failures with proposed models, more or less well documented results that contradict these models, or lack of easy rules of thumb to follow, such new information is often ignored. In the research presented below, we have tried to confirm and extend some of the previous work on the effects of climatic conditions on ascospore release under laboratory and orchard conditions. Adaption of this information into practical disease management is discussed. Earlier and ongoing work on ascospore dicharge in *V. inaequalis* was reviewed by Gadoury *et al.* (1994). The present paper is comprised by the most recently published and ongoing work at our respective institutes at Ås, Norway and Geneva, New York. Our research focus has been on climatological conditions affecting diurnal and seasonal distribution of ascospore release.

Effect of light on ascospore discharge

Light is second only to the presence of free water in regard to the magnitude of the effect upon ascospore release in *V. inaequalis*. A diurnal pattern of ascospore release in *V. inaequalis*, with a suppressed release in darkness, has previously been reported by many authors (Frey and Keitt 1925, Keitt and Jones 1926, Baumeister 1954, Hirst *et al.* 1955, Hirst and Stedman 1962, Brook 1966, 1969a, 1969b, Pinto de Torre *et al.* 1984, MacHardy and Gadoury 1986, Aylor and Sutton 1992, Warner and Braun 1992). The suppression of ascospore release is not absolute, and MacHardy and Gadoury (1986) in New Hampshire trapped 0-9% of the spores between 18:00 h in the evening and 07:00 h in the morning. Warner and Braun (1992) in Ontario, Canada, trapped 13% of the season's ascospores between 20:00 h in the evening and 05:00 h in the morning.

Brook (1969a, 1969b) was the first to report that far red light stimulates ascospore release in *V. inaequalis*. The most stimulatory wavelength area was within 710-730 nm. Palm (1988) reported that few ascospores of *V. inaequalis* were trapped when the light intensity was below 2000 lux. Field studies indicate that some stimulation of ascospore release by light occurs shortly before sunrise, but the maximum rate of release is not reached until several hours later (MacHardy and Gadoury 1986). MacHardy and Gadoury found over a 4-year period in New Hampshire, that the maximum in ascospore release occurred between 11:00 and 12:00 h, which is 5-6 hours after sunrise. However, in laboratory studies, Hirst and Stedman (1962) and Brook (1969b) reported that most of the release will occur within three hours of initial wetting.

In several unpublished and at least two published reports (Gjærum 1954, Warner and Braun 1992), the pattern of diurnal periodicity of ascospore release and the suppression of ascospore release by darkness has not been consistently reproduced in the laboratory. Thus, while field experiments have been remarkably consistent both worldwide and over a period of several decades, laboratory experiments were less comprehensive, and results were often inconsistent with field observations. The consequent uncertainty has, in some cases, delayed the adoption of revised criteria for predicting apple scab infection periods.

Effect of low temperatures on ascospore discharge

The rate of ascospore release in *V. inaequalis* is reduced at temperatures below 10°C (Hirst and Stedman 1962, MacHardy and Gadoury 1986), and the reduction is greatest when temperatures approach freezing (Hirst and Stedman 1962). Above 10°C there may be little or no effect of temperature on the rate of release (Seem *et al.* 1979). Both Hirst and Stedman (1962) and MacHardy and Gadoury (1986) observed that cumulative distributions of ascospore release were shifted in time approximately 2-3 hours when temperatures during natural rain events were below 10-12°C. However, the quantitative impact of temperatures between 0 and 10°C on the rate of ascospore release is poorly understood and has not been considered in current scab management programs.

Effect of dew on ascospore discharge

Several authors have reported that dew has little or no effect on ascospore release in *V. inaequalis* (Frey and Keitt 1925, Keitt and Jones 1926, Wiesman 1932, Hirst and Stedman 1962, Preece 1964, Brook 1969a, MacHardy and Gadoury 1986). However, Moore (1958) found large numbers of ascospores of the apple scab fungus in dew droplets from overwintering apple leaves on the ground.

Seasonal distribution of ascospore maturation and release

Seasonal distribution of ascospore release in *V. inaequalis* has been studied by many authors (Childs 1917, Stover and Johnson 1924, Frey and Keitt 1925, Schneiderhan 1925, Keitt and Jones 1926, Wiesman 1935, Weber 1934-35, Fjelddalen 1948, Weber and Jørgensen 1953, Gjærum 1954, Hirst *et al.* 1955, Hårdh 1955, Szkolnik 1969, 1974, Brook 1976, Gadoury and MacHardy 1982, Norin 1989, Ylämäki 1989). The primary season for ascospore release typically lasts 6-10 weeks, but the major period of spore release can be much shorter. The first ascospores are normally mature and ready to be released at bud break of the apple tree. Depending on the frequency of rainfalls, the peak in release is usually from the tight cluster stage to the petal fall stage of fruit bud development.

Models based on temperature sums or temperature sums combined with moisture data have been developed to predict maturation of ascospores in *V. inaequalis* (Massie and Szkolnik 1974, Gadoury and MacHardy 1982, James and Sutton 1982b, Lagarde 1988). Gadoury and MacHardy (1982) developed a model to estimate the cumulative percentage of matured ascospores, based on degree-day (DD) accumulation starting at the first appearance of mature ascospores. The model predicts 50, 95, and 99% spore maturation at 250, 420, and 490 DD (base temperature 0°C), respectively. The model was developed in New Hampshire, an area characterized by frequent rain events during spring and early summer.

It is known that periods of extreme dry weather can delay ascospore maturation in *V. inaequalis* (Keitt and Jones 1926, Wilson 1928, James and Sutton 1982a, 1982b, O'Leary and Sutton 1986, Schwabe *et al.* 1989). James and Sutton (1982a) in North Carolina, found that dry periods in spring delayed ascospore maturation in the field. In the laboratory, the pseudothecial development rate was retarded at a water saturation deficit greater than 85%, and there was no pseudothecial development in dry leaves. No pseudothecial development was found if rainfall was 0.25 mm or less and hours of 100% RH was 12 or less per day (James and Sutton 1982b).

Materials and methods

Much of the work presented in this paper has been described in detail previously (Gadoury *et al.* 1994, 1996, 1998, Stensvand *et al.* 1994, 1997, 1998). Thus, experiments are only briefly described below.

A wind tunnel for laboratory studies
The construction and operation of a wind tunnel and spore trap to study ascospore release of *V. inaequalis* in the laboratory was described by Gadoury *et al.* (1996). By means of this apparatus, the environment of an orchard during a rain event was recreated, and rainfall, temperature, light intensity, and quality, humidity, and air flow could be accurately controlled. By means of the wind tunnel we studied the effects of the following on the suppression of ascospore release in darkness: relative humidity, maturation and accumulation of ascospores in the pseudothecia, light quality, and light intensity. Futhermore, we studied changes in the rate of ascospore release over time and the rate of ascospore release at temperatures between 1 and 8°C.

Field studies on ascospore release during simulated rain events
Three plots of approximately 4 m^2 each were surrounded by wire mesh to contain apple leaves during winter in level grass fields. Severely scabbed leaves were placed within these enclosures in autumn of each year of the study (3 years). Three to four days before bud break of the cultivar McIntosh, canopies were suspended approximately 2 m above each plot to shield the enclosed leaves from rainfall. At the center of each enclosure, a Burkard 7-day volumetric spore sampler (Burkard Manufacturing, Ltd., Rickmansworth, Hertfordshire, England) was installed. The samplers operated continuously throughout the study. The covered enclosures were exposed to simulated rain every 3, 6, or 9 days by installing a single sprinkler head at the center of each plot, which dispensed water evenly over the floor of the enclosure at the rate of 9.3 cm/h. To prevent interplot interference in trapping airborne ascospores, no two plots were wet at the same day. Simulated rain aplications began 21:00 h and continued until 15:00 h the following day (18 h total duration). A datalogger at the site provided hourly measurements of temperature and leaf wetness within the covered enclosures.

Ascospore trapping in the field in Norway
Burkard 7-day volumetric spore samplers were installed in different apple growing regions of Norway during the primary inoculum seasons of 1989 to 1998. Recorded data were studied to find relationships between climatological parameters and diurnal or seasonal fluctuations in spore release. A degree-day model previously developed in New Hampshire to predict ascospore maturity (Gadoury and MacHardy 1982) was evaluated, and an adaption of the model to actual spore release is suggested.

Results and discussion

Effect of low RH on the suppression of ascospore release by darkness
In two populations of *V. inaequalis* (from Sweden and New York) the use of relatively dry air in the wind tunnel resulted in a substantially greater percentage of ascospore release during darkness. A dry air stream passing over the leaf samples represented an unnatural environment for ascospore release, since rainfall was coincident with very low humidity. The observed breakdown of the normal suppression of ascospore release during darkness was equally unnatural. The use of humidified air supply resulted in a response typical of the pattern reported in several orchard studies of ascospore release during natural rain, i.e. suppression of ascospore release during darkness and stimulation during light. Unnaturally low RH may have been a confounding factor in the aberrant patterns of ascospore release observed in some previous laboratory and greenhouse studies (Gjærum 1954, Warner and Braun 1992).

Effect of maturation and accumulation of ascospores in the pseudothecia on ascospore release in darkness

Increasing the duration of incubation without opportunity for ascospore release resulted in an increasing percentage of ascospores released during darkness in wind tunnel tests. Incubation of leaf samples for extended periods resulted in the maturation, and perhaps senescence, of the majority of the season's total inoculum. We incubated moist leaf samples collected at green tip for up to 11 days at 20°C. This corresponded to the accumulation of 220 degree-days (base = 0°C), enough to mature 40-70% of the season's total ascospore supply (Gadoury and MacHardy 1982), in addition to what had matured in the samples prior to incubation. With each successive day of incubation, a larger percentage of the total ascospore discharge occurred during darkness in the wind tunnel.

The percentage of ascospores released during darkness in different populations of *V. inaequalis* from various apple growing countries throughout the world, was directly proportional to the percentage of asci containing morphologically mature ascospores. Populations with higher numbers of mature asci were collected at later phenological stages of apple, which are associated with the depletion of the ascospore supply (MacHardy 1996). While this trend was possibly exaggerated in the international collection because of maturation of pseudothecia during shipment of leaf samples, the association of elevated ascospore releases during darkness with later phenological stages was, however, consistent.

From the above, it is evident that the suppression of ascospore release during darkness breaks down as populations of pseudothecia senesce. In genereal, the longer the interval between bud break and collection of the leaf samples from the various sites, the higher the percentage of ascospores released during darkness. Among populations of pseudothecia borne on scabbed apple leaves collected at the green tip stage of apple fruit buds, ascospore release during a 3 hour dark interval in the wind tunnel tests averaged 3.8% and ranged from 1.4 to 7.5% (n = 10). Comparable release during darkness was 7.9% from a single population collected at 1 cm green, 14.8% from a single population at tight cluster, 14.9% at pink (n= 3), 62.3% at bloom (n = 3), and 38.6% (n = 2) at petal fall.

In all 3 years of our field study under controlled conditions, a substantially higher percentage of ascospores were discharged during darkness at bloom than at green tip. Lack of suppression of ascospore release was not evident until more than 80% of the season's ascosporic inoculum had been trapped. The seasonal rate of ascospore maturation was not affected by the length of the interval between wetting events in any year of the study. As was noted in the wind tunnel studies, a higher percentage of ascospores were released during the dark interval (21:00 to 07:00 h the following day) at bloom than at green tip. Loss of suppressive effects of darkness on ascospore discharge did not appear to be a continuous or linear response until near the end of the ascospore maturation process in our field experiments. Once 80% or more of the ascospores matured, the potential existed for an increasing percentage of the available ascospores to be released during darkness. The potential increased rapidly once 80% of the ascospores had been trapped, but the response occurred as the absolute inoculum dose was rapidly shrinking (i.e., as the last 20% of the ascospores were discharged). However, while there was a clear relationship between our experimental creation of a senescent population and elevated ascospore release during darkness in a wind tunnel and during simulated rain in the field, no such phenomenon was reported in previous extensive studies of ascospore release during continuous or discontinuous natural rain events in New Hampshire (MacHardy and Gadoury 1986), Geneva and Highland, New York (Gadoury *et al.* 1995), and North Carolina (Aylor and Sutton 1992).

Ascospore release as affected by light quality and intensity

Colored filters yielding peak transmittance in the violet or red portions of the visible spectrum were employed to isolate the wavelengths responsible for the stimulation of ascospore release. In two populations from widely seperated geographic regions (Sweden and New York), we obtained a stimulation of ascospore release in response to light in the waveband from approximately 625 to 725 nm. Release during exposure to light within this waveband was not significantly different ($p = 0.05$) from that observed in daylight-balanced illumination. Wavelengths below 625 nm did not stimulate ascospore release, and release was not significantly different from that observed in darkness ($p = 0.05$). Thus, the earlier observations of Brook (1969a, 1969b) in New Zealand on the effects of red light on ascospore release in *V. inaequalis* can probably be applied to other geographic regions.

Linear regression analysis indicated that the rate of ascospore discharge was directly proportional to light intensity between 0.25 and 5.2 $\mu W/cm^2$ (recorded at 725 nm) in samples from two sites. The percentage of ascospores released during the first hour of illumination at 0.25 $\mu W/cm^2$ was not significantly different from percentage of ascospores released during darkness, but was significantly greater at 1.2 $\mu W/cm^2$. In samples from another site, ascospore release during the first hour of illumination at 0.5 $\mu W/cm^2$ was 9,9% (SE = 1.46) compared with 1.2% (SE = 0.79) in darkness during the same interval.

Under orchard conditions, the rate of ascospore release increases immediately after sunrise at temperatures above 10°C, but the peak rate of release is not reached until nearly 3-6 h later (MacHardy and Gadoury 1986). The minimum intensity of light required to stimulate ascospore release is relatively low compared with the typical intensity appearing the first hours after sunrise. For example, during a rain event on 24 May 1992 in Geneva, New York, light intensity at 725 nm reached the threshold level we found for stimulation of ascospore release (0.5 $\mu W/cm^2$) between 05:00 and 05:15 h, but it had increased to 4 $\mu W/cm^2$ by 07:00 h and 12 $\mu W/cm^2$ by 09:00 h.

Both global radiation and spectral distribution was recorded at Ås, Norway during a rainy day (May 7, normally early to mid part of the primary inoculum season). Sunrise was 04:05. At 720 nm, light intensity in an open field was 0.02 $\mu W/cm^2$ at 04:00 h, and it reached 0.63 $\mu W/cm^2$ at 05:00 h, which is above the light intensity that stimulated ascospore release in our lab studies. Palm (1988) indicated that few ascospores of the apple scab fungus are released at light levels below 2000 lux. In natural daylight 2000 lux equals approximately 8 W/m^2 global radiation. On May 7 at Ås, this level of global radiation was reached between 05:00 and 05:30 h, which was 1-1.5 h after sunrise.

Changes in rate of ascospore release over time

In one experiment, the number of ascospores discharged per minute was recorded from leaf samples exposed to simulated rain and daylight-balanced illumination in the wind tunnel for 2 h at 6°C, followed by 4 h at 20°C to harvest the remaining matured ascospores. The rate of ascospore release increased over time at 6°C, after an initial lag period of 30 min during which there was little or no ascospore release. The increase in rate of discharge was nonlinear and was sustained over the 2 hour duration of the 6°C phase of the experiment. Thus, the delay in reaching the peak rate of ascospore release after sunrise may be due to the combined impact of increasing light intensity after sunrise and the intrinsic increase in the rate of discharge over time. As discussed below, the magnitude of the time-dependent increase in the rate of ascospore discharge may itself be dependent upon temperature.

In another experiment leaf samples were exposed to simulated rain for a total of 9 h at 20°C and were illuminated either coincident with the start of wetting or beginning 2, 3, or 6 h later. When illumination of the leaf sample was delayed after the onset of simulated rain, an increasing percentage of the ascospores was discharged during the first hour of the

experiments. The duration of the dark interval preceding illumination can thus affect the rate of ascospore release once light is available, and a more rapid response to light could be expected as the duration of wetting during darkness is increased.

Ascospore release at low temperatures in the laboratory and field

Ascospore release was monitored in the wind tunnel at 1, 2, 4, 6, and 8°C in daylight-balanced light. To determine the total potential release from a leaf sample, experiments consisted of a low-temperature phase, lasting 6 h at 1°C or 3 h at 2-8°C, followed by 3 h at 20°C to harvest the ascospores remaining in the sample after the low-temperature phase.

Low temperatures resulted in a lag phase in the cumulative distribution of ascospore release, during which few or no ascospores were detected. The time until first detection of a given quantity of inoculum was inversely proportional to temperature. In two of three experiments at 1°C, the initial release of ascospores occurred after 131 and 153 min. In the third experiment at 1°C, no ascospores were detected until the temperature was raised during the final 3 h of the experiment. The mean time required to exceed a cumulative catch of 1% was 143 min. at 2°C, 67 min. at 4°C, 56 min. at 6°C, and 40 min. at 8°C. At 4, 6, and 8°C, the mean times required to exceed a cumulative catch of 5% were 103, 84, and 53 min., respectively. There was a reduction in the rate of release from 8 to 1°C, and consequently there was a reduction in the number of ascospores released at any given time.

During 16 episodes under field conditions in Norway when temperature was below 10°C and rain and leaf wetness started during night (after sunset) and continued until or beyond 24:00 h the following night, few spores were released until 4-5 hours after sunrise. The cumulative diurnal percentage of spores released (from midnight) at sunrise and 1, 2, 3, 4, and 5 hours after sunrise (± std. dev.) was 0.34 (± 0.43), 0.65 (± 1.02), 1.05 (± 1.73), 2.29 (± 3.13), and 3.73 (± 3.74), respectively. The mean temperature for each of the episodes varied from 1.7 to 8.2°C. Eight of the episodes had mean temperatures below 5°C, but there were no differences in release pattern below or above 5°C. For 7 episodes of rain and leaf wetness below 10°C which started during night and continued until the following afternoon, the release pattern was similar. If the percentage of spores released between midnight and 15:00 h was set to 100%, the cumulative percentage of spores released at sunrise and 1, 2, 3, 4, and 5 hours after sunrise (± std. dev.) was 0.89 (± 1.22), 1.11 (± 1.22), 1.37 (± 1.87), 1.73 (± 2.13), 2.88 (± 2.55), and 7.39 (± 8.22), respectively.

Our studies of the effects of temperature on ascospore release confirmed and extended major findings of earlier works (Hirst and Stedman 1962, MacHardy and Gadoury 1986, Seem *et al.* 1979).

Ascospore release during dew

At one location (Ås) in Norway in 1990, 1992, and 1997, and another one (Svelvik) in 1992, a total of 14.8, 1.4, 0.27, and 26.9%, respectively, of the season's total spore release was trapped during periods of dew. Dew followed by ascospore release was observed 22 days at the two locations. During one night with dew at Ås in 1990 and two with dew at Svelvik in 1992, approximately 13 and 20 %, respectively, of the season's total numbers of spores were observed.

Dew alone has never before been reported to be associated with high numbers of airborne ascospores of *V. inaequalis*, although some ascospore release by *V. pirina* has been reported during dew (Latorre *et al.* 1985, Spotts and Cervantes 1994). From previous work, it was concluded that unless dew followed rain before leaves had dried, dew alone had little importance in liberating spores (Hirst and Stedman 1962, MacHardy and Gadoury 1986). Similar results and conclusions were reported from studies conducted in Connecticut (Miller and Waggoner 1958), Wisconsin (Frey and Keitt 1925, Keitt and Jones 1926), and

Switzerland (Wiesman 1932). Brook (1969a) stated that both low temperatures and darkness before dew formation might depress the rate of spore release during dew and concluded that few ascospores are released, not because they fail to become airborne. However, Moore (1958) found numerous ascospores trapped in a water film on overwintered leaves and speculated that asci might remain submerged in thick water films, and thereby release ascospores into the dew film rather than into the air.

Minimum temperatures during nights with dew and high spore release varied from 5.7 to 12.5°C. Considerable ascospore release has been observed at these temperatures in Norway and other countries. Thus, temperatures seems an unlikely factor to either suppress or favor ascospore release during the period of our study.

High number of spores were trapped prior to sunrise, and on an average, 48.4% of the spores were trapped prior to 0400 h in the morning. Episodes in which more than 1% of the season's inoculum was released during dew occurred around bloom of apple, which is the peak period for ascospore discharge, and followed more than 2 days of fair weather (clear, warm days and cool, humid nights). From our lab studies, we have shown that the normal suppression of ascospore release during darkness can be negated by promoting ascospore maturation over extended periods without opportunity for ascospore discharge. Furthermore, our field studies under controlled conditions showed that the normal periodicity of ascospore release in orchards may break down as populations senesce and the last 5-10% of the ascospores mature. Thus, the accumulation of mature ascospores during extended periods with abundant dew (but without rain) and/or senescence of the pathogen population might occasionally lead to significant ascospore release during dew, given that temperature, thickness of the dew film, and absence of stimulatory light levels are not always limiting. Most of our observations of dew and high ascospore trapping occurred when degree-day accumulation would suggest that ascospore maturation was in an exponential phase (Gadoury and MacHardy 1982). Additionally, seven to eight rain-free days preceded the two largest ascospore trappings. Some combination or interaction of these factors may have been responsible for the relatively large numbers of ascospores trapped during occasional dew periods.

Seasonal distribution of ascospore release

Forecasts of a model previously developed in New Hampshire (Gadoury and MacHardy 1982) to estimate ascospore maturity, were compared to observed release. The model predicts 50, 95, and 99% spore maturation to occur at 250, 420, and 490 degree-days (DD), respectively. In locations and years with frequent rain events throughout the season for ascospore release, actual spore release followed predicted maturation closely. Protracted dry periods with no or little rain not only delayed ascospore release, but also maturation, and consequently extended the season for ascospore release. These observations are in agreement with those made by previous authors (Keitt and Jones 1926, Wilson 1928, James and Sutton 1982 a, 1982b, O'Leary and Sutton 1986, Schwabe *et al.* 1989). The mean DD accumulation (base temperature 0°C from the green tip phenological stage of the apple flower bud) at the time when the seasonal spore trapping had accumulated to 50, 95, and 99% was 367, 664, and 746, respectively. The most extreme year was 1992, when DD accumulation reached 1305 and 1092, respectively, at two locations at the time 95% of the season's spores were trapped. By halting DD accumulation during dry days, it was possible to improve the accuracy of the model. The best estimate of the spore maturation was made by halting DD accumulation after more than 4 days without rain.

Recommendations for practical management of apple scab

From the above it can be concluded that light intensity prior to sunrise on rainy days has no further stimulatory effect on ascospore release in *V. inaequalis* than complete darkness. Thus, the protracted dawn and dusk occurring under northern latitudes does not stimulate increased spore release compared to darkness. The light level that occurs between sunrise and one hour thereafter should be considered as the trigger of increased spore release in the morning. For practical scab management; time of sunrise coincides with the time of an increase in ascospore release in the morning.

The numerous revisions to Mills' criteria with regard to day and night release of ascospores are only appropriate for use in commercial orchards where potential ascospore dose (Gadoury and MacHardy 1986) is relatively low. Many commercial orchards harbor relatively small populations of *V. inaequalis* (Gadoury and MacHardy 1986). The percentage of available ascospores released by night rains is generally a small part of the potential release (Aylor and Sutton 1992, Brook 1969a, 1969b, 1975, MacHardy and Gadoury 1986). The important question in regard to control of apple scab is whether or not 5 to 10% of the maximum airborne ascospore dose in a well-managed commercial orchard represents a threat to the crop.

MacHardy and Gadoury (1989) provided an estimate of the maximum airborne ascospore dose that is likely to occur in a well-managed commercial orchard at night. Further evidence of the low risk of infection at low airborne ascospore doses was provided in a 6-year study by MacHardy *et al.* (1993), in which trees in low-inoculum commercial orchards were not sprayed for one to five infection periods, without regard to timing of the wetting events and without significant increases in fruit infection. Similar observations were made in low inoculum orchards in Norway (Stensvand and Amundsen 1997). Where the potential ascospore dose is relatively high, severe fruit infection could result from ascospores released during the night (MacHardy *et al.* 1993). MacHardy *et al.* recommend a potential ascospore dose of 1000 ascospores per m2 orchard area as a threshold level of inoculum below which infection periods could be ignored during early host growth. This threshold level, which approximates that 5% of the shoots are infected (Stensvand and Amundsen, unpublished data), could also be used conservatively now to identify orchards where timing of rain can be used to refine forecasts of infection.

Until the potential of late-season breakdown of the suppression of ascospore release during darkness can be more clearly defined, we would also suggest that elevated levels of ascospore release during night-time rain events should be considered a possibility once 90% of the ascospore population has matured, an event approximately coincident with the accumulation of 400 degree-days (base = 0°C) after bud break (Gadoury and MacHardy 1982). In very dry weather, during which substantial degree-day accumulation could occur, but during which the rate of ascospore maturation might be retarded (MacHardy 1996), the petal-fall stage of fruit bud development could be substituted for the above suggested degree-day threshold.

Because ascospore release is suppressed both in darkness and at low temperatures, there is a potential for additive effects of light and low temperatures. For example, if a cold rain event began during late afternoon, when darkness was only a few hours away, significant ascospore release might be delayed until dawn, whereas at warmer temperatures release could begin immediately after wetting. Additionally, in situations in which airborne ascospore dose would ordinarily be near a threshold level for significant infection under the most favorable temperatures, the reduction of airborne ascospore dose at temperatures near freezing may result in an absence of detectable infection even though the minimum environmental criteria for ascosporic infection are met or exceeded.

It is important to distinguish between the effects of temperature on the discharge of ascospores and the effects of temperature on the infection process. Low temperatures will delay the arrival and reduce the dose of ascosporic, but not conidial, inoculum. The extension of mimimum infection times due to the delay of ascospore release is most likely to be significant at temperatures of 2°C or less. At temperatures below 2°C, ascospore release is virtually nil for several hours. Even after release begins, the rate of ascospore release remains extremely low. Of course, at extremely high inoculum levels, the absolute number of ascospores released could be significant, even though it represents a small proportion of the total inoculum available. However, since many commercial orchards harbor little overwintering ascosporic inoculum (Gadoury and MacHardy 1986) detectable disease may not follow infection periods at temperatures near freezing, due to delayed ascospore release, reduced rate of release, and consequent low airborne ascospore dose. No such disparity is anticipated for conidial inoculum: no previous studies have demonstrated reduced production, release, or dispersal during cold wetting periods, neither is a disparity anticipated for ascosporic infection at high inoculum levels.

At temperatures between 0 and 10°C both our data and previous results (MacHardy and Gadoury 1986) have shown that ascospore release is delayed for several hours after sunrise compared to at higher temperatures. In practical scab management in low inoculum orchards, we therefore suggest to delay counting hours to add up to a predicted infection until 2 hours after sunrise if temperature is below 10°C.

The conditions that promote large releases of airborne ascospores during dew are complex, as well as very uncommon. In management of apple scab, long-term (e.g., >24 h) survival of ascospores on dry leaves is assumed to be nil (MacHardy 1996). The sequential occurrence of specific weather conditions, for example (i) fair-weather days, (ii) cool nights with abundant dew formation, (iii) significant release and dispersal of airborne ascospores, and (iv) poor drying conditions or additional hours of leaf wetness due to fog or rain, may be required for dew-released ascospores to constitute a threat of infection. Absent the foregoing, release during dew may be more likely to deplete the ascospore supply without a consequent increase in the overall risk of disease.

If rain events occurred frequently under orchard conditions in Norway, the New Hampshire degree-day model for ascospore maturation was very accurate in predicting actual spore release. However, the model was not accurate when there were protracted dry periods. We suggest an improvement of the model; dry days (i.e. less than 0.2 mm rain and less than 12 h wet leaves due to fog or dew) beyond the 4th dry day should be subtracted from the degree-day accumulation. This will give a better estimate of the spore maturation and actual release and much more exactly pinpoint the end of the primary inoculum season than by using the model under dry conditions as it is.

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A waterbath method for detection of potential ascospore discharge of *Venturia inaequalis*

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Abstract : Potential ascospore release of *V. inaequalis* was monitored using a waterbath method during six release seasons (1994-1999). Overwintered leaves were shaken with water and the ascospores discharged into the water were quantified microscopically with a cytometer. Centrifugation to increase ascospore concentration could be omitted when a high volume cytometer was used eliminating the need for a costly centrifuge system. Ascospores were quantified weekly before and during the annual period of primary infections and results evaluated together with data obtained from volumetric spore traps and scab infected trees. In the orchard the infection rates were determined with a bioassay using apple seedlings as indicators. Data from the waterbath method correlated well with that obtained from the spore traps with advantage that ascospores could be detected earlier at the beginning of the season. Spore titers increased with the main aerial spore peaks and decreased with the end of the ascospore discharge in the orchard. The correlation of ascospore concentrations (waterbath method) with subsequent ascospore discharges (spore trap) caused by rain indicated the applicability of the method for prognosis. In the last two release seasons aliquots of leaves were preincubated at 20 °C and high humidity for one and two weeks and were compared to the actual samples, respectively. A preview of the maturation process of ascospores could be assessed. In 1999 time course experiments of ascospore discharge into water were performed and some stimuli for discharge were characterized.

Key words : *Venturia inaequalis*, apple scab, ascospore discharge, epidemiology, discharge into water

Introduction

Apple scab caused by *Venturia inaequalis* (Cke.) Wint. can be controlled by the regular application of protective fungicides and/ or by curative fungicides in response to discrete infection periods. Warning systems are based on the predictions of scab development according to the infection criteria of Mills (1944) that define the period of leaf wetness necessary for infection. Rate of infection is also determined by amount of ascospore inoculum present (Kohl *et al.* 1994; Anagnostakis and Aylor 1991). Accordingly, basic research on the effects of various environmental factors on ascospore release by *V. inaequalis* has been performed using advanced laboratory devices (Gottwald *et al.* 1997; Stensvand *et al.* 1997; Gadoury *et al.* 1996). Models and methods have been developed to assess the potential inoculum (Gadoury and MacHardy 1986), the maturity of ascospores, and the inoculum concentration in the air. The first significant ascospore release into the air is difficult to assess by microscopic evaluation of the maturity of the pseudothecia, asci and ascospores (Gadoury *et al.* 1992, James and Sutton 1982). Field measurements of ascospore concentration in the air using volumetric spore traps is appropriate for a continuous recording, but requires considerably more effort. Using a simple wind tunnel apparatus, ejected ascospores were quantified from a stream of air over moistened overwintered leaves as an alternative (Stephan 1987; Aylor 1996). Aylor (1996) detected a lag between the cumulative curves of airborne

ascospores monitored with a volumetric spore trap and the release potential of ascospores tested in spore release towers. During the ascospore release season of 1993, Kohl *et al.* (1994) used the release of ascospores from scabbed leaves into water (Gadoury and MacHardy 1982, Sutton and Jones 1976) to develop a simple quantitative method. This easy to perform waterbath method is well suited to detect the beginning and the end of ascospore release with high sensitivity. Furthermore, the results correlated with the detection of airborne ascospores and with data from a laboratory jet spore sampling method, which is comparable to wind tunnel method. The objective of the current study was to simplify the waterbath method and to substantiate the preliminary results over six further ascospore release seasons. The time course of ascospore release potential was evaluated with airborne ascospores data and monitoring of infected indicator trees in the orchard.

Material and methods

Detection of infection periods in the orchard

Potted apple seedlings were used as trap plants to detect primary infections during the six ascospore release seasons (1994-1999). Sets of 15 potted 'Golden Delicious' seedlings were placed around overwintered leaf depots in the orchard during each infection period (Kohl *et al.* 1994). Disease assessment of exposed seedlings was done after an incubation time in the greenhouse of about one month.

Aerial ascospore quantification

Two 7-day recording volumetric spore traps (Burkard Ltd., Rickmansworth, England) with single leaf depots (Kohl *et al.* 1994) were operated continuously during the periods of ascospore release in 1994-1999. Spores were counted microscopically (500x) by scanning transects across the long axis of the tape at 2 mm (1 h) intervals. The daily average values of recorded ascospores by both traps are presented.

Potential ascospore release, waterbath method

Leaf depots overwintered on the orchard ground consisted of scabbed leaves of cv. 'Golden Delicious' in trays (55x30x7 cm) covered with wire mesh. During the periods of ascospore release in 1994-1999 leaf samples were removed weekly and dried overnight in the laboratory. Leaves were bisected along the mid veins and strong veins were discarded with the petioles. The leaf material was reduced to pieces of about 1-2 cm, 1 g of which was agitated in 50 ml distilled water in a 100-ml Erlenmeyer flask for one hour on a reciprocating shaker (rotation frequency: 50/min; radius: 1 cm). The liquid was filtered through Miracloth (Calbiochem) and 40 ml centrifuged for 15 min at 12,100 g (JA 20, Beckman, 10,000 rpm). The pellet was suspended in 1 ml water and the spores quantified with a Neubauer-cytometer. Additionally, in 1997-1999 a Kolkwitz planktoncytometer (0.5 ml, Hydro-Bios, Germany) was used. Immediately after shaking the leaves, the planktoncytometer was filled with the spore suspension. Ascospores were allowed to settle onto the 1mm-square grid for 10 min and ascospores from at least 20 squares were counted. For each sample time point waterbath experiments were replicated twice and the average values presented. Preincubation experiments were performed with leaf aliquots removed from the depot one or two weeks before the standard sampling dates, respectively. The wetted aliquots were incubated at 20 °C in the dark in a 600 ml beaker closed with foil. At the bottom of the beaker a wet cellulosic plug maintained a high humidity during the incubation period. Acoustic energy was transmitted by an ultrasonic bath (Bandelin, Sonorex TK 52) to the Erlenmeyer flask or by loudspeaker impulses from an electronic metronome directed to a polyethylen bag or plastic vessel containing the leaves and water. Time course experiments were performed by removing

0.6 ml aliquots at different times for determination of spore concentration with the planktoncytometer. Alternatively at the different times the spore suspension was decanted completely for determination of spore concentration followed by renewal of water, respectively.

Results

The cumulative numbers of airborne ascospores determined by volumetric spore traps and the cumulative potential release of ascospores determined by waterbath method are presented in Figure 1. The waterbath method detected ascospore concentrations, similar to that detected airborne. In periods with no significant ascospore releases in the orchard (spore trap) the waterbath method detected the presence of ascospores ready for discharge. In all years spore titers could be determined within the period of detected scab infections. Moreover, the concentrations of ascospores could be determined before and after the appearance of primary infections. Before and after the periods of primary infections, the waterbath method, compared to the volumetric spore trap method, yielded more reliable data. In 1997 the potential ascospore release was confirmed with the planktoncytometer. There were no essential differences in spore concentration pattern, obtained with the Neubauer-cytometer and by centrifugation.

Preincubation of leaves for 14 d in 1998 and 7 d/14 d in 1999 caused a shift of maturity curves (Figs. 2,3). Preincubation for 14 d showed a shift of about 2-3 weeks in 1998 and of about 2-4 weeks in 1999. Overall counts of ascospores were higher (especially in 1998) and sensitivity increased at the beginning of the primary seasons.

Time course of ascospore discharge into water is shown in Figure 4. Complete exchange of water resulted in less variation and showed clearly the trend of further discharge within time. Further discharge did not even cease when leaf samples were shaken with water for 20 h.

Shaking the leaves in the dark did not result in a significant reduction of ascospore discharge. The effect of shaking, ultrasonic and loudspeaker impulses are presented in Figure 5. Ultrasonic led to an increase of temperature and this high energy input was not effective for induction of ascospore discharge. Loudspeaker impulses which caused only slight vibration of the water induced the discharge according to frequency and sound permeability (plastic vessels versus plasticbags).

Discussion

Laboratory spore quantification methods suited for prognosis of ascospore release in the orchard should fulfill several criteria. Sampling procedures and methods should be easy and rapid to perform with minimal instrumentation. Furthermore, a simple time schedule with few sampling dates should yield sufficient numerical data during the relevant period of primary infections and also before and after this period. Particularly, the beginning of the ascospore release season should be detected with high sensitivity. The time course of ascospore release potential during the primary season should reflect the actual spore release in the orchard detected by spore traps. The method for assessment of potential ascospore release presented in this study essentially fulfilled the mentioned criteria. In 1997, instrumentation was reduced considerably by application of the planktoncytometer, which allowed equivalent ascospore quantification without prior centrifugation. The simple time schedule of weekly samplings resulted in sufficient data before, during and after the primary season where the primary infection season was defined by the first and last detected infection. For influence of weather conditions and the time schedule of leaf sampling and the degree of cumulative potential

ascospore release to lag behind the cumulative airborne ascospores detected by spore traps see Kollar (1998).

In a similar study Aylor (1996) compared the time course of the release of *V. inaequalis* ascospores recorded with a volumetric spore trap in the field with potential release determined every 3 to 5 days from scabbed leaves under standard conditions in spore release towers in the laboratory. There was a significant lag between the cumulative curves obtained from the volumetric spore trap and the spore tower. Furthermore, at the beginning of the seasons, the potential ascospore releases detected with the spore tower were low compared to the concentrations obtained by the spore trap. Much of the overall lag was explained by a natural decline in the number of source leaves. However, the spore release tower method itself could have contributed to the observed lag. In the study reported here, the waterbath method showed a high sensitivity at the beginning of the season and compared to spore tower methods has additional advantages. It is quick and easier to perform with minimal instrumentation and, therefore, also suited for a large number of leaf samples. In addition, orientation of pseudothecia in the leaves, which may be different even within one curled leaf, and the degree of attrition or fragmentation of leaves, create no artifacts.

Experiments with preincubation of leaf samples, time course of ascospore discharge and its stimulation add to the knowledge to improve or to adapt the standard method presented here to the individual needs.

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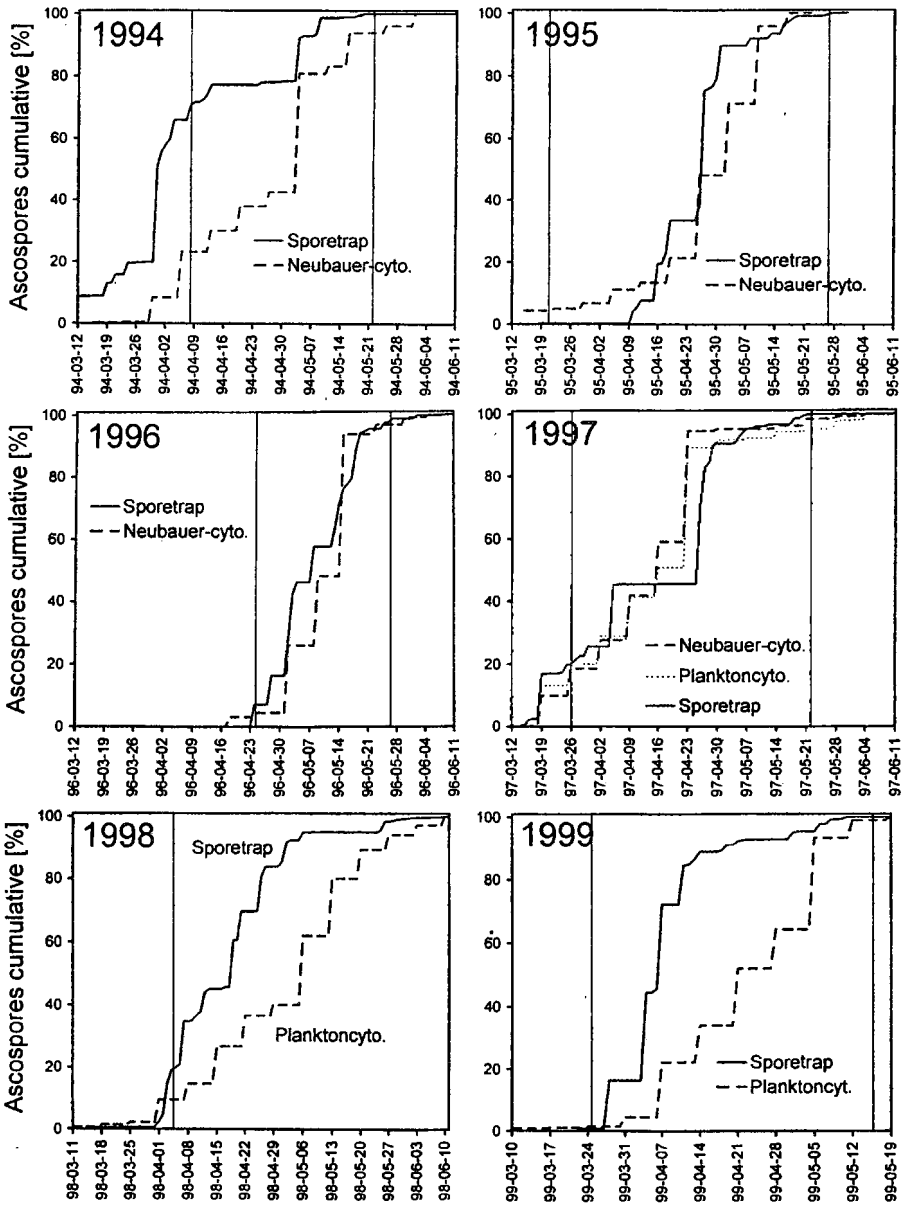

Figure 1. 1994 -1999. Comparison of cumulative numbers of airborne ascospores (sporetrap) and potential release of ascospores (waterbath method). Overwintered leaves were sampled weekly, shaken with water and ascospores were quantified with a planktoncytometer (1997-1999) or after centrifugation with a Neubauer-cytometer (1994-1997). The vertical lines indicate the detected periods of primary infections using exposed apple seedlings.

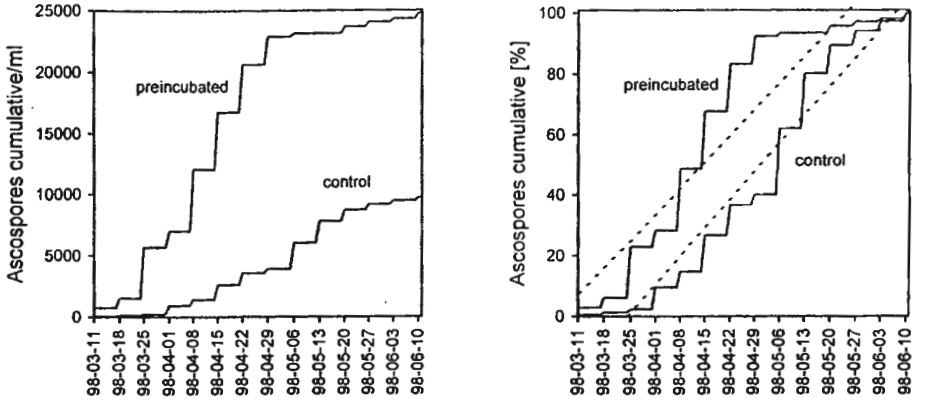

Figure 2. 1998. Ascospores cumulative after waterbath method with preincubated (14 d, 20 °C, wet in the dark) leaf samples versus control (no preincubation). Absolute spore concentrations (left) and expressed as percentage (right, trendlines included).

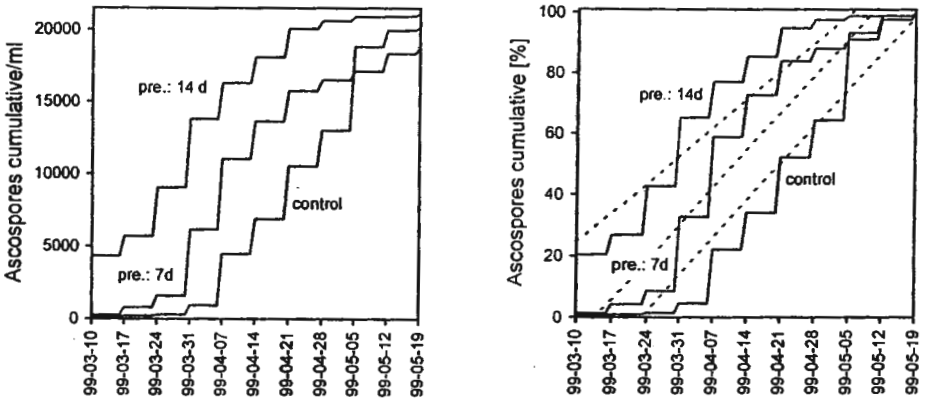

Figure 3. 1999. Ascospores cumulative after waterbath method with preincubated (7d/14 d, 20°C, wet in the dark) leaf samples versus control (no preincubation). Absolute spore concentrations (left) and expressed as percentage (right, trendlines included).

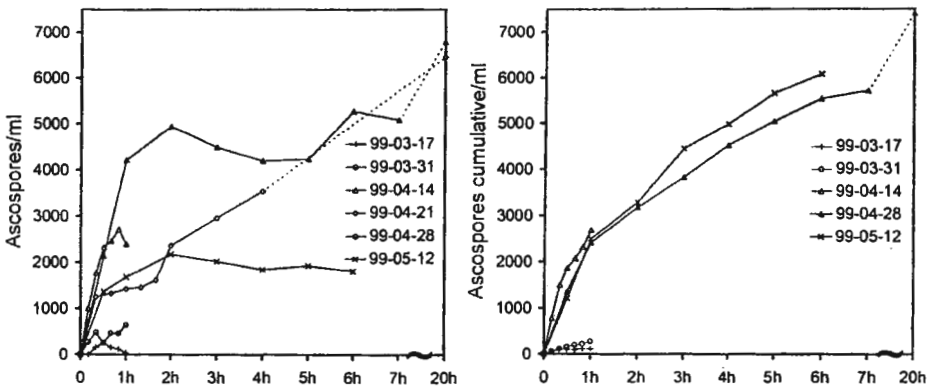

Figure 4. Time course of ascospore discharge into water (waterbath method) determined with planktoncytometer. Aliquots for ascospore quantification were removed from samples after the time intervals without renewal of water (left) or followed by a complete exchange of water (right).

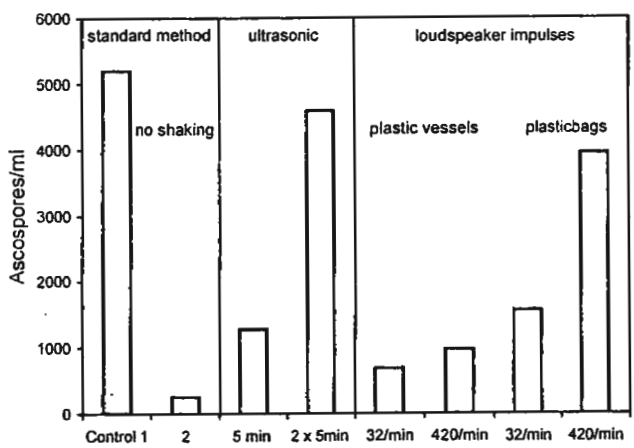

Figure 5. Induction of ascospore discharge into water (waterbath method) by shaking (control 1) or not (2), ultrasonic (Erlenmeyer flasks containing leaves and water immersed in an ultrasonic bath), loudspeaker impulses from an electronic metronome directed to plastic vessels or -bags containing the leaf samples and water.

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Relationship between assessment of scab on apple leaves in autumn and ascospore production the following spring

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Abstract : A study was conducted to determine if a late winter/pre-bud-break laboratory procedure to predict an orchard's level of "scab-risk" based on trapping ascospores from all scabbed leaves found on 600 shoots the previous autumn could replace the current procedure that predicts the level of "scab-risk" based on an autumn assessment of scab incidence on 600 shoots. A model to predict ascospore productivity from ascospores trapped at *n* degree-days (DD) after a biofix (date of first matured ascospores) was developed under prescribed laboratory temperature and humidity conditions. To determine if all scabbed leaves found on 600 shoots was a large enough sample to represent ascospore productivity in an orchard, the actual ascospore productivity on a leaf set comprised of all scabbed leaves found on 600 shoots in autumn was compared with ascospore productivity predicted by the model. Four replicated collections of scabbed leaves from 600 shoots in each of six predominantly McIntosh commercial orchards provided 24 leaf sets for testing. Two lines of evidence are suggestive that the proposed procedure will not be reliable : (i) ascospore productivity was not predicted with 95% confidence in 21 of 48 tests, and (ii) 7-fold to 361-fold differences in ascospore productivity among the four replicated assessments in the six orchards was much too great to consider basing a prediction of "scab-risk" on a sample size of 600 shoots. The reliability of the model improved when the four replicate assessments were pooled: ascospore productivity on all scabbed leaves found on the pooled 2400 shoots was within the model's predicted 95% confidence limits of ascospore production in all six orchards. However, a procedure that requires examining 2400 shoots and trapping ascospores from all scabbed leaves found on those shoots would not be practical. In five orchards, scab incidence in each of the four replicate assessments classified the orchard as either low "scab-risk" or moderate "scab-risk". In one orchard, three assessments classified the orchard as "moderate-risk," while the fourth assessment was slightly below the threshold for a "moderate-risk" orchard. With this reliability and other considerations, scab incidence on 600 shoots remains the most useful and reliable measure to predict an orchard's level of "scab-risk.". Analysis of the four replicate assessments in the six orchards discussed here and in orchards examined similarly the previous year showed that within 95% confidence intervals, the potential ascospore dose (PAD) calculated from one assessment of scab on 600 shoots was within 10% of the true mean in orchards with PAD <1000. Also, the 1-fold to 3-fold difference in predicted PAD in the four replicated assessments in the six orchards was within a range that is acceptable for the "PAD" action threshold.

Key words : *Venturia inaequalis*, potential ascospore dose, PAD, spore trapping, integrated pest management, IPM

Introduction

A fundamental assumption of nearly all apple scab management programs throughout this century is that the supply of ascosporic inoculum in every orchard is always great enough to justify the repeated application of fungicides beginning shortly after bud break and scheduled to insure that the leaves and fruit are always protected, at least until the supply of ascospores is exhausted. This fungicide program may have been necessary before the development of modern fungicides and air blast sprayers and the planting of cultivars on semi-dwarfing and dwarfing rootstocks, but it cannot be justified for all orchards now. Several studies conducted

during the past 15 years in the northeastern United States (MacHardy 1994, 1998a, 1998b, MacHardy *et al.* 1993.) have demonstrated that many orchards had managed scab so effectively, as identified by the low incidence of leaf scab in autumn, that one to several early-season infection periods could be left unprotected the next season without resulting in an unacceptable incidence of scabbed fruit at harvest. These orchards had 50 or less scabbed leaves on 600 shoots assessed in autumn. They are designated "low-risk," and are distinguished from "moderate-risk" orchards in which foliar scab incidence in autumn was so high that scab the following spring could be controlled only if the fungicide program targeted all infection periods, including the early ones.

The significance of identifying a "low-risk" (low ascosporic inoculum) orchard is that several tactics can be employed that reduce the recommended seasonal fungicide dose without increased risk to the crop. One tactic, for example, is to delay the first fungicide application to control scab until after three infection periods but before the fourth infection period, or at the pink phenological stage (whichever comes first) if $\leq$50 scabbed leaves are found on 600 shoots assessed in autumn. A seasonal reduction in fungicide dose of 20-40% has been common in "low-risk" orchards that used this tactic.

The prediction of a "low-risk" orchard based on an assessment of foliar scab in autumn has been demonstrated successfully in numerous orchards over 15 years (MacHardy, 1998a, 1998b), but we have been concerned that it does not take into account several factors that may increase or decrease, perhaps significantly, the actual ascospore dose that develops in spring. It doesn't, for example, consider that although a lesion is still present, the fungus under the lesion may have been eradicated by fungicides used throughout the season. Nor does the assessment consider lesion age, time of leaf fall, presence or absence of snow cover, temperature and moisture conditions, leaf nutrient and pH status, phylloplane organisms, and apple cultivar that have been reported to affect the production and survival of pseudothecia (MacHardy 1996a). Also not taken into account are infections that may occur after the assessment or infections that are latent at the time of assessment, perhaps because of fungicides applied during the growing season that inhibited, but did not kill, the pathogen.

To address our concern, we conducted a study designed to develop a procedure that would replace the autumn assessment of lesion incidence with a late-winter/early-spring assessment of ascospore productivity. The approach we selected was similar to an approach that has been used successfully by entomologists for many years: (i) trap the pest and count the number trapped, (ii) develop an action threshold that relates the number trapped to the amount of damage, and (iii) recommend appropriate management practices based on decision-rules associated with the action threshold. The advantage of a late-winter/early-spring assessment over the standard autumn assessment would be that it would take into account nearly all of the factors discussed above that impact the development and survival of pseudothecia.

The procedure we envisioned was to have a grower collect all scabbed leaves on 600 shoots, overwinter them on the orchard floor in plastic mesh "sandwiches," bring the leaves into a room maintained at normal room temperature in late-February, incubate them until approximately 125 DD had accumulated, trap and stain the ascospores using an ELISA procedure (Berkett, *et al.* 1992; Gotlieb *et al.* 1995.), relate the total ascospores trapped to a "scab-risk" action threshold that would indicate the orchard's level of "scab-risk," and select the appropriate mix of tactics based on the level of "scab-risk."

For our proposed procedure to be feasible, we needed to (i) determine if all scabbed leaves found on 600 shoots was a large enough sample size to represent ascospore productivity in the orchard and (ii) develop and validate a laboratory-derived model that would identify an orchard's level of "scab-risk" based on one trapping of ascospores from all scabbed leaves found on 600 shoots at 125 DD after the biofix.

We were concerned that testing all scabbed leaves collected from 600 shoots would be too time-consuming and labor-intensive to be acceptable to growers, so a study was conducted to determine if single-lesion leaves could be ignored. Theoretically, if a lesion is the result of infection by a single ascospore, then leaves with a single lesion should not become fertile.

Also, most scabbed leaves in an orchard that had been well- or moderately-managed for scab have only one lesion, so ignoring single-lesion leaves would increase the feasibility of our proposed procedure considerably.

The experimental unit selected to obtain a measure of ascospore productivity was all scabbed leaves on 600 shoots. This allowed a direct comparison of ascospore productivity and potential ascospore dose (PAD), i.e., the predicted ascospore dose per meter square of orchard floor (Gadoury and MacHardy 1986), in each orchard, because the amount of foliar scab on 600 shoots in autumn is the most important variable in the equation for calculating PAD. The replicated assessments of PAD in an orchard allowed us to determine the reliability of predicting PAD from an assessment of foliar scab on 600 shoots.

The objectives of our study were to (i) determine the relationship between lesion density on a leaf and lesion fertility and ascospore productivity, (ii) determine the reliability of predicting PAD from an assessment of foliar scab on 600 shoots, and (iii) determine the reliability and feasibility of an indoor spore-trapping procedure to predict the level of "scab-risk."

Material and methods

Determine the relationship between lesion density on a leaf and lesion fertility and ascospore productivity

When collecting the scabbed leaves found on 600 shoots in each replicate assessment, each leaf was identified as having 1, 2-5, or >5 scab lesions. Leaves in each lesion category were kept separate, and data were collected on the number of ascospores trapped per lesion category so that the relationship between the number of lesions and lesion fertility and ascospore productivity could be determined.

Determine the reliability of predicting PAD from an assessment of foliar scab on 600 shoots

While examining the 600 shoots in each replicated assessment in an orchard, data needed to calculate PAD were recorded: lesion incidence and density on each shoot, number of leaves/shoot, and leaf litter density (Gadoury and MacHardy 1986). The PAD calculated for each of the four replicated assessments in an orchard was compared with the "PAD" action threshold to determine the reliability of one assessment of 600 shoots to predict a PAD that was above or below the "PAD" action threshold.

Determine the reliability and feasibility of an indoor spore-trapping procedure to predict the level of "scab-risk"

STEP 1 : develop a model to estimate the percentage of the season's ascospores matured. In autumn, 1994, three sets of severely scabbed leaves were collected from McIntosh trees in one orchard in Vermont (VT) and one orchard in New Hampshire (NH). In each state, one set was overwintered in the orchard where the leaves were collected and the other two sets were overwintered at two other orchards. On 1 February 1995, all leaf sets were brought into the laboratory, placed in moist chambers (relative humidity >90%), and incubated at 5 C. After each set had been incubated for 55 DD (base 0 C), pseudothecial squash mounts were prepared at 3-4 day intervals and examined for matured ascospores. During laboratory incubation, the leaves were kept moist by periodically misting the leaf sets. When the biofix (i.e., first matured ascospores) for a leaf set was identified, 20 leaf disks (one

from each of 20 leaves) were cut with a #15 cork borer, placed in separate plastic mesh packets, and incubated in a moist chamber for 150 DD at 5C (30 days), 200 DD at 10 C (20 days), and finally at 20 C. Twice each week until the supply of ascospores was exhausted, each disk was placed on a "funnel" spore trap to collect the matured ascospores. For each test day, the cumulative DD and the total ascospores trapped were recorded. Ascospore productivity on each leaf disk, and for the 20 leaf disks in a set, was calculated by totaling the ascospores recorded for all trappings. The relationship between cumulative DD and percent of total ascospores trapped provided the data to develop a model to estimate ascospore maturity with 95% confidence bands (Figure 1) using the procedure described by Gadoury and MacHardy (Gadoury and MacHardy, 1982).

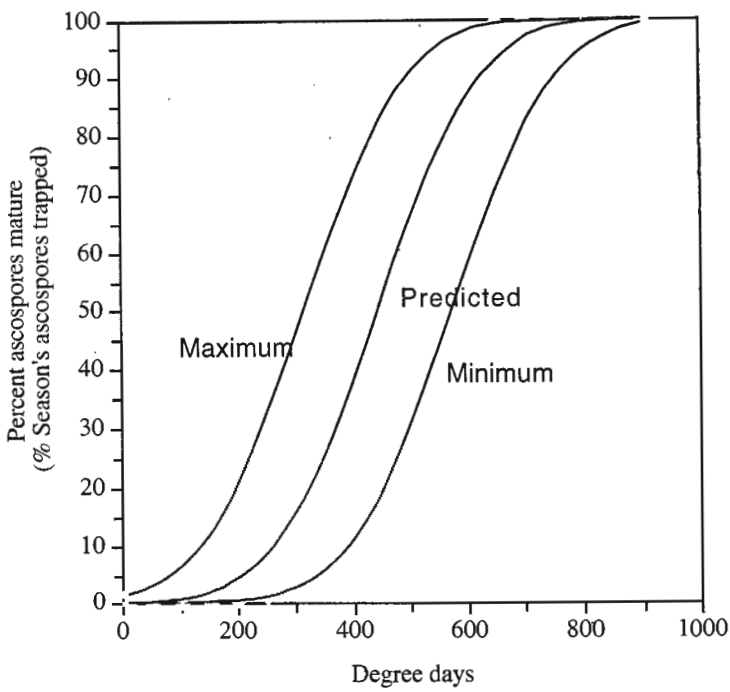

Figure 1. Model to predict maturation of *Venturia inaequalis* ascospores developed from scabbed McIntosh apple leaves incubated under prescribed temperature and humidity conditions in the laboratory in February and March after overwintering on the orchard floor

STEP 2 : use the ascospore maturation model to validate the proposed procedure to predict an orchard's level of "scab-risk" based on one trapping of ascospores at a specified number of DD from the biofix. The ascospore maturity model (Figure 1) predicts the percentage of the season's ascospores that have matured at any given DD after the biofix. If *% season's ascospores trapped* is substituted for *% season's ascospores matured*, the model can be used to predict, with 95% confidence, ascospore productivity based on a single trapping of ascospores at a known number of DD from the biofix. This idea was tested at 100 and 200 DD as a means to validate the model. The model predicts that 95% of the time, the cumulative ascospores trapped at 100 DD should be within 0.04 to 6.2% of all ascospores trapped and at 200 DD should be within 0.4 to 20.0% of all ascospores trapped. These

percentages were used to calculate the predicted 95% confidence limits of ascospore productivity from the cumulative ascospore trappings at 100 and 200 DD. At 200 DD, for example, the model predicts that 4% of the total ascospores trapped for the season will be trapped, although 95% of the time the ascospores trapped will be within the range 0.4 to 20.4% of the total ascospores trapped from a leaf set. Thus, if 50 ascospores are trapped up to 200 DD, ascospore productivity of the leaf set is predicted to be 1,250 (50 ÷ 0.4). However, 95% of the time, the ascospore productivity that could account for 50 ascospores trapped at 200 DD would be within the range 245 to 12,500. The model was validated if the actual ascospore productivity of a leaf set was within the 95% confidence limits of the predicted ascospore productivity.

Twenty-four sets of scabbed leaves, four sets from each of six predominantly McIntosh commercial orchards (three orchards in Vermont and three orchards in New Hampshire), were collected in autumn 1994. Each set was comprised of all scabbed leaves found on 600 shoots. The leaves were placed in plastic mesh "sandwiches," and kept overwinter on the orchard floor until 1 February 1995 when they were brought into the laboratory, incubated and tested for ascospore production, as described above. With each leaf set, the minimum and maximum ascospore productivity that could, 95% of the time, account for all ascospores trapped up to 100 DD and 200 DD was calculated, as explained above, and compared with the actual ascospore productivity. If the actual ascospore productivity was within the predicted range of ascospore productivity, this was validation that one trapping of ascospores at 100 or 200 DD after a biofix could predict the level of "scab-risk" in an orchard with 95% confidence.

STEP 3 : determine if all scabbed leaves found on 600 shoots is a large enough sample size to represent ascospore productivity in an orchard.
Data collected to validate the ascospore productivity procedure (see preceding paragraph) were used to determine if all scabbed leaves found on 600 vs 2400 shoots is a reliable sample size to represent ascospore productivity in an orchard.

STEP 4 : determine the reliability of predicting "scab-risk" based on the total scabbed leaves found on 600 shoots.
The incidence of scabbed leaves found on 600 shoots in each of the four replicates per orchard was compared to established incidence levels associated with "low-risk" and "moderate-risk" orchards (MacHardy 1998a, 1999b, MacHardy *et al.*, 1999) to determine if 600 shoots is a sufficient sample size to predict "scab-risk."

Results

Determine the relationship between lesion density on a leaf and lesion fertility and ascospore productivity in commercial orchards well-managed and moderately managed for scab

Lesion density (Table 1). Leaves with 1 lesion account for 37 to 82%, leaves with 2-5 lesions account for 12 to 35%, and leaves with >5 lesions account for 2 to 28% of the total scabbed leaves collected in the six orchards. When all 14,400 shoots examined in the six orchards were pooled, leaves with 1, 2-5, or >5 scab lesions accounted, respectively, for 61, 23, and 16% of the scabbed leaves observed.

Lesion fertility (Table 1). Of the 91, 82, 143, 295, 234, and 341 scabbed leaves collected, respectively, in the six orchards, no ascospores were trapped from 88 (97%), 72 (88%), 125 (87%), 266 (90%), 214 (91%), and 315 (92%) leaves, respectively. Thus, of 1,186 scabbed leaves tested, only 106 leaves (9%) were fertile.

Lesion fertility on leaves with 1, 2-5, or >5 lesions (Table 1). The percentage of leaves with 1, 2-5, or >5 scab lesions on which fertile lesions developed, i.e., ascospores were trapped, ranged from 2 to 10%, 8 to 30%, and 18 to 40%, respectively, in the six orchards. For all leaves with 1, 2-5, or >5 scab lesions collected from the 14,400 shoots assessed for scab in the six orchards, fertile lesions developed on 5, 10, and 20%, respectively.

Ascospore productivity per fertile leaf on leaves with 1, 2-5, or >5 lesions (Table 1). The mean ascospore productivity per fertile leaf with 1 lesion ranged from 341 (VT-12) to 10,977 (NH-17); with 2-5 lesions it ranged from 850 (NH-16) to 6,402 (VT-14); and with >5 lesions it ranged from 510 (NH-16) to 6,840 (VT-14). The greatest difference in ascospore productivity was in leaves with one lesion. In two orchards, ascospore productivity per fertile leaf on leaves with one lesion was low (341 and 929 ascospores were trapped) compared with the other four orchards in which ascospore productivity per fertile single-lesion leaf was relatively high, (2,468 to 10,977 ascospores were trapped).

The contribution of leaves with 1, 2-5, or >5 lesions to the ascospore productivity of all leaves tested (Table 1). Leaves with 1, 2-5, or >5 scab lesions contributed 2 to 98%, 2 to 77%, and 1 to 61%, of the total ascospores trapped, respectively, in the six orchards.

Ascospore productivity on leaves with 1, 2-5, or >5 lesions (Tables 1 and 2). Ascospore productivity on individual leaves with 1, 2-5, or >5 lesions ranged from 160 to 30,670, 236 to 27,366, and 265 to 44,383, respectively. The mean ascospore productivity on all leaves with 1, 2-5, or >5 lesions was 4,864, 4,062, and 3,331, respectively. The greatest difference in ascospore productivity per fertile leaf was in leaves with 1 lesion. The VT-12 and NH-15 orchards were very similar in the number of scabbed leaves collected in each lesion category and in the predicted PAD, but there was a 18-fold difference in the number of ascospores trapped per fertile leaf from leaves with one lesion, i.e., 341 ascospores in VT-12 and 6,256 in NH-15.

Determine the reliability of predicting PAD from an assessment of foliar scab on 600 shoots (Table 3)

The predicted PAD in the four replicate assessments in the six orchards ranged from 143 to 312, 117 to 441, 836 to 1,792, 1,335 to 1,775, 2,109 to 3,690, and 2,364 to 3,644, respectively. The PAD predicted for the six orchards ranged from 177 to 2,912. A predicted PAD of 600 or less places an orchard in the "low-risk" category (MacHardy 1998a, 1999b). Thus, the two orchards with a predicted PAD of 209 and 220 are classified as "low-risk;" the four orchards with a predicted PAD of 1,248, 1,541, 2,688, and 2,912 are classified as"moderate-risk" (MacHardy 1994, 1998). In each of the six orchards, the predicted PAD for the four replications was either below or above the 600 PAD threshold that distinguishes a 'low-risk" orchard from an orchard with a "moderate" or "high" scab-risk.

Determine the reliability and feasibility of an indoor spore-trapping procedure to predict the level of "scab-risk."

Validate the indoor spore-trapping model to predict ascospore productivity (Figure 1, Tables 4-6). When the test leaf set was comprised of all scabbed leaves found on 600 shoots, the actual ascospore productivity was outside the predicted ascospore productivity 95% confidence limits in 18 of the 48 tests. All four replicated tests were within the ascospore productivity 95% confidence limits in only one orchard (NH-17), and only for the 200 DD test. When the test set to calculate the predicted ascospore productivity was comprised of all scabbed leaves found on the pooled 2400 shoots examined in an orchard, the actual ascospore

productivity of those leaves was within the predicted ascospore productivity 95% confidence limits in all 12 tests (Table 6).

Determine if all scabbed leaves found on 600 shoots is a large enough sample size to represent ascospore productivity in an orchard (Table 3).
Ascospore productivity on the scabbed leaves collected in the four replicate assessments in the six orchards ranged from 0 to 2,275, 85 to 30,711, 8,538 to 62,754, 592 to 111,268, 5,645 to 72,984, and 7,613 to 46,068, respectively. The differences in ascospore productivity in the four replicate assessements ranged from 7-fold in VT-13 to 361-fold in NH-15,

Determine the reliability of predicting "scab-risk" based on the total scabbed leaves found on 600 shoots (Table 3).
The number of scabbed leaves on 600 shoots in the four replicated assessments in the six orchards ranged from 21-25, 17-24, 30-44, 63-83, 47-77, and 77-97, respectively. The current threshold for characterizing an orchard as "low-risk" is 50 or fewer scabbed leaves per 600 shoots (MacHardy *et al.* 1999). For all but one orchard (VT-14), all the replications within an orchard were consistently (i.e., 100%) below threshold or all were above threshold. In VT-14, three replications were above threshold (i.e., 57, 78, and 53) and one was slightly below (i.e., 47).

Table 1. Relationship between the number of scab lesions on a leaf in autumn, 1994 and lesion fertility and ascospore production next spring in six commercial orchards in New Hampshire and Vermont

Orchard/PAD[a]	No. of lesions	No. scabbed leaves	% of total scabbed leaves	Ascospore production				
				No. leaves fertile	% leaves fertile	Total spores trapped[b]	% of total spores trapped	Spores trapped per fertile leaf
VT-12	1	73	80	2	3		23	341
PAD = 209	2-5	13	14	1	8		77	2,275
	>5	5	6	0	0		--	--
						2,957		
NH-15	1	67	82	5	8		70	6,256
PAD = 220	2-5	10	12	3	30		23	3,479
	>5	5	6	2	40		6	1,125
						44,230		
NH-17	1	80	56	3	4		32	10,977
PAD = 1,248	2-5	34	24	7	21		43	6,267
	>5	29	20	8	28		25	3,132
						101,864		
NH-16	1	239	81	24	10		98	8,106
PAD = 1,541	2-5	51	17	4	8		2	850
	>5	5	2	1	20		0.3	510
						198,454		
VT-14	1	86	37	2	2		2	929
PAD = 2,688	2-5	82	35	6	7		31	6,402
	>5	66	28	12	18		67	6840
						122,356		
VT-13	1	176	52	3	2		6	2,468
PAD = 2,912	2-5	88	26	8	9		33	5,102
	>5	77	22	15	19		61	5,049
						123,959		

[a]PAD based on an autumn assessment of 2400 extension shoots at each site.

[b]Total ascospores trapped from all scabbed leaves tested in all lesion-density categories.

Table 2. Comparison of ascospores trapped in spring from leaves with 1, 2-5, or >5 visible scab lesions in autumn[a]

Lesions per leaf	Ascospores trapped for season[b]		
	Minimum	Maximum	Mean
1	160	30,670	4,846
2-5	236	27,366	4,062
>5	265	44,383	3,331

[a]Leaves were collected in autumn, 1994 in six commercial apple orchards in New Hampshire and Vermont.

[b]ANOVA indicated no significant difference in mean ascospores trapped between lesion categories.

Table 3. Relationship between the number of scabbed leaves on 600 shoots assessed for scab in autumn, 1994, the predicted potential ascospore dose (PAD), and ascospore productivity next spring.

Orchard	Replication number	Total scabbed leaves		Estimated PAD		Ascospore productivity		
		600 shoots	2400 shoots	Each replication	All replications	Each replication	All replications Total	Mean
VT-12	1	24		143		341		
	2	21		166		2,275		
	3	25		312		341		
	4	21		215		0		
			91		177		2,957	739
NH-15	1	24		184		85		
	2	17		117		30,711		
	3	22		441		4,925		
	4	19		138		8,509		
			82		220		44,230	11,058
NH-17	1	32		836		14,186		
	2	30		1,071		62,754		
	3	44		1,792		8,538		
	4	37		1,295		16,386		
			143		1,248		101,864	25,466
NH-16	1	63		1,542		592		
	2	71		1,335		111,268		
	3	78		1,511		45,938		
	4	83		1,775		40,656		
			295		1,541		198,454	49,613
VT-14	1	57		2,148		72,984		
	2	77		3,690		35,756		
	3	53		2,804		5,645		
	4	47		2,109		7,971		
			234		2,688		122,356	30,589
VT-13	1	78		2,394		46,068		
	2	89		3,644		56,581		
	3	77		2,364		13,697		
	4	97		3,245		7,613		
			341		2,912		123,959	30,989

Table 4. Validation of a laboratory-derived model to predict ascospore maturation determined by comparing actual ascospore productivity with predicted ascospore productivity calculated from the cumulative ascospores trapped at 100 DD from the biofix[a]

Site	Replicate	Cumulative ascospores trapped to 100 DD	Ascospore productivity			Acceptable[e]
			Predicted[c] 95% confidence range		Actual[d]	
			Minimum	Maximum		
VT-12	1	12	193	30,000	341	YES
	2	0	0	0	2,275	NO
	3	0	0	0	341	NO
	4	0	0	0	0	YES
VT-13	1	130	2,093	325,000	46,068	YES
	2	1,274	20,511	3,185,000	56,581	YES
	3	4	64	10,000	13,697	NO
	4	1	16	2,500	7,613	NO
VT-14	1	1,065	17,147	2,662,500	72,984	YES
	2	198	3,188	495,000	35,756	YES
	3	0	0	0	5,645	NO
	4	6	97	15,000	7,971	YES
NH-15	1	0	0	0	85	NO
	2	7	113	17,500	30,711	NO
	3	1,154	18,579	2,885,000	4,925	NO
	4	72	1,159	180,000	8,509	YES
NH-16	1	0	0	0	592	NO
	2	418	6,730	1,045,000	111,268	YES
	3	20	322	50,000	45,938	YES
	4	44	708	110,000	40,656	YES
NH-17	1	0	0	0	14,168	NO
	2	166	2,673	415,000	62,754	YES
	3	334	5,377	835,000	8,538	YES
	4	52	837	130,000	16,386	YES

[a]All scabbed leaves removed from 600 shoots, overwintered on the orchard floor until 1 February, then incubated under prescribed humidity and temperature conditions.

[b]Cumulative ascospores trapped up to 100 degree-days (DD) after the first matured ascospores detected (biofix).

[c]At 100 DD, the ascospore maturation model developed under prescribed laboratory incubation conditions predicts that the cumulative ascospores trapped from all scabbed leaves collected from 600 shoots in autumn and incubated similarly should be within 0.04 to 6.2% of all ascospores that will be trapped from those leaves 95% of the time. The minimum and maximum figures are the predicted minimum and maximum cumulative number of ascospores that should be trapped, 95% of the time, at the end of maturation.

[d]Trapping stopped when no ascospores were trapped for two successive trapping events.

[e]The relationship between actual and predicted ascospore productivity was acceptable (YES) when the actual ascospore productivity was within the predicted 95% confidence range and unacceptable (NO) when the actual ascospore productivity was outside the predicted 95% confidence range.

Table 5. Validation of a laboratory-derived model to predict ascospore maturation determined by comparing actual ascospore productivity with predicted ascospore productivity calculated from the cumulative ascospores trapped at 200 DD from the biofix[a].

Site	Replicate	Cumulative ascospores trapped at 200 DD[b]	Ascospore productivity			Acceptable[e]
			Predicted[c] 95% confidence range		Actual[d]	
			Minimum	Maximum		
VT-12	1	28	140	7,000	341	YES
	2	53	265	13,250	2,275	YES
	3	0	0	0	341	NO
	4	0	0	0	0	YES
VT-13	1	1,548	7,740	387,000	46,068	YES
	2	14,890	74,450	3,722,500	56,581	NO
	3	201	1,005	50,250	13,697	YES
	4	86	430	21,500	7,613	YES
VT-14	1	11,316	56,580	2,829,000	72,984	YES
	2	1,712	8,560	428,000	35,756	YES
	3	15	75	3,750	5,645	NO
	4	187	935	46,750	7,971	YES
NH-15	1	0	0	0	85	NO
	2	53	265	13,250	30,711	NO
	3	1,758	8,790	439,500	4,925	NO
	4	160	800	40,000	8,509	YES
NH-16	1	62	310	15,500	592	YES
	2	894	4,470	223,500	111,268	YES
	3	46	230	11,500	45,938	NO
	4	100	500	25,000	40,656	NO
NH-17	1	74	370	18,500	14,168	YES
	2	8,190	40,950	2,047,500	62,754	YES
	3	640	3,200	160,000	8,538	YES
	4	818	4,090	204,500	16,386	YES

[a]All scabbed leaves found on 600 shoots, overwintered on the orchard floor until 1 February, then incubated under prescribed humidity and temperature conditions.

[b]Cumulative ascospores trapped up to 200 degree-days (DD) after the first matured ascospores detected (biofix).

[c]At 200 DD, the ascospore maturation model developed under prescribed laboratory incubation conditions predicts that the cumulative ascospores trapped from all scabbed leaves found on 600 shoots in autumn and incubated similarly should be within 0.4 to 20.0% of all ascospores that will be trapped from those leaves 95% of the time. The minimum and maximum figures are the predicted minimum and maximum cumulative number of ascospores that should be trapped, 95% of the time, at the end of maturation.

[d]Trapping stopped when no ascospores were trapped for two successive trapping events.

[e]The relationship between actual and predicted ascospore productivity was acceptable (YES) when the actual ascospore productivity was within the predicted 95% confidence range and unacceptable (NO) when the actual ascospore productivity was outside the predicted 95% confidence range.

Table 6. Validation of a laboratory-derived model to predict ascospore maturation determined by comparing actual ascospore productivity on all scabbed leaves found on 2400 shoots examined in autumn 1994 with predicted ascospore productivity calculated from the cumulative ascospores trapped to 100 and 200 DD from the biofix[a]

Site	DD	Cumulative ascospores trapped[b]	Ascospore productivity			Acceptable[e]
			Predicted[c] 95% confidence range		Actual[d]	
			Minimum	Maximum		
VT-12	100	12	193	30,000	2957	YES
VT-13	100	1,409	22,685	3,522,500	123,959	YES
VT-14	100	1,269	20,431	3,172,500	122,356	YES
NH-15	100	1,233	19,851	3,082,500	44,230	YES
NH-16	100	482	7,760	1,205,000	198,454	YES
NH-17	100	552	8,887	1,380,000	101,846	YES
VT-12	200	105	525	26,250	2957	YES
VT-13	200	16,725	83,625	4,181,250	123,959	YES
VT-14	200	13,230	66,150	3,307,500	122,356	YES
NH-15	200	1,971	9,855	492,750	44,230	YES
NH-16	200	1,102	5,510	275,500	198,454	YES
NH-7	200	9,722	48,610	2,430,500	101,864	YES

[a]Cumulative ascospores trapped up to 100 degree-days (DD) after the first matured ascospores detected (biofix).

[b]All scabbed leaves removed from 2400 shoots, overwintered on the orchard floor until 1 February, then incubated under prescribed humidity and temperature conditions.

[c]At 100 and 200 DD, the ascospore maturation model, developed under prescribed laboratory incubation conditions, predicts that the cumulative ascospores trapped from all scabbed leaves collected from 600 shoots in autumn and incubated similarly should be within 0.04 to 6.2% and 0.4 to 20.0%, respectively, of all ascospores that will be trapped from those leaves 95% of the time. The minimum and maximum figures are the predicted minimum and maximum cumulative number of ascospores that should be trapped, 95% of the time, at the end of maturation.

[d]Total ascospores trapped for all scabbed leaves found on 2400 shoots examined in autumn.

[e]The relationship between actual and predicted ascospore productivity was acceptable (YES) when the actual ascospore productivity was within the predicted 95% confidence range and unacceptable (NO) when the actual ascospore productivity was outside the predicted 95% confidence range.

Discussion

Scab buildup, lesion fertility, and ascospore productivity in commercial orchards well-managed and moderately-managed for scab

The study provided new information on the buildup of scab, lesion fertility, and ascospore productivity in orchards well- to moderately-managed for scab, and a composite profile for orchards well- to moderately-managed for scab, based on the six commercial orchards, follows: there will be less than 100 scabbed leaves on 600 shoots, most scabbed leaves will have only one lesion, approximately 10% of the scabbed leaves will become fertile, and ascospore productivity will be similar on scabbed leaves with 1, 2-5, and >5 scab lesions. These findings have been incorporated into our current autumn assessment procedure. For example, we now give each scabbed leaf equal "weight," regardless of the number of lesions, when assessing 600 shoots to determine "scab-risk," because on average, ascospore productivity was similar on leaves with 1, 2-5, or >5 lesions.

Theoretically, single-lesion leaves should not become fertile if they are the result of infection by a single ascospore, and ignoring leaves with one-lesion would increase considerably the feasibility of the procedure investigated here, but the results show clearly that they cannot be ignored. Why ascospores may be produced on single-lesion leaves has been discussed previously (MacHardy, 1996b).

Reliability of predicting potential ascospore dose from an assessment of foliar scab on 600 shoots

This is the first report of a study that investigated the reliability of assessing 600 shoots to predict PAD in an orchard. Analysis of the four replicate assessments in the six orchards discussed here and in orchards examined similarly the previous year showed that within 95% confidence intervals, the PAD calculated from one assessment of scab on 600 shoots was within 10% of the true mean in orchards with PAD <1000.

The 1-fold to 3-fold difference in predicted PAD in the four replicated assessments in the six orchards is within a range that is acceptable for the "PAD" action threshold, which states that tactics to reduce the overall fungicide dose can be employed if the predicted PAD is ≤600 (MacHardy 1994). In two orchards (VT-12 and NH-15), the predicted PAD was <600 in all replicated assessments; thus, any one of the four replicated assessments in each orchard gave the same result as all four: the orchard was below the "PAD" action threshold. The PAD in all replicated assessments in the remaining four orchards was above the "PAD" action threshold, i.e., the PAD ranged from 836 to 3644; thus, any one of the four assessments in each of the four orchards would have predicted a PAD above the "PAD" action threshold. These results indicate that one assessment of 600 shoots will give a reliable prediction of PAD.

Relationship between the predicted PAD and ascospore productivity. There was poor correlation between PAD calculated from an assessment of 600 shoots and ascospore productivity on the scabbed leaves collected from those shoots : differences in ascospore productivity on scabbed leaves collected in the four replicated assessments of 600 shoots ranged from 7-fold to 361-fold in the six orchards, whereas differences in PAD ranged from 1-fold to 3-fold. Based on these data, it would appear that the autumn calculation of PAD or an assessment of scab incidence are not a reliable predictor of "scab-risk." Our interpretation, however, is that ascospore productivity on a scabbed leaf is too variable to base a prediction of an orchard's "scab-risk" on the 100 or less scabbed leaves that would occur on 600 shoots in an orchard well- or moderately-managed for scab. Pseudothecial density is one of components in the equation to calculate ascospore dose, and it is computed as the product of lesion fertility and the number of mature pseudothecia per fertile lesion leaf (Gadoury and MacHardy, 1986). As Gadoury and MacHardy (1986) noted, variability in pseudothecial density (and, thus, in ascospore productivity) was much greater when less than 5% of the leaves had scab lesions and there were one to two lesions per scabbed leaf. The incidence and severity of scab in the six orchards in the present study were in these categories, so variability in the mean number of mature pseudothecia formed per visible lesion may explain some of the great differences in ascospore productivity among the four collections in an orchard. What is revealed in these differences in ascospore productivity is that a much greater sample size is needed, e.g., collect scab leaves from 2400 or more shoots, to take into account the variability in ascospore productivity that occurs among individual scabbed leaves in an orchard with a low incidence and severity of foliar scab.

The reliability and feasibility of an indoor spore-trapping procedure to predict the level of "scab-risk."

Reliability of predicting the level of "scab-risk" from the number of scabbed leaves found on 600 shoots in autumn. In five orchards, the number of scabbed leaves observed in each of the four replicate assessments of 600 shoots in an orchard classified the orchard as low "scab-risk," placing it below the "reduce-fungicide" action threshold, or as moderate "scab-risk," requiring the fungicide program recommended to all orchards (MacHardy *et al.* 1999). In the sixth orchard, three replications were above threshold of 50 scabbed leaves that distinguish "moderate-risk" and "low-risk" orchards (i.e., 57, 78, and 53) and one was slightly below (i.e., 46). With this reliability and other considerations, scab incidence on 600 shoots remains the most useful and reliable measure to predict "scab-risk" despite our concern discussed in the Introduction

In orchard NH-17, the number of scabbed leaves in each of the four replicated assessments of 600 shoots placed the orchard in the "low-risk" category whereas each of the four calculations of PAD placed the orchard in the "moderate-risk" category. This discrepancy is explained by the equation to calculate PAD, in which lesion density has a much greater influence on the calculation of PAD than incidence. The number of leaves with sheet scab In NH-17 was unusually high for an orchard with a low incidence of foliar scab. The lesion density of a leaf classified as sheet scab was set at 15 lesions, but based on our present knowledge of ascospore productivity on leaves with multiple lesions, we know that this figure is overestimating ascospore production on those leaves and that the calculation of PAD is too high. Thus, the orchard should be considered a "low-risk orchard as identified by the number of scabbed leaves on the 600 shoots.

Feasibility of an indoor spore-trapping procedure to predict the level of "scab-risk." For the proposed procedure to replace the autumn assessment procedure as a predictor of "scab-risk," the actual ascospore productivity of a leaf set should lie within the 95% confidence limits of the ascospore productivity predicted by the number of ascospores trapped from the leaf set 125 DD after the biofix. Two lines of evidence are suggestive that the proposed procedure will not be reliable: (i) the ascospore productivity of all scabbed leaves found on 600 shoots in autumn was not predicted with 95% confidence from the cumulative ascospores trapped to 100 and 200 DD in 18 of 48 tests,. and (ii) 7-fold to 361-fold differences in ascospore productivity among the four replicated assessments in the six orchards were much too great to consider basing a prediction of "scab-risk" on ascospore productivity on scabbed leaves found on 600 shoots. Ascospore productivity of all scabbed leaves found on the pooled 2400 shoots, however, was within the predicted 95% confidence limits of ascospore maturity in the six orchards tested, but realistically, a procedure that requires examining 2400 shoots and trapping ascospores from all scabbed leaves found on those shoots would not be practical.

Even if a grower was willing to examine 2400 shoots, he/she may not be willing or have the time to handle and incubate the leaves inside a room. Inside incubation could be minimized if the collected leaves remained in the orchard until 125 DD after the biofix (bud-break) and were then brought into the laboratory and tested for ascospore production. This would allow pseudothecia on the collected leaves to mature under natural conditions. The test would occur relatively early in the primary scab season, but it is questionable how useful a prediction of "scab-risk" would be at that time. One advantage of a late-winter prediction of "scab-risk" using the proposed indoor procedure is that it would allow the grower more time to plan the fungicide strategy and order fungicide for the coming growing season.

Another approach would be to place one or more spore traps in an orchard and trap ascospores discharged into the orchard air during a rain event that occurred close to 125 DD after bud break. Three advantages of this approach over the laboratory approach are that (i) it

takes into account all factors after leaf fall that influence the sexual stage and the production and maturation of pseudothecia and ascospores under completely natural conditions, (ii) it samples a larger proportion of the orchard population of the pathogen, and (iii) the actual airborne ascospore density of the pathogen in the orchard is calculated and used to predict "scab-risk." Two main disadvantages, particularly in a moderately- or well-managed orchard, are that the airborne ascospore density may be (i) below the trapping efficiency of the spore trap and (ii) too variable throughout the orchard to obtain meaningful trapping data without using many spore traps. Other disadvantages are that it would be labor-intensive and time-consuming during one of the busiest times of the year for a grower and the grower must develop skills and expertise in operating the spore traps, using a microscope, and identifying and counting ascospores. The latter problem would be lessened if the ELISA technique were available, but an ELISA kit designed for use by growers (Berkett *et al.*, 1992, Gotlieb *et al.*, 1995) is not available commercially at this time. Finally, the usefulness of the technique is marginal considering the assessment of "scab-risk" would not occur until after the primary scab season had begun, as explained in the preceding paragraph.

A significant advantage of the autumn assessment of "scab-risk" over assessments made in late-winter/early-spring or during the primary scab season is that it can be used in conjunction with a "sanitation" action threshold that justifies the use of sanitation practices against the overwintering stage of the pathogen (MacHardy 1998a, 1998b, MacHardy and Sutton, 1995). The two to four hours required to assess 600 shoots has been a limitation in this procedure, but this problem has been overcome by the development of a sequential sampling procedure in which the level of "scab-risk" can by predicted after examining as few as 100 shoots on 10 trees (MacHardy *et al.* 1999). Potential errors in performing the technique and identifying scab lesions on senescing leaves in late autumn are being addressed by grower training workshops and the production (in progress) of a video demonstrating the procedure accompanied by laminated sheets of colored photographs showing leaves with autumn symptoms of scab and blemishes, insect damage, and expressions of senescence and environmental influences that appear on leaves in autumn that can be confused with scab.

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Cellulase-and pectinase-zymograms of various *Venturia inaequalis*- and *V. pirina*-isolates

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Abstract : The secretion of endo-cellulases and -pectinases of *V. inaequalis* could be a prerequisite factor for permitting the scab fungus to exist on the living plant. The pectinase production is high even when very few mycelia have developed, and this enzyme is produced regardless of the composition of the artificial medium. In contrast to the constitutive production of pectinase, the cellulases are very specifically induced. Monoconidial isolates were obtained from different apple cultivars, *Malus* spp. and *Pyracantha* sp. .The geographic origins were Germany (49 isolates), Italy (1 isolate) and Columbia (9 isolates). Exemplary two isolates of *Venturia pirina* were included. Enzyme production *in vitro* was performed in liquid static cultures and culture filtrates were used for enzyme preparations. For constitutive pectinase secretion isolates were grown in a defined glucose medium, whereas cellulase production was induced in potato dextrose broth supplemented with cellulosic sheets. Zymograms were obtained after separation of proteins according to their pI by IEF followed by a specific staining of the enzyme activities, respectively. Cellulase zymograms showed a complex banding pattern with up to 15 isoforms whereas pectinase zymograms showed a single band at pI 10. No significant differences were detected for pectinase and cellulase zymograms within all *V. inaequalis*-isolates. Some differences in cellulase banding were found towards *V. pirina* and within the pear scab isolates. The high uniformity of the cell wall-degrading enzymes may be the result of the coevolution of the host plant and the fungal pathogen.

Key words : *Venturia inaequalis, Venturia pirina*, pectinase, cellulase, cell wall-degrading enzymes, zymogram, isozymes

Introduction

Once the plant cuticle is penetrated by *V. inaequalis* pectinases followed by cellulases may be the first important enzymes secreted. In its biotrophic phase the fungus is limited to a position between the cuticle and the outer epidermal cell walls where degradative enzymes may have a key function for the release of nutrients, the removal of physical barriers, and for induction of host responses by enzyme-generated products of plant cell wall. Kollar (1994) examined the specific induction of extracellular cellulase production by chemotrophic and topographic signals. A complex isozyme pattern was detected and cellulases were isolated from leaf lesions of naturally infected apple trees. In contrast to the very specific requirements of *V. inaequalis* for cellulase induction, pectinase production was strictly constitutive and the pectinase activity focused in a single band at pH 10 (Kollar, 1998). The cellulase pattern from 19 *V. inaequalis* isolates from cultivar 'Golden Delicious' were essentially identical, and differences were restricted mainly to quantitative variability (Kollar, 1994). The pectinase of these *V. inaequalis*-isolates did not show any differences in characteristics of degradative activity or the activity band detected after isoelectric focusing. The high uniformity of the cell wall-degrading enzymes could be a consequence of biotrophic interaction, because the host plant may impose particularly stringent biochemical requirements on a fungal pathogen and exert strong selection against any variation. The aim of this study was to add to the knowledge

of variability of cell wall-degrading enzymes from *V. inaequalis* - and *V. pirina* - isolates , which may depend on extremes of geographical origin and/or different host plants.

Material and Methods

Media, growth conditions and fungal strains

The monoconidial isolates of *V. inaequalis* and *V. pirina* are listed in Table 1. Liquid static 250 ml-cultures for production of enzymes (Kollar, 1994, 1998) were maintained at 20 °C for 40 to 45 days. Inoculation was performed with mycelial plugs of the respective fungal isolate. Production of cellulases was achieved by complex medium (PDB, Difco) supplemented with dialysis tubing (Serva, regenerated cellulose 29 mm). For pectinase production a defined medium was prepared with Murashige and Skoog basal salt macronutrient and micronutrient solution at the recommended dilution (Sigma). Prior to autoclaving, thiamine HCl (vitamin B_1) was added to a final concentration of 0.4 ppm together with 2 % glucose.

Preparation of enzymes and isozyme analysis

Preparation of enzymes and isozyme analysis were performed according to Kollar (1994, 1998). Culture filtrates were centrifuged for reduction of mucous substances and melanoproteins and, after lyophilization, fractionated ammonium sulfate precipitation with a final saturation of 80 % was used to concentrate the proteins. For electrophoresis of isozymes, samples were desalted either by gel filtration (cellulases) and by dialysis or ultrafiltration (pectinases). Pectinase/Cellulases were separated according to their pI by IEF on ultrathin 150 µm layers of polyacrylamide gel (Servalyt Precotes, 3-10/3-6, Serva) 125 x 125 mm, at 4 °C (Isobox HE 950, Hoefer) as recommended by the manufacturer. After prefocusing to 500 V, samples were loaded with an applicator strip (Serva), slot 7 x 1 mm, at the anode/cathode. For pectinase/cellulase detection, gels were transferred to pectin/CMC-agarose. IEF gels were blotted on the substrate agarose layer for 60 min. After removal of the gel, the substrate agar was incubated overnight at ambient temperature. Pectinolytic/cellulolytic activity was visualized with 1 % cetyltrimethylammonium bromide (Serva) / 0.1 % Congo red.

Results and discussion

All isolates of *V. inaequalis* and *V. pirina* displayed constitutive pectinase production. Isoelectric focusing followed by the zymogram technique revealed a single activity band at pI 10. Isozyme patterns of the different *V. inaequalis* isolates showed considerable uniformity for the cellulases. Variation was mainly restricted to quantitative differences revealing more or less strong bands for each isozyme (Fig. 1.). *V. pirina* cellulase zymograms (Fig.1.) detected mainly quantitative differences as compared to isolates of *V. inaequalis*. Isolate V.p.2 showed two new bands between isozyme 2, 3 and 3, 4, respectively. During the biotrophic phase of parasitism a strong selection pressure may favour the development of an effective host-adapted enzyme system. Generally, isozyme uniformity is suggested to be a feature of obligate parasites because the host-plant may impose particularly stringent biochemical requirements on a fungal pathogen and exert strong selection against any variation. The uniform isozymes presented for *V. inaequalis* and *V. pirina* may indicate some common features with obligate parasites. However, this uniformity was surprising because an adaption to the various host plants may occur and especially the strong segregation of isolates (e.g. Columbia /Germany) may result in a genetic drift.

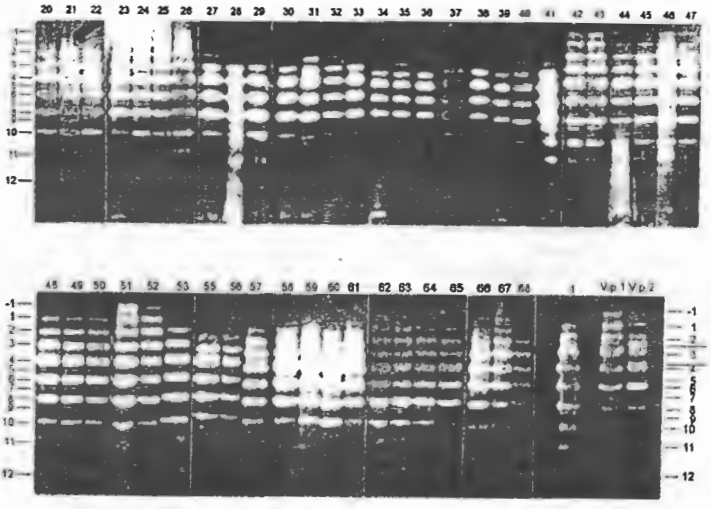

Figure 1. Cellulase isozyme pattern analysis of *Venturia inaequalis*-and *V. pirina*-isolates. Lanes 20-68 represent the isolates V20-V68 in Table 1., lane 1 shows the pattern of the reference strain V1 (*V. inaequalis*) versus the *V. pirina* strains Vp1,2 listed in Table 1.

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Table 1. *Venturia inaequalis* (V) - and *V. pirina* (V.p.) -isolates

Isolate	host-plant	geographic origin
V20, 21	'James Grieve'	at a range of 30 km from institute orchard (i.o.)
V22	*M. floribunda*	i.o.
V23	*M. adstringens* 'Hopa'	i.o.
V24	*M. purpurea* 'Royal red'	i.o.
V25	*M. koreana*	i.o.
V26	*M. pumila* 'Darthmouth'	i.o.
V27	*M. scheideckerii*	i.o.
V28	*M. ioensis* 'John Downie'	i.o.
V29	*M. micromalus*	i.o.
V30	*M. atrosanguinea*	i.o.
V31	*M. toningoides*	i.o.
V32	*M. moerlandsii* 'Profusion'	i.o.
V33	*M. aldenhamensis*	i.o.
V34	*M. moerlandsii* 'Nicoline'	i.o.
V35, 36	*M. robusta* var. *persicifolia*	i.o.
V37	*M. virginia crab*	i.o.
V38	*M. purpurea* 'Eleyi'	i.o.
V39	'Golden Delicious'	Italy
V40	*M. ioensis* var. *spinosa*	i.o.
V41	*M. sylvestris*	i.o.
V42, 43, 44	'Golden Dorsett'	Columbia
V45, 46	'Anna'	Columbia
V47, 48	'Ein Shemmer'	Columbia
V49, 50	'Emilia'	Columbia
V51, 52	'Prima'	i.o.
V53	*M. floribunda*	i.o.
V54, 55, 56	*Pyracantha* sp.	i.o.
V57	'Mc Intosh'	i.o.
V58	'Gloster'	i.o.
V59	'Zuccalmaglio'	i.o.
V60	'Boskoop'	i.o.
V61	'Cox Orange'	i.o.
V62	'Delbard Jublié	i.o.
V63	'Rubinette'	i.o.
V64	'James Grieve'	i.o.
V65	'Idared'	i.o.
V66	'Jonathan'	i.o.
V67	'Tydemann's Red'	i.o.
V68	'Jonagold'	i.o.
V.p.1	'Vereinsdechant'	i.o.
V.p.2	'Gute Luise'	i.o.

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Development and evaluation of a forecasting system for scheduling fungicide sprays for control of brown spot (*Stemphylium vesicarium)* of pear

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Abstract : Brown spot of pear caused by *Stemphylium vesicarium*, is a fungal disease of increasing economic importance in several pear-growing areas of Europe, including Girona in Spain, Emilia-Romagna in Italy, Bouches du Rhône in France, Holland and Portugal. Efficient control of brown spot of pear is achieved only with protectant fungicide sprays applied, at 7- to 15-day intervals depending on the type of fungicide. A commercial schedule for disease control which requires a high number of fungicide applications consists of starting sprays after petal fall and end some weeks before harvest. However, some fungicide sprays are unnecessary because environmental conditions are not always suitable for infections. A forecasting model named STREP based on the effect of wetness duration and mean temperature of wetness periods on disease severity, was developed for detecting moments and intensity of infection risk during the vegetative period of pear. The model was evaluated for disease prediction in 40 field trials and was validated for scheduling fungicide sprays during 3 years in eleven orchard plot and mesoscale trials in different climatic areas. The STREP model was a useful tool for rational control of brown spot of pear, because minimized the number of fungicide sprays and maintained the same levels of control compared to the fixed spray commercial schedule. A software including the STREP model will be available soon for processing weather, pathogen and host information to provide disease risk and recommend threshold values for decision making in fungicide application programs for brown spot disease control.

Key words : brown spot of pear, reduced spray, fungicide savings

Introduction

Brown spot is an important fungal disease of pear caused by *Stemphylium vesicarium* (Wallr.) E. Simmons (teleomorph *Pleospora allii* (Rabenth. Ces.& De Not) which affects several pear growing areas of Europe. Infections occur on leaves, fruits and twigs, and the most susceptible pear cultivars are Abate Fetel, Passe Crassane, Alexandrine and Conference (Ponti *et al.* 1982, Blancard *et al.* 1989, Cavanni *et al.* 1994, Montesinos *et al.* 1995b).

Control of brown spot of pear (Fig. 1) is achieved with preventive sprays of carbamate fungicides (e.g. thiram) applied at 7-day intervals or with carboximides (e.g. procymidone) sprayed at 15-day intervals (Brunelli *et al.* 1986, Vilardell 1988, Ponti *et al.* 1992, Brunelli *et al.* 1994). The commercial schedule for disease control consists of starting fungicide sprays after petal fall and end applications a few weeks before harvest. However, some applications of fungicides may be unnecessary because environmental conditions were not always suitable for fruit or leaf infections by *S. vesicarium* (Montesinos and Vilardell 1992).

An infection model, named STREP, was developed for prediction of infections of *S. vesicarium* on pear (Fig. 2). STREP was validated for disease prediction during two years in Girona (Spain) and Emilia-Romagna (Italy) in 40 field trials covering a wide range of wetness durations and temperature (Montesinos *et al* 1995a).

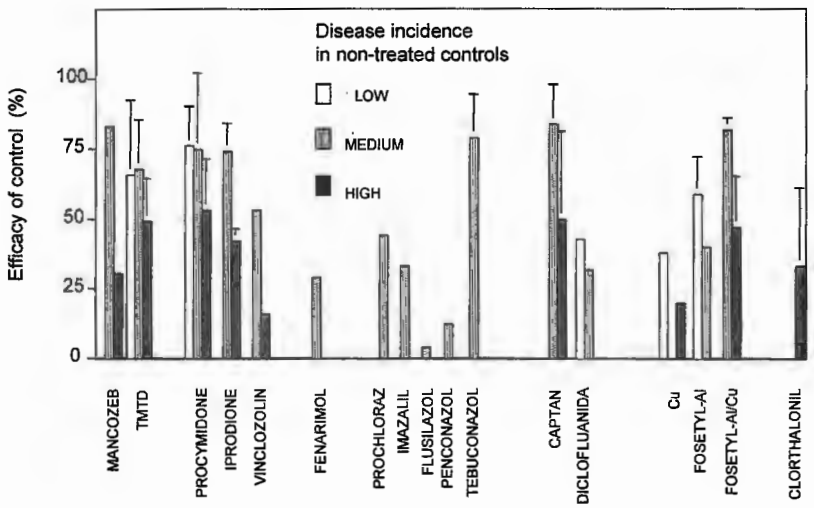

Figure 1. Efficacy of several fungicides in orchard plot trials for control of brown spot of pear. Data correspond to 15 trials performed from 1987 to 1995 in commercial orchards of cultivars Passe Crassane, Conference and Abate Fetel, naturally affected by the disease. Products were applied between June and September by spraying fungicides at fixed spray schedules and at the dose recommended for each product.(Data from Montesinos *et al* 1996).

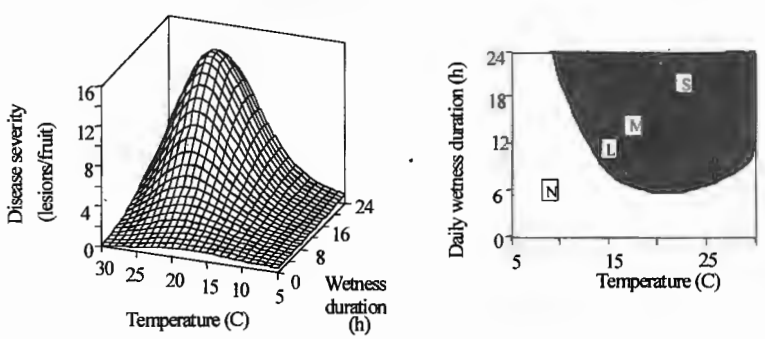

Figure 2. Response surface obtained from experimental data for the effect of wetness duration and temperature of wetness periods on brown spot of pear (A), and disease risk chart (B). (Data from Montesinos *et al.* 1995a).

The objective of this work was to evaluate the STREP model for scheduling fungicide applications in reduced fungicide use programs in comparison to the standard commercial schedule based on fixed sprays.

Materials and methods

The brown spot forecasting system (STREP) is based on an empirical model which determines when environmental conditions are favorable for infection of S. *vesicarium* and disease development. Daily wetness duration (W) and mean air temperature of wetness periods (T) are used to compute a daily infection risk (S) according to the following equation:

$$Log_{10}(S) = - 1.70962 + 0.00289\ T + 0.04943\ W + 0.00868\ T\ W - 0.002362\ W^2 - 0.000238\ T^2W$$

S and 3-day cumulative daily infection risk (SA) were calculated every 24 h. SA was computed by totaling S values for the past 3 days, and was used as action threshold for spraying fungicides in the field trials.

The environmental parameters were monitored with automatic weather stations. Mean temperature and relative humidity, duration of wetness and total rainfall were recorded by the dataloger at hourly intervals. For each day, the 24-h period considered for calculations started at 8:00 h (GMT) of the previous day and finished at 8:00 h (GMT) of the current day. Every 24-h period S (daily infection risk) and SA (3-day cumulative daily infection risk) were calculated.

Field trials were conducted in pear orchards in Girona (Spain) during years 1995, 1996 and 1997. The orchards were naturally affected by brown spot disease. For identification through this report, number codes are assigned consistently to each trial (Table 1).

Table 1. Orchard plot trials performed in Girona for evaluation of STREP model in reduced fungicide use programs for control of brown spot.

Trial	Year	Orchard location	Pear Cultivar	Fungicide
2	1995	Riudellots	Passe Crassane	thiram
4	1996	Perello	Passe Crassane	thiram
5	1996	Riudellots	Passe Crassane	thiram
7	1997	Estanyol	Conference	thiram
9	1997	Perelló-1	Passe Crassane	thiram
10	1997	Perelló-2	Passe Crassane	kresosim-methyl

Experiments were performed in orchard plots of 80 to 100 trees. The orchard trials were done with pear cultivars Conference and Passe Crassane, which were highly susceptible to the disease. Fungicides used depend on the trial and were the carbamate thiram (200 g a.i./hl of Pomarsol Forte, Bayer), and the strobilurine analog kresoxim-methyl (10 g a.i./hl of Stroby, Basf). Fungicides were applied till runoff point in plot trials with an engine operated portable sprayer (Stihl model SR400, Waiblingen, Germany). Thiram was assumed to provide 7 days of protection and kresoxim-methyl 15 days, except when rainfall surpassed 20 mm. When more than 20 mm rain fell after spraying within the protection period considered, trees were sprayed again.

Fungicide treatments consisted of fixed or under STREP guidance spray application schedules, and non-treated controls. In the fixed spray schedule, fungicides were applied at 7-day (thiram) or 14-day (kresoxim-methyl) intervals. Treatments in the fixed spray schedule started after petal fall, usually between the last week of April and the third week of May. In guided spray schedules, the fungicides were applied when the selected SA action threshold

(0.4 or 0.6) was reached according to the STREP model. Fungicide applications finished two weeks before harvest. Harvest was performed in late August for cultivars Conference and in the second half of October for cultivar Passe Crassane. Treatments within each plot were arranged in a randomized complete block design with 4 repetitions of 4-5 trees per treatment depending on trial. On fruits, disease incidence (% of fruits) and severity (lesions per fruit) were assessed on 20 fruits per tree each 15 to 20 days during the vegetative period. On leaves mean disease incidence per tree was calculated from the values of each of the four shoots and used in statistical analysis. Treatment efficacy was calculated as the relative percentage of disease reduction in relation to the non-treated control.

Data at harvest were analyzed using PC-SAS package (SAS System v.6.12, SAS Institute Inc. North Carolina, USA). The effect of treatments was determined by means of ANOVA for a randomized complete block or completely randomized design using the GLM procedure and contrasts. Means comparisons were performed with Tukey's test. Linear regression was performed with REG procedure.

Results

The typical pattern of daily infection risk dynamics during the vegetative period in relation to weather parameters consisted of three periods (Fig. 3). A first period (from April 15 to June 15) was characterized by several long daily wetness duration (frequently higher than 10 h), low mean daily temperatures of wetness periods, relatively constant daily duration of relative humidity higher than 90% and several rains. A second period (from

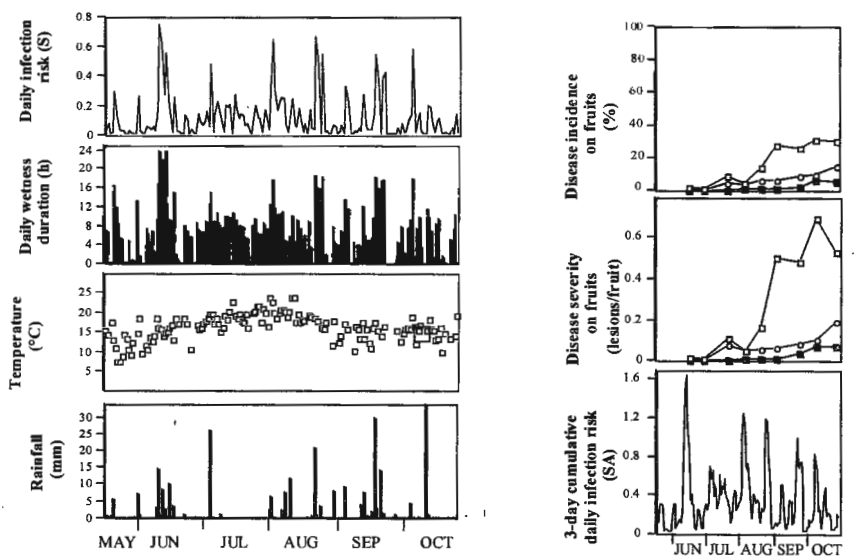

Figure 3. Dynamics of daily infection risk (S), daily wetness duration, mean temperature during the wetness period, and rainfall in trial 2 (Left panel). Brown spot disease progress curves on fruits for non-treated controls (□), and treated with thiram at fixed spray schedule (■), and thiram at STREP threshold SA=0.4 (o), in relation to the dynamics of the 3-day cumulative daily infection risk (SA) (Right panel).

June 15 to August 15) consisted of relatively dry weather conditions and was characterized by short daily wetness periods (frequently less than 10 h), high mean daily temperatures of wetness periods (15 to 27 °C), and few rains. The third period (from August 15 to October 15) was relatively wet and was characterized by long daily wetness duration, and frequent daily periods of high relative humidity, decreased temperatures of wetness periods and frequent rains. Patterns of disease progress in relation to infection risk and treatments are shown in Fig 3.

Disease incidence at harvest was significantly lower in fungicide treatments, either STREP scheduled or fixed, than in non-treated controls ($P<0.01$) (Table 2). Disease incidence observed in fruits sprayed according to a SA threshold of 0.4 was not significantly different from the fixed spray schedule in 4 out of 5 trials. The savings in number of fungicide sprays in STREP guided schedules compared to the fixed spray schedule were 26-50% with thiram. In one case (trial 10), in which kresoxim-methyl was used, a lower disease control in the SA 0.4 schedule than in the fixed spray schedule was observed, but still a significant reduction of disease levels compared to untreated control was obtained.

Table 2. Disease incidence (DI) and number of fungicide applications (NT) during the 1995-1997 growing seasons for each trial according to the spray schedule used.

Trial	Non-treated control	Fixed schedule[x]		STREP schedule			
				SA=0.4		SA=0.6	
	DI (%)	DI (%)	NT	DI (%)	NT	DI (%)	NT
2	30.5	5.5	22	--		15.2 s[y]	7
4	65.7	24.6	23	28.6 ns	17	32.9 ns	12
5	65.1	21.1	24	24.2 ns	12	40.9 s	7
7	87.0	34.7	16	37.7 ns	11	56.0 ns	7
9	96.5	52.7	20	59.2 ns	14	--	--
10	96.4	53.2	10	73.7 s	8	--	--

[x] Fixed, commercial fungicide spray schedule applied at a fixed interval (7 or 14 days depending on fungicide); SA0.4 or 0.6, sprays according to the STREP model.

[y] Significance according to ANOVA with contrasts comparing fixed and STREP scheduled fungicide sprays; ns, not significant; s, significant ($P<0.05$).

Disease incidence on fruits did not differ significantly from the fixed spray schedule in 1 out of 4 orchard plot trials sprayed according to SA threshold of 0.6. In trials 2, 5, and 7 disease incidence in plots sprayed according to the SA threshold of 0.6 was significantly higher than in fixed spray schedule, but was significantly lower ($P<0.05$) than in non-treated controls.

Discussion

Duration of wetness and temperature of wetness periods observed in most days during the course of the present study were not favorable to infections by *Stemphylium vesicarium*. This is supported by previous studies in which optimal conditions for leaf and fruit infection by *Stemphylium vesicarium* on pear were found above 10-12 h and temperatures of the wetness periods higher than 15 °C (Montesinos *et al.* 1995a). Also, high relative humidity has little influence in spore germination of *S. vesicarium*, since this only occurs when values are too high and dew formation exist (Montesinos and Vilardell 1992).

STREP predicts different levels of infection risk through the growing season of pear and indicates the start of disease progression curve. The distribution of infection risk through the growing season of pear follows a pattern characterized by two high risk periods (period I from middle April to middle June, and period III from middle August to middle September) separated by a low risk period (period II from late June to late July). This pattern was in agreement with the pattern of progress of disease incidence on fruits and leaves at harvest on trees left unprotected by fungicides for 4-week periods distributed within the growing season (Montesinos *et al* 1992, 1995a).

The STREP model performance was good at SA 0.4 in plots in commercial production conditions. Globally, the savings in number of fungicide sprays applied using STREP compared to the fixed spray schedule were 26 to 50% when used fungicides with a 7-day protection period (thiram). Fungicide spray savings obtained with STREP were similar to those reported for other models like FAST, CU-FAST or TOM-CAST for *Alternaria solani* on tomato (Pennypacker *et al.* 1983, Madden *et al.* 1988, Keinath *et al* 1996), for apple scab (Ellis *et al.* 1984) and to a previous report using FAST for rational control of brown spot of pear (Montesinos and Vilardell 1992).

Reduced fungicide spray programs based on STREP may be not sufficient to decrease fungal inoculum on leaves and fruits when disease pressure in the orchard is high, and a stronger strategy should be adopted in this cases. A procedure which can be recommended is based on a long term program using first fixed spray schedules and after, less restrictive action thresholds as soon as disease pressure decrease in subsequent years.

The results obtained showed consistently that the STREP model is a useful tool for rational control of brown spot of pear. The use of STREP with SA action thresholds of 0.4 reduced the number of fungicide sprays and maintained the same levels of efficacy of control compared to the commercial fixed spray schedule. Therefore the model is suitable to be used in practice. However, implementation of the model in warning stations will require to account for effects of spore inoculum levels, phenological stage and pear cultivar susceptibility which are factors affecting disease.

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Isolation of strains of *Erwinia amylovora* resistant to oxolinic acid

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Abstract : Due to the appearance of resistance to streptomycin in Israel, the only bactericide that is recommended to control fire blight since 1997, is oxolinic acid (Starner). During 1998 and 1999 samples of infected flowers and shoots collected from pear orchards that were sprayed with 0.15% starner 20% wp, were examined for the appearance of resistant strains of *Erwinia amylovora*. The minimal inhibitory concentration of oxolinic acid for *Erwinia amylovora* was determined as 1µg/ml. No resistant strains were detected in 22 orchards that were sampled during 1998. In 1999 23 samples were collected from 5 orchards. In two orchards resistant strains were isolated on CCT plates containing 1 and 5 µg/ml oxolinic acid. The resistant strains were confirmed as *Erwinia amylovora* by PCR reaction and in immature pear fruit assay. The strains were able to grow also on 10 µg/ml oxolinic acid. In one orchard the resistant strains were also resistant to streptomycin. In pathogenicity assays conducted with immature pear fruits that were dipped in 0.15% starner 20% wp, the resistant strains were pathogenic.

Keywords : Starner, fire blight

Introduction

Fire blight caused by the bacterium *Erwinia amylovora* was first detected in Israel in 1985 (Zutra *et al.* 1986, Shabi and Zutra 1987). Since then, the disease has been observed in pear, apple, quince and loquat orchards all over the country (Shabi and Zutra 1989, Zilberstaine *et al.* 1996). Streptomycin was used for controlling the disease since 1986. Streptomycin-resistant *E. amylovora* strains were first detected in Israel in 1991 in an isolated pear orchard in the south. During 1995 a severe fire blight epidemic occurred in two regions in the north. Resistant strains of *E. amylovora* to streptomycin were identified in the major pear, apple and loquat growing regions in Israel (Manulis *et al.* 1998). Since 1997 streptomycin has been removed from recommendation and instead oxolinic acid 300 µg/ml (Starner 20% wp, Sumitomo Chemical Co., Japan) has been recommended exclusively for fire blight control. Oxolinic acid (S-0208) is a synthetic bactericide used for control of bacterial diseases on rice and vegetables such as cabbage, potato and onion. It is not widely used against fire blight.

In Israel oxolinic acid is the only bactericide used against fire blight in pear orchards since 1997. The recommended concentration is 0.15% of starner 20% wp, 5-6 sprays during the blooming period in the spring. In this study we describe isolation of resistant strains of *E. amylovora* in two pear orchards.

Materials and methods

Isolation of bacteria

Isolations of *Erwinia amylovora* were made from infected flowers or shoots. From each orchard at least 3 samples were tested. Each sample was suspended in 5 ml sterile water and ground with a homogenizer (Pro200, Pro Scientific Inc., USA). Hundred µl of the homogenate were plated on CCT agar medium (Ishimaru and Klos, 1984) and on CCT + 1 µg/ml oxolinic

acid (formulated 20% wp, or pure grade, Sigma). The plates were incubated for 3 days at 28°C.

***Identification of* Erwinia amylovora**

Characteristic colonies of *E. amylovora* that grew on CCT and CCT + 1 μg/ml oxolinic acid were identified by PCR using the primers and amplification conditions described by Bereswill *et al.* 1992. The isolated bacteria were tested for pathogenicity on slices of immature pear fruits as described previously (Norelli and Gilpatrick, 1982). Pathogenicity tests with the resistant strains were also conducted on immature pears that were dipped in a solution of 300 μg/ml oxolinic acid (formulated 20%).

Results and discussion

Determining the minimal inhibitory concentration (MIC)

Twenty different strains of *E. amylovora* isolated from pear, apple, loquat or quince from different regions in Israel were examined on CCT plates containing 0.5, 1, 5, 10 or 20 μg/ml oxolinic acid. Seven strains were able to grow on 0.5 μg/ml but none of the strains grew on 1 μg/ml or above. Therefore the MIC was determined as 1 μg/ml and this was the concentration that was chosen for examining the appearance of resistant strains.

The MIC is similar to that determined for other phytopathogenic bacteria such as *Erwinia carotovora* subsp. *atroseptica* and *E. c.* subsp. *carotovora* (0.4 μg/ml). In contrast phytopathogens which belong to the species *Pseudomonas syringae* such as pvs. *lachrymans*, *maculicola* or *tabaci* have higher MIC (25 μg/ml). The MIC for phytopathogens from the species *Xanthomonas campestris* is 6.3 μg/ml (Hikichi *et al.* 1989).

Isolation of resistant strains

During 1998 twenty-two orchards were examined for the presence of resistant strains. No resistant strains were detected. In 1999, 23 samples were collected from 5 orchards. In two orchards in the north, resistant strains were isolated on CCT plates containing 1 and 5 μg/ml oxolinic acid. The resistant strains were confirmed as *E. amylovora* by PCR reaction and in pear fruit assay. The strains were able to grow also on 10 μg/ml oxolinic acid. In one orchard the resistant strains were also resistant to streptomycin. In pathogenicity assay conducted with immature pear fruits that were dipped in 0.15% starner 20% wp, the resistant strains were able to produce ooze and to cause blackening of the pear slices. Figure 1 show the results obtained with two strains; M2 resistant to oxolinic acid and 209 resistant to streptomycin but sensitive to oxolinic acid. In the presence of oxolinic acid only the oxolinic acid-resistant strain (M2) was pathogenic, whereas the streptomycin-resistant strain (209) was not. Similar results were obtained with resistant strains isolated from the second orchard (results not shown).

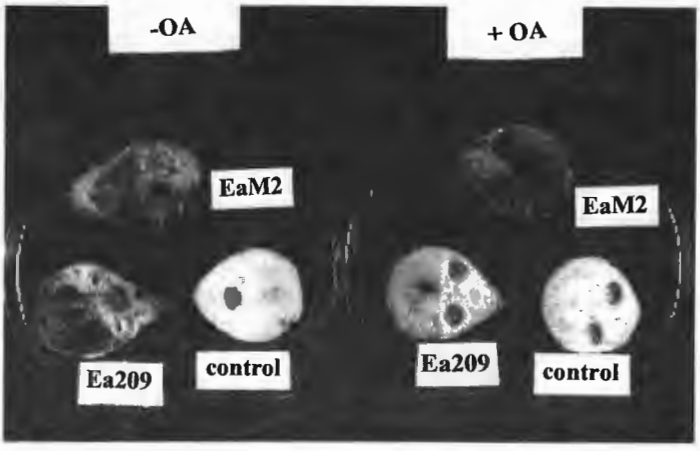

Figure 1. Immature pear fruit assays carried out with and without oxolinic acid
+OA - pear fruits dipped in 0.15% Starner 20% wp.
EaM2- *Erwinia amylovora* strain resistant to oxolinic acid.
Ea209- *Erwinia amylovora* resistant to streptomycin and sensitive to oxolinic acid.
Control- water

This is the first report on isolation of *E. amylovora* strains resistant to oxolinic acid. In Japan resistant strains of *Burkholderia glumae* and *Acidovorax avenae* which are pathogenic on rice were reported (Morikawa *et al.* 1997, Hikichi *et al.* 1998). The resistance was also demonstrated in field experiments (Morikawa, personal communication). Further characterization of the oxolinic acid-resistant strains of *E. amylovora* will be conducted in the future.

The appearance of resistant strains of *E. amylovora* in two orchards may indicate that the population of this pathogen has the potential to develop, in the future, resistance that will not be controlled by 300 μg/ml oxolinic acid.

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SSR analysis of apple scab lesions

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Abstract : The analysis of populations of plant pathogenic organisms using molecular DNA markers has gained remarkable attention within the recent years. Knowledge about diversity within and between populations is important to develop and manage plant protection strategies. SSR (simple sequence repeats, microsatellites) markers are highly informative and reproducible and allow the analysis of a large number of samples within a relatively short time. Previous studies of *V. inaequalis* populations have been performed using single spore isolates of the fungus. We show in this publication that DNA extraction directly from lesions on apple leaves followed by SSR analysis give reproducible results and allow to omit the single spore cultivation step, therefore enabling an analysis of a large number of samples.

Key words : Microsatellite, SSR, *Venturia inaequalis*, single spore isolates

Introduction

In the recent years, the increasing consumer's interest for more ecologically produced food raised the pressure for producers to reduce the input of plant protection chemicals. One way to respond to these demands is the use of crops that are resistant to their main diseases. The pathogens causing these diseases are not static but are exposed to evolutionary forces. The knowledge about these forces and their implications on the pathogen population structure is essential for developing plant protection strategies oriented to keep the plant resistance durable (Wolfe and Gessler, 1992).

Research on population dynamics is not new. However, the techniques that allow the analysis of DNA polymorphisms have become increasingly important and remarkably improved the knowledge about the variability of plant pathogen populations. However, the shortcoming of all such studies is the small sample unit and intrinsic unreliability of particular types of markers. Among the molecular DNA markers that reveal pathogen variability, Simple Sequence Repeats (SSRs, also called microsatellites) have been recognized to be extremely useful. Although their developement (Tenzer *et al.* 1999) takes a relatively long time compared to RAPDs or RFLPs, they allow the screening of a large number of samples (isolates) within a relatively short time and are highly reproducible and informative. But a bottleneck still remains in relation to the number of samples in the test set, because single spore isolates need a large input of work and time to be produced. Moreover, in certain cases, such as obligate biotrophic parasites, pure cultures are not obtainable.

Venturia inaequalis (Cooke.) Winter amend. Aderhold causes apple scab, the most important disease on apples (*Malus* x *domestica*) in temperate regions with cool, moist weather in early spring. The disease is nowadays mainly controlled by fungicides, with up to 15 applications per season. Already 100 years ago Aderhold (1899) recognized the variability among *V. inaequalis* isolates. His work was followed by several other publications in the early 20th century (Schmidt 1940, Wiesmann 1931). Of course these works were restricted to the investigation of morphological and physiological variability. Later on, evidence for differences in pathogenicity was provided (Koch *et al.* 1996, Sierotzki *et al.* 1994). However,

until very recently the implications of these findings had been neglected, and the *V. inaequalis* populations were generally regarded to be homogeneous, at least in respect to pathogenicity. The study on several *V. inaequalis* populations from diverse european regions showed that the populations are becoming more different with the increasing distance between them (Tenzer & Gessler 1999).

Studies on plant pathogens are often difficult to perform due to the biotrophic nature of the organism, which means that the pathogen has to be isolated and cultivated on plants. This is an enormously tedious and laborious work, and usually allows only the sampling of relatively small number of isolates. In the case of *V. inaequalis*, single spore isolates can be produced in order to get enough fungal material for DNA extraction. However, this procedure needs a lot of time and work. It would be therefore desirable to have a method at hand which allows to omit the step of multiplication of the pathogen, and therefore would allow the analysis of a large number of samples within short time. Especially in the field of population studies, it is very useful or even a requirement to have a large sample size in order to make reliable statements.

Due to the length of their primer sequences, SSR markers (microsatellites) are highly specific for their target sequence. We can therefore assume that, starting from a mixture of host and pathogen DNA, the specificity of the SSR primers allows clear identification of microsatellite alleles originating from the target organism.

The usefulness of this method was showed by analysing lesions of many trees in an untreated orchard. As a possible application of the procedure, we analysed lesions of two neighbouring *Malus floribunda* 821 trees and 5 samples of Vf resistant cultivar Topaz, which showed heavy scab symptoms in the years 1998 and 1999. Up to now, it is not known whether the lesions are caused by one or several *V. inaequalis* genotypes, and if they do recombine with the surrounding population.

Material and Methods

Fungal material

Single spore isolates of *V. inaequalis* were kindly provided by I. Tenzer and used as a control. Lesions from the field originated from an untreated orchard in Wädenswil, Switzerland. The host plants consisted of two progenies (about 300 plants each), Fiesta x Discovery and Iduna x A679-2. Leaves with lesions were collected from trees chosen at random, where only one isolate per tree was taken.

DNA extraction

The lesions were excised, dried with silicagel and stored at 3 °C. For DNA extraction, lesions were cut to about 1 cm^2 in size and put to an 2 ml Eppendorf tube containg 400 µl of sterile glass beads. The tubes were put in a -80°C freezer for at least 1 h in order to facilitate homogenisation, which was performed in a cell homogenisator (B. Braun, Melsungen, Germany) for 30 seconds. The tubes were then transferred on ice to prevent warming up of the leaf material. 600 µl of lysis buffer (Qiagen Lysis Buffer AP, Cat. #19078) preheated to 65°C were added and the tubes well shaken to dissolve the plant/fungal material. The tubes were then immediately put in a water bath at 65°C for 15 minutes, followed by the addition of 200 µl of ice-cold precipitation buffer (Qiagen Precipitation Buffer, Cat #19079) and incubation on ice for 10 minutes. One volume of Chloroform-Isoamylalcohol (24:1) was added and the tubes well shaken. After centrifugation in an Eppendorf centrifuge for 20 min, the upper phase was transferred to a new tube and 0.7 volumes of Isopropanol were added for DNA precipitation. The tubes were then centrifuged for 20 minutes, the pellet washed with 70 %

ethanol and air dried. The dry pellets were then suspended in 50 µl H_2O bidest. and the DNA concentration determined.

Microsatellite PCR amplification

PCR of the microsatellites as well as electrophoresis were performed after Tenzer *et al.* (1999). The microsatellite primers used were developped by Tenzer *et al.* (1999).
Reaction products were analysed on a sequencing gel as described by Gianfranceschi *et al.* (1998).

Results

39 lesion samples of an untreated orchard were analysed using several microsatellite primer pairs (Figs. 1, 2). Almost all DNA samples gave a clear signal. Although some PCR artefacts were also visible on the gel, the SSRs are easily recognisable by the presence of secondary bands, so called stutter bands. The markers were also in the size range of the alleles amplified from single spore isolate DNA. The control samples containing the DNAs of the parents of the two progenies did not produce any signal. Some samples did not produce a signal on all tested microsatellite loci.

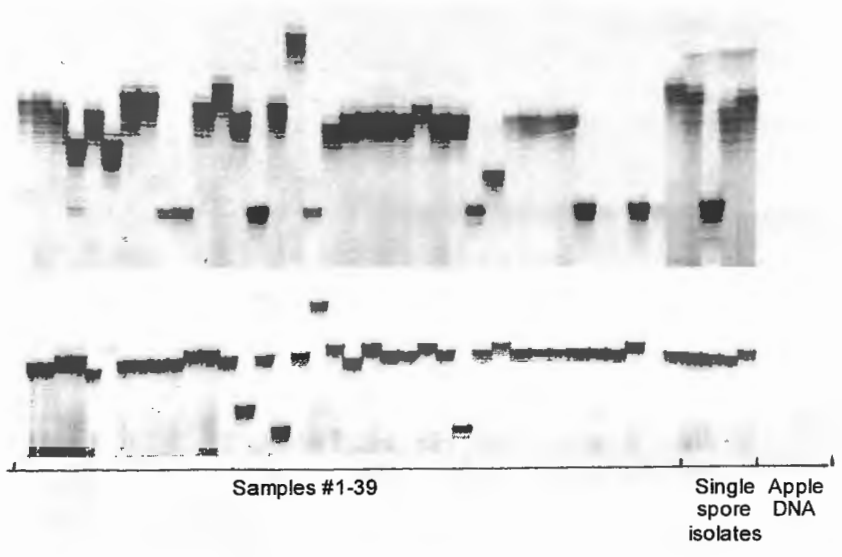

Figure 1. Microsatellite loci 1tc1a (upper) and 1tc1b (lower) on 39 samples from an orchard with the offspring of 2 apple crosses. 4 single spore isolates and the parental apple DNA (cvs. Fiesta, Discovery, Iduna, A679-2) as control/reference.

Lesions from *M. floribunda* 821 and from Vf resistant cultivar Topaz were sampled in 1999, and analysed for their allelic state of 4 microsatellite loci. All samples showed one allele of the same size (Fig. 2, not all data shown), whereas the control samples from a neighbouring untreated orchard produced many different alleles (Fig. 3, not all data shown).

A special case is microsatellite 1tc1g. This marker showed more than one allele on some samples, but never on samples originating from Vf carriers (Fig. 3).

All lesion samples from *M. floribunda* 821 collected in 1998 showed the same allele pattern as the samples collected in 1999 (Fig. 4).

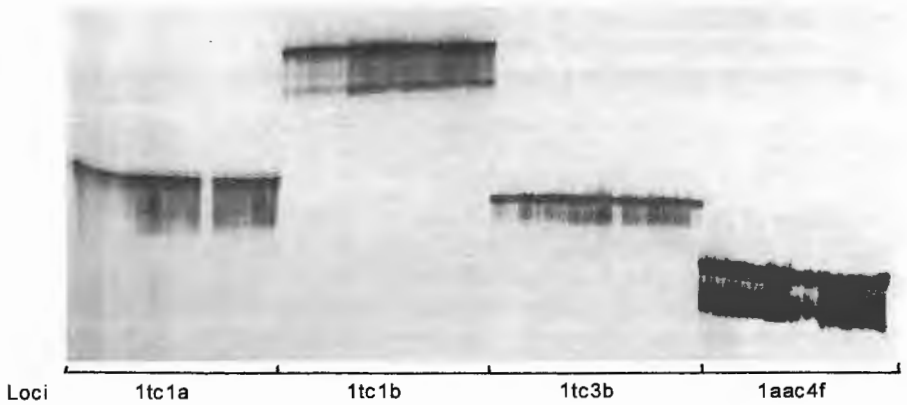

Figure 2. Five samples of cv. Topaz and 11 samples originating from *Malus floribunda* 821 tested on 4 microsatellite loci.

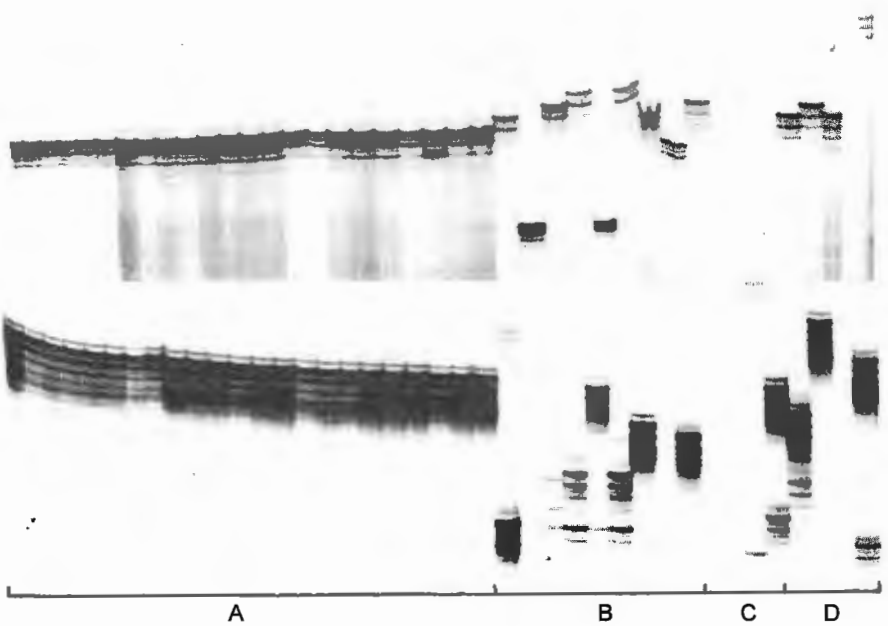

Figure 3. Microsatellite markers 1tc1a (upper) and 1tc1g (lower). A: 5 and 18 samples originating from cv. Topaz and *M. floribunda* 821, respectively. B: 9 samples from a neighbouring orchard. C: Apple DNA from cvs. Fiesta, Discovery and *M. floribunda*. D: 5 single spore isolates.

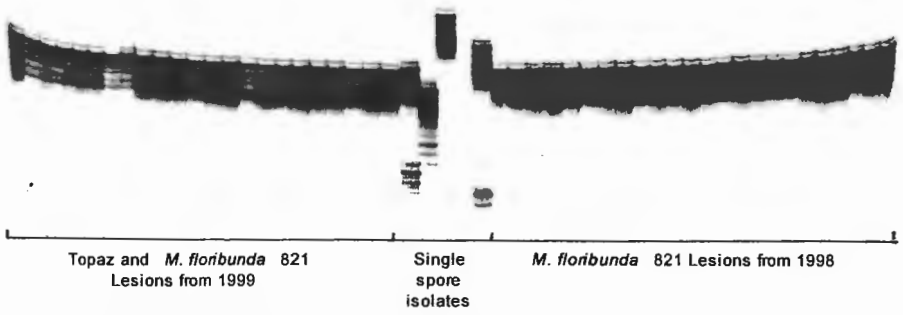

Figure 4. Microsatellite locus 1tc1g. Samples originating from cv. Topaz (first 5 lines), *Malus floribunda* 821 collected in 1998 and 1999. Five single spore isolates as reference.

Discussion

The extraction of DNA directly from scab lesions on apple leaves is a fast way to produce a large number of samples within a relatively short time. Using a simple DNA extraction protocol, a lab trained person can easily extract 50 to 100 samples per day. Compared to the production of single spore isolates, this means a massive reduction in work and time required to produce a large sample size which is necessary for population studies. The band patterns that were amplified using directly extracted lesion DNA were as clear and good as the signals produced with single spore isolate DNA. Some samples showed signals only for some loci. One reason for this may be incorrect DNA concentration, since it is not possible to quantify the amount of fungal DNA in a mixture. Nevertheless, we adjusted the DNA content of all samples to the usual 1ng/µl, which worked fine in almost all cases. On well sporulating lesions, the number of *V. inaequalis* spores propably equals or even surmounts the number of underlying plant cells, which results in an about 1:1 proportion of fungal and plant DNA. The fact of missing alleles may also be due to null alleles, *i.e.* alleles which do not produce a signal at all.

The DNA samples from the parents of the progenies in the untreated orchard usually showed no amplification products. This was to be expected from the specific nature of the microsatellite primer sequences. In cases where both the fungal and the apple DNA would produce signals, the bands originating from the apple genome could easily be identified by comparison with the banding patterns of reactions with pure apple DNA.

The analysis of about 40 lesions of Vf carriers *M. floribunda* 821 and Topaz from the years 1998 and 1999 indicated that there is only one strain abundant in Wädenswil, which causes the lesions on the analysed Vf resistant apple trees. Although this cannot really be proven, the indication is very strong, since a lot of different alleles could be found in the surrounding scab populations. The lesions from 1998 showed as well only one allele: the same as 1999. The explanation for this may either be asexual overwintering of scab or recombination between mating types within the Vf overcoming pathotypes. We think that the probability for the latter case is rather small, because it would require mating types with identical SSR allele patterns.

The amount of DNA extracted from lesions was always enough to make at least 20 PCR reactions. However, in most cases there was enough DNA to make at least hundred reactions, especially if the lesions were heavily sporulating. Since the direct extraction from leaves, *i.e.*

omission of single spore cultivation allows the to create a large sample number, the fraction of samples with insufficient DNA can be neglected, if a lot of reactions have to be made.

Among the several molecular markers available for pathogen population studies, SSR markers are certainly the most useful and informative ones, since they are codominant, *i.e.* multiallelic. Compared to RFLPs they have the advantage that only a small amount of DNA is required to perform the analysis. SSR markers take some time until they are developped. On the other hand, once the primers are developped, a large number of samples can be analysed within short time. Due to the specificity of microsatellite markers the pathogen does not have to be 'purified', *e.g.* by single spore isolate production. This not only facilitates the whole analysis. It also excludes some kind of selection, which occurs for instance when scab samples are taken from treated orchards. These fungi often grow very bad and will therefore not be included in the final test set.

Although in this paper we reported exclusively about *V. inaequalis*, SSR analysis from extracts of lesions may be especially useful for strictly biotrophic fungi, where the cultivation of isolates is a very cumbersome step.

Acknowledgments

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First report on the presence of *Venturia inaequalis* race 7 in French apple orchards

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Abstract : In France, *Venturia inaequalis* strains able to overcome the *Vf* gene resistance were observed for the first time in 1995. The cultivar Judeline showed a severe attack in a cider apple orchard in Normandie, while the cultivar Judaine was scab free. These two cultivars are *Vf* resistant. The pathogenicity of one strain from Judeline (1163) was compared to that of the race 1 and 7 reference strains (104 and 1066), and to that of one Dutch strain (1127) isolated from the cultivar Vanda (*Vf* resistant) ; the test was performed on a range of 8 apple cultivars, including some cultivars of Judeline and Judaine pedigrees. The results showed that the strains 1127 and 1163 were similar to the race 7 reference strain, virulent to *Malus floribunda* 821 and avirulent to Golden Delicious. These 3 strains were virulent to Judeline and avirulent to Judaine. The analysis of the pedigree of Judeline and Judaine showed that Priam, *Vf* resistant parent of the 2 cultivars, was resistant to the 4 tested strains, while Jonathan (parent of Priam) and Reinette du Mans (parent of Judaine) were resistant to strain 1163 and susceptible to strains 1127 and 1066. These results suggest three hypotheses to explain the resistance of Judaine. It could be due to the *Vg* gene from Golden Delicious, which could be inherited in Priam and transmitted to Judaine and not to Judeline. In the same manner, a resistance gene or QTL could be transmitted from Jonathan to Judaine. But this resistance could also be inherited from Reinette du Mans, which is an old French cultivar slightly susceptible to scab. Its tolerance could be under polygenic control. This study confirms that some *Vf* resistant cultivars possess additionnal resistance : major genes (like *Vg*) or QTLs, which can be interesting in presence of *V. inaequalis* strains virulent to the *Vf* gene.

Key words : apple scab, virulence, *Malus x domestica,* resistance breakdown

Introduction

The emergence of *Venturia inaequalis* strains able to overcome the resistance of the *Vf* gene was first reported in Germany, in an experimental orchard (Parisi *et al.*, 1993). This new race, called race 6, was virulent to most of the recently selected *Vf* resistant cultivars, but avirulent to the progenitor *Malus floribunda* 821 (Parisi and Lespinasse, 1996). The resistance of this clone is more complex that the single *Vf* gene, and could be induced at least by two major genes (Bénaouf *et al.*, 1997).

After this first record, the presence of race 6 was not reported in other countries. Another kind of strain virulent to the *Vf* gene was found in England, in an ornemental apple of *M. floribunda* (Roberts and Crute, 1994), and called English race or race 7. Its characteristics were to be virulent to *M. floribunda* 821, but avirulent to Golden Delicious. The resistance of this cultivar to race 7 is due to a single gene, named *Vg* (Bénaouf and Parisi, 1997). This explains why race 7 was not virulent to all the recently selected *Vf* cultivars ; some of them could have inherited the *Vg* gene from Golden Delicious, which has been frequently used in the apple breeding programmes.

The first report of the presence of *V. inaequalis* strains virulent to the *Vf* gene in commercial orchards dates from 1994, in The Netherlands (Schouten and Schenk, 1997). These strains belong to race 7 (Parisi, unpublished data).

In 1995, for the first time in France, scab symptoms were found on a *Vf* resistant cultivar. This occured in a cider apple orchard, in Normandie, planted with 3 susceptible cultivars : Douce Moën, Clos Renaux and Judor, and 2 *Vf* resistant cultivars : Judeline and Judaine. The cultivar Judeline showed a very high scab attack, while Judaine remained scab free.

In 1997, a test of the pathogenicity of 2 strains from Judeline gave a first evidence that these 2 strains belong to race 7. Here we report the results of a second test, which compared the pathogenicity of one strain from Judeline to those of 2 reference strains (race 1 and 7), and a race 7 strain from The Netherlands.

Materials and methods

Venturia inaequalis strains

Four strains were tested. Their origins and characteristics are given in Table 1. The method used for inoculum production was described by Parisi *et al.* (1993).

Table 1. Origin and characteristics of the tested strains

Strain	Origin	Cultivar	Characteristics
104	France, 1978	Golden Delicious	Race 1 reference strain
1066	France, 1993	*Malus floribunda* 821	Race 7 reference strain [a]
1127	The Netherlands, 1994	Vanda	Race 7[b]
1163	France, 1995	Judeline	Unknown

a Monoconidial strain from FI 1 isolate, found in England (Roberts and Crute 1994)
b L. Parisi, unpublished results

Plants

The host range was comprised of 7 cultivars of *M. x domestica* and *M. floribunda* clone 821. Their characteristics (susceptibility to scab, resistance genes) are given in table 2.

Table 2. Characteristics of the clones of *Malus* included in the host range

Malus x domestica cultivars	Characteristics
Gala	Scab susceptible
Golden Delicious	Scab susceptible in field conditions, *Vg* gene
Jonathan	Scab susceptible
Reinette du Mans	French local cultivar, slightly susceptible to scab
Judeline	*Vf* resistant
Judaine	*Vf* resistant
Priam	*Vf* resistant
Malus floribunda 821	Progenitor of *Vf* resistant selections

Gala is the susceptible control. The other *Malus* cultivars and species were tested because they were involved in the pedigrees of Judeline and Judaine. These two cultivars are *Vf* resistant apple-juice cultivars, selected by INRA. They are half-sib, both derived from Priam crossed with Golden Delicious for Judeline and Reinette du Mans for Judaine (Figure 1).

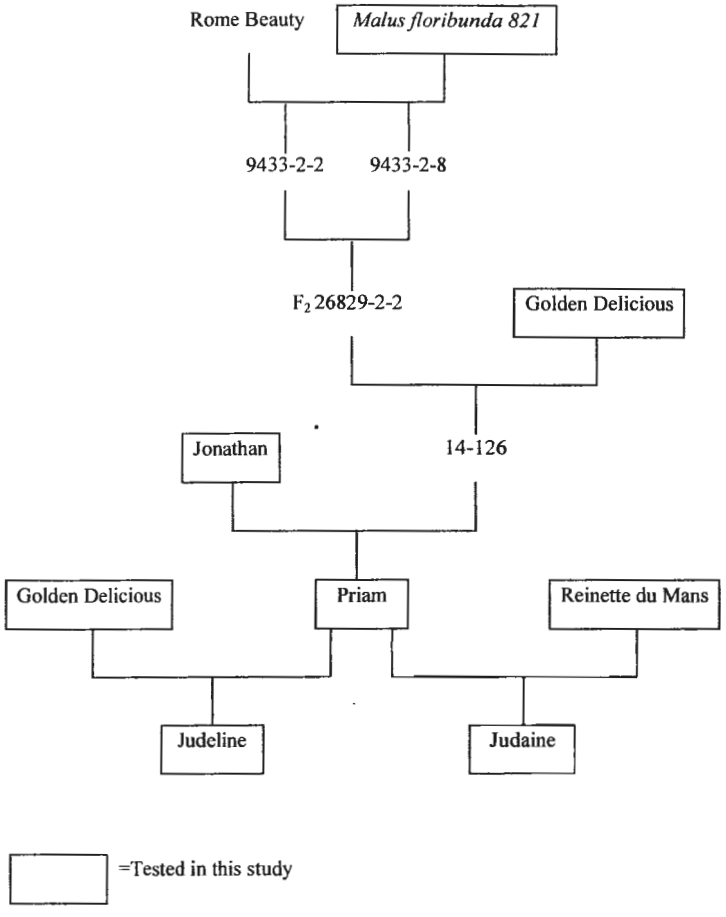

Figure 1. Pedigrees of the cultivars Judeline and Judaine

The trees were grafted onto M9 rootstocks and grown in pots in the greenhouse. For the tests, 4 to 5 trees per cultivar were inoculated with each strain ; each tree had 1 to 3 growing shoots when inoculated.

Inoculation

The experiments were done in a growth chamber. The size of the growth chamber did not permit all the cultivars inoculated with the 4 strains to be tested together. Two different experiments were done, with 2 strains of *V. inaequalis* tested for each experiment on the complete host range. The protocol for the inoculation and the incubation of the plants was described by Parisi and Lespinasse (1996).

Symptoms assessment

The symptoms were assessed 18 or 19 days after the inoculation, following the experiment. For each shoot, all the leaves were observed for the presence of symptoms, the class of the symptoms, and when scab lesions were visible, the percentage of leaf area with sporulating lesions. For the class of symptoms, the scale of Chevalier *et al.*(1991) was used. This scale consists of 6 classes :

Class 0 : no symptom,
Class 1 : hypersensitivity (pin-points pits),
Class 2 : resistance, chlorosis and/or necrosis without sporulation,
Class 3a : weak resistance, chlorosis and/or necrosis with slight sporulation,
Class 3b : weak susceptibility, chlorosis and/or necrosis with sporulation
Class 4 : susceptibility, abundant sporulation without chlorosis and/or necrosis.

The disease severity was assessed on a scale derived from Croxall *et al.* (1952), which takes into account the percentage of leaf area with sporulating symptoms (las) :

Class 0: no symptom, class 1: $0 < las \leq 1$, class 2: $1 < las \leq 5$, class 3 : $5 < las \leq 10$, class 4 : $10 < las \leq 25$, class 5 : $25 < las \leq 50$, class 6 : $50 < las \leq 75$, class 7 : $75 < las \leq 100$

For each cultivar/strain interaction, the incidence : percentage of scabbed leaves and the severity : median of scores of all the scabbed leaves were calculated, and all the classes of symptoms plotted.

Results

The results (Table 3) shows that the strain 104 (race1) is virulent to Gala and Golden Delicious, and avirulent to the 3 *Vf* resistant cultivars and the clone 821 of *M. floribunda.* The resistance of this clone to race 1 was expressed as a class 1 symptom. These results are in agreement with the previous tests of the pathogenicity of this strain (Parisi and Lespinasse, 1996, Bénaouf *et al.*, 1997). In addition, the results showed that strain 104 was virulent to Jonathan and Reinette du Mans.

The 4 strains tested were virulent to Gala (Table3). However, the incidence of disease obtained with the strains 104 and 1066 was higher than that obtained with the strains 1127 and 1163. With the strain 1127, symptoms of different classes were obtained. This could indicate a difference of aggressiveness between the strains (104 and 1066 could be more agressive to Gala than 1127 and 1163). Because the 4 strains were tested in 2 different experiments, a stastistical analysis of the results was not possible; this difference of aggressiveness must be confirmed.

The strain 1163 from Judeline had the same behaviour to Golden Delicious (avirulent) and *M. floribunda* 821 (virulent) as the race 7 reference strain 1066, and the Dutch strain 1127. The resistance of Golden Delicious to these 3 strains was expressed as a class 2 to 3a symptom. However, the strain 1163 was avirulent to Jonathan and Reinette du Mans, while the strains 1066 and 1127 were virulent to these 2 cultivars.

The 4 tested strains were avirulent to Judaine and Priam, while the 3 strains 1066, 1127 and 1163 were virulent to Judeline.

Table 3. Scab incidence, severity, and classes of symptoms observed in the interactions between 7 cultivars of *M. x domestica* and *M. floribunda* 821 and 4 strains of *V. inaequalis*.

Strains	104			1066		
Cultivars of *M. x domestica*	I[a]	S[b]	Classes	I	S	Classes
Gala	33.8	3	4	26.8	4	3b,4
Golden Delicious	36.3	4	4	0	0	2,3a
Jonathan	27.8	3	4	25	3	4
Reinette du Mans	32.6	3	4	29.8	3	4
Judaine	0	0	1	0	0	2
Judeline	0	0	3a	29.9	3	4
Priam	0	0	3a	0	0	2,3a
M. floribunda 821	0	0	1	36.8	3	4

Strains	1127			1163		
Cultivars of *M. x domestica*	I	S	Classes	I	S	Classes
Gala	3.7	3	2,3a,3b,4	10.8	4	3b,4
Golden Delicious	0	0	2	0	0	2
Jonathan	3	2	1,2,4	0	0	2,3a
Reinette du Mans	21.2	3	4	0	0	1,2
Judaine	0	0	1,2	0	0	2
Judeline	8.2	2	3b	7.2	4	3b,4
Priam	0	0	1,2	0	0	1,2
M.floribunda 821	13.6	3	3b,4	14.6	4	3b,4

a I=incidence
b S=severity

Discussion

The first *Vf* resistance breakdown observed in France, on the cultivar Judeline, seems to be due to race 7. However, the pathogenicity of one strain from Judeline (1163) differs from that of the race 7 reference strain (avirulent to Jonathan and Reinette du Mans).

The fact that Judaine was resistant to all the strains avirulent to the *Vg* gene suggests that its resistance could be due to the *Vg* gene, inherited from Priam. Judeline has apparently not inherited this resistance gene, even if the last cross of its pedigree involved Priam and Golden Delicious (Figure 1), both probably heterozygous *Vg/vg*. This gene was not selected in the plant breeding programmes.

However, the orchard resistance of Judaine could be also due to resistance factors inherited from Jonathan, Reinette du Mans, or both. The last is an old local French cultivar, considered slightly susceptible to scab (Olivier *et al.*, 1984). Its field tolerance could be under polygenic control. However, in our test, this cultivar exihibited a complete resistance expressed as classes 1 or 2 symptom. In the absence of information concerning the genetic determinism of the resistance of these 2 cultivars, and the frequency in the orchard of strains similar to 1163, it is impossible to predict the stability of the Judaine resistance.

This work shows clearly that two cultivars selected for *Vf* resistance (Judaine et Judeline) can have different genetic backgrounds which induce different reactions when *Vf* is overcome. We must increase our knowledge of these genetic backgrounds, to be able to develop strategies for *Vf* resistant cultivar plantings. A first step could be to obtain molecular markers

of the *Vg* gene, to verify the hypothesis that the resistance of several *Vf* cultivars to race 7 could be due to *Vg*.

Race 7 of *V. inaequalis* seems to spread in Europe more quickly than race 6 ; we do not know why. If we suppose that this is coincidental, there is no need to cumulate the *Vf* and *Vg* genes ; probably the fungus will be able to quickly overcome both the genes. Conversely, if we suppose that the association of the virulence to *Vf* and avirulence to *Vg* gives a selective advantage to the fungus, this kind of strategy could be attractive. This strategy could be enhanced by the addition of other resistance factors ; this work confirms that they are more frequent in apple cultivars that initially supposed (Sierotzki *et al.*, 1994). It could be useful to identify more precisely these factors and evaluate their utility for plant breeding programmes.

Acknowledgements

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Genetic diversity of the apple mildew fungus *Podosphaera leucotricha* : DNA-markers give surprising results, artefacts or relevant role of sexuality ?

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Abstract : Genetic variability of *Podosphaera leucotricha* was assessed with RAPD markers. Each sample consisted of conidia brushed off from a single primary infection site developed early spring from a bud. From 190 samples enough DNA could be gained for the necessary PCR. With selected RAPD-markers bands the 190 samples could be differentiated in 95 haplotypes. Even in single trees various haplotypes were found. On the other hand the same haplotypes were found on different cultivars. This would indicate that the sexual form has a more relevant form as we assumed and many of the primary infection originated from ascospore infections. An other explanation would be that the variability detected through the pattern of RAPD-markers is an artefact

Introduction

Mildew caused by the fungus *Podosphaera leucotricha*, is in Switzerland a secondary problem, as the fungicides used to control the main disease, apple scab, give also a good control of mildew. With the availability of scab resistant cultivars, fungicide treatments are left out. Mildew can develop without restriction on mildew-susceptible cultivars. Therefore apple breeding is currently incorporating resistance also against this pathogen into the new varieties (see also Goerre *et al.* 1999). However it is not known if the fungus can overcome any host resistance and select after genetically recombination new virulent races. The fungus is thought to live overwhelmingly in the asexual phase, overwintering as mycelium in the buds (Cunningham 1923, Siebs 1959), as attempts to germinate ascospores or cause infections with ascospores usually failed (Woodward 1927, Fischer 1956, Wartenberg 1960, Korban and Riemer 1990). In the study here presented, we wanted to analyse the variability of populations of the apple mildew pathogen *P. leucotricha.* The goal was to understand the evolution of pathogen populations in a perennial culture system in relation to the cultivars and sites, and estimate a possible gene flow inside an orchard between sub-populations on different cultivars and between different orchards in relation to locations. We postulate that "isolates" showing a different RAPD-marker banding pattern, are genetically different deriving from different ascospores and "isolates" having identical RAPD-marker pattern will be clones, single differences may be interpreted as mutation event in a clonal lineage.

Material and Methods

A methodology to gain DNA directly from the fungal samples collected on the leaves, has been elaborated so that, with a high probability, we can have sufficient DNA for PCR and RAPD analysis. We attempted to set up a system to multiply the fungus in a "*in vitro*"-system on detached apple leaves so that isolates derived from few conidia could be made and maintained. Although we succeeded in particular cases, the system is not reliable yet. For this

reason currently we gain fungal DNA directly by scraping the fungal conidia and mycelia from natural infected leaves. Each sample consists of the leaves originated from the same bud and is collected early season (April). As the fungus overwinters in the buds, we assume therefore that a sample consists of a single genotype. When, for specific purpose such as repeated testing, more DNA was requested, a multiplication step on young trees in the greenhouse was made.

DNA was extracted following the methodology of Sierotzki *et al.* (1994, 1998), however as starting material, the spores were frozen at –20°C and not lyophilised. Amplification, electrophoreses and statistical analysis used, are described in Tenzer and Gessler (1999).

Only polymorphic bands which could be clearly scored as absent or present in several repeats were used.

Result and Discussion

In spring-summer 1995 and 1996 188 samples consisting of leaf-bunches infected with *Podosphaera leucotricha* were collected. From 123 samples not sufficient quantities of DNA could be extracted. From 65 samples enough DNA of sufficient quality was extracted. PCR was performed with 213 primers, out of these 17 primers gave easy scorable and clear polymorphic bands on a restricted number of sample size. Finally 18 polymorphic bands obtained with 7 primers were used to genotype all samples. The 65 samples originated from three sites. Site one consisted of three trees (one Golden Delicious and two different scab resistant selections) isolated on a terrace in the middle of Zürich. Site two (Eschikon) is a small collection of various cultivars and breeding selections ca 10 years old with trees interwoven, the next orchard being ca 500 m distant. The third site is in Ticino near Cadenazzo and is also a collection of mostly scab resistant cultivars. The trees from which samples were taken stood as fare apart as possible mostly over 20m. These sites and their particularities were chosen because no fungicide treatments were ever made and the mildew population was therefore never subjected to a fungicide selection pressure nor to a bottleneck situation were by chance a large proportion of the variability could be loosed. The 65 samples could be differentiated into 21 groups (= haplotypes or genotypes).

On the site "terrace" the 33 samples were assigned to 3 haplotypes (16, 9,8), the 3 trees present are small (2m high) and highly isolated from any infection source. Samples were close to each other, still three clearly distinct genotypes were present. The 16 samples from the site Eschikon represented 8 haplotypes, one recurring 6 times, three twice and the other once; the 16 samples from Cadenazzo 12 haplotypes. Except two all haplotypes were specific to the site. The haplotype dominant on site terrace found there 16 times, was found also 6 times in Eschikon. As our collaborators often move between these two sites, this could indicate that we eventually transported and diffused this clone. An other haplotype was found twice in Eschikon and once in Cadenazzo. This may be a bias and by using more markers the isolates may be distinguished. Even if the sample size is small, the three populations have a high allele variability inside each population and the populations are statistically distinct. No association to particular cultivars was found, identical haplotype could even be found on neighbour trees of different cultivars.

In 1997 nine trees of different cultivars at four different sites (Stadel, Wädenswil, Horgen and Güttingen) were sampled. From 125 samples enough DNA for analysis could be extracted. Four primers, already used prior (1996), gave good profiles and 17 polymorphism were detected. However repeating reactions, 9 had to be left out as they were not consistent. The primer OP D05 allowed the use of four polymorphism out of 5 (Fig. 1), primer OP AA06 and OP Q12 only one out of 4 and OP E07 two out of four. Of these 8 loci only two were the same as used in 1966 as the others used in 1996 were or not detectable any more or not

consistent, six were new. We do not know the reason of this except that the first part (1996) was executed by the second author and work 1997 by the third author.

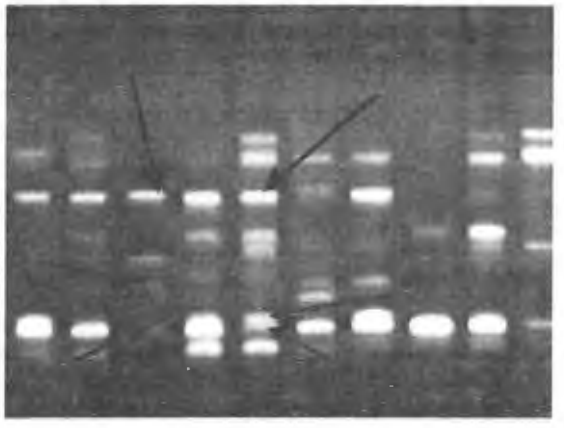

Figure 1. RAPD-PCR patterns of *Podosphaera leucotricha* isolates (origin Stadel) created with primer (OPD05) 5'-TGA GCG GAC A-3'. Isolate in lane three has the markers D5-400, D5-570 and D5-900, but not D5-410. Isolate in lane 5 has D5-400, D5-410, and D5-900, but not D5-570. Other polymorphism were not scored.

With 8 RAPD loci, created with 4 primers 74 haplotypes could be discriminated. Still some loci were not scorable (considered missing data) so 33 of the 74 haplotypes were differentiated through less than 8 loci. 42% of the samples were haplotypes present only once. Other 18% of the samples were haplotypes found twice. Three haplotypes were found often (8, 8 and 9 times) (20% of the samples). No statistical correlation between haplotypes and site or cultivars occurred (Tab. 1).

Genetic diversity (G_{ST}) between the single trees varied between 0.05 and 0.44; between the sites from 0.041 up to 0.22 with an average of 0.146, which clearly is higher than the values (0.04) for apple scab measured between populations more apart (Tenzer and Gessler 1997). This may indicate that migration events (past and present) are rare and populations had time to drift apart, suggesting that bottleneck situation limiting the size of a population may be frequent and/or population sizes small.

Genetic diversity inside the sites (H_s) was between 0.15 and 0.35 with an average of 0.25. This is a value which is close to values found for the sexual reproducing pathogen *V. inaequalis* (0.26 to 0.33) (Tenzer and Gessler 1999), and contradicting the assumption that a population is made up from one or few clones.

Differences in RAPD-alleles between two isolates may be due to a single mutation in an isolate originating from the same clone by asexual reproduction (same clonal lineage), most frequent would be single difference, rare two difference. Sexual recombination between isolates having different banding patterns would lead to any combination with most frequent number of difference between the offsprings being half the differences between the parents. A sexual recombining population would therefore present a frequency of differences between each isolate to any other related to the number and frequency of polymorphic markers considered. We made pairwise comparison between all haplotypes which had no missing data

(41) counting in each case the number of differences in 8 RAPD markers. Most frequent were 3 differences (Fig. 2). This indicates that sexual reproduction is the main reason for the creation of different haplotypes. However over half of seemingly clear scorable bands (17) had to be left out, as we were not able to score them constantly when repeating PCR. In fig. 1 a typical result is presented, the profiles of the isolates seem clearly different. However, as only 25% of the polymorphism could be used by an other experimentator an year later and 43 isolates (33 haplotypes) presented missing data, some doubt remain when using RAPD-markers in such studies and obtaining results, which differ from the general assumption. Currently we try to develop more reliable markers.

Table 1. Distribution of the haplotypes of *Podosphaera leucotricha* as determined with 8 RAPD-markers.

Site	Cultivar	Haplotype ID number																				No of unique haplo-types	Totale isolates	No. haplo-types per tree
		3	5	7	8	14	19	21	22	24	25	27	30	33	37	39	43	48	52	58	61	65			
Stadel	Johnathan I								1		1			2			2					2	14	22	19
	Johnathan II	1		5												2						1	4	13	8
	Ontario																		1	1			2	4	4
	Sauergrauch						3	1	1														0	5	3
Wäden.	Florina						2	1		1		1	1	1				2					3	12	10
	MacFree						2	1				2								1	2		6	14	11
Horgen	Florina			1	1	1	1	1		1	2		1						1				9	19	18
Güttin.	Florina	1	1	1	1	2									1								9	16	15
	Johnatan		1	2	6	2			2						1								6	20	12
Sum		2	2	9	8	5	8	4	4	2	3	3	2	3	2	2	2	2	2	2	2	3	53	125	

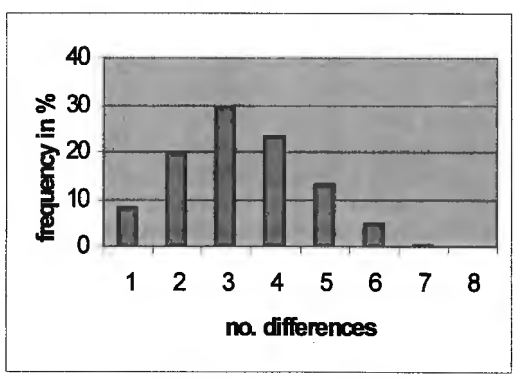

Figure 2. Frequencies of number of differences comparing pairwise 41 haplotypes of *Podosphaera leucotricha* based on 8 polymorphic RAPD-bands.

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Rural cultivars as an important source of resistance genes

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Abstract : Some cultivars from Carpathian villages have good horizontal resistance against common pear and apple disease agents, particularly, *Venturia inaequalis, Podosphaera leucotricha,* etc. It is worthwhile to use these cultivars as source of resistance genes.

Key words : rural cultivars, resistance genes.

Introduction

South slopes of Carpathians are covered with forests, woods and shrubs. The most reach flora diversity is at the foothills of the mountains. Here many species of wild fruit and berry plants grow. Among them *Malus sylvestris Mill., Pyrus communis L., Cerasus avium (L.) Moench., Rosa canina L., Sorbus aucuparia L., Prunus spinosa L., Corylus avellana* L., *Castanea sativa* Mill., *Crataegus oxyacantha L. p. p. , Rubus caesius L., Rubus idaeus L.,* and more rarely *Prunus domestica L., Persica vulgaris Mill. Cornus mas L., Padus racemosa (Lam.)Glib., Sorbus domestica L.* can be met. Rural cultivars and wild forms of fruit plants in Subcarpathia were not enough studied. Somewhat general characteristic of them was given in works of Cherneki (1974), Zayats & Sikura (1993), Zayats (1998, 1999).

Climatic conditions of Carpathians are favorable for the development of fungal diseases. The plant grown here are often under the epiphytotic stress. Because of this matter, the local varieties of plants were selected against fungal and bacterial pathogens pressure and they are tolerant to the most of fungal diseases. Thus, cultivars developed here have very good horizontal resistance to *Venturia inaequalis* and *Podosphaera leucotricha*, the causal agents of apple scab and powdery mildew.

Material and Methods

The material collected from local peasants was observed in laboratory under light microscope and tested for diseases resistance by using grading system developed in Nikitsky Botanical Garden by I. Ryabow (1969). The symptoms of the diseases were read into field conditions onto grafted material. Other commercially important characteristics were tested as discribed in Dospehov (1973), Moyseychenko (1988).

Results and discussion

During several expeditions five promising cultivars of apple were collected : 'Zimnica', 'Dobrokvaska', 'Kamyanka', 'Dimyanka' and 'Baracke'.

The former two cultivars have fruits with sweet-sour taste and the latter three are bearing sweet fruits with nice apple flavor. Only 'Dimyanka' is early autumn-harvesting cultivar. The rest are winter apples.

Observation of fruits in laboratory showed well developed wax layer onto the fruits and leaves. This covering forms a water-repellent surface and thereby prevent formation of a film

of water in which the germination of fungi or multiplication of bacteria may take place. Thick cuticules decrease mechanical damage during cultivation and harvesting procedures. Flesh density and juice content in ripe fruits are high in 'Zimnica', 'Kamyanka' and 'Dobrokvaska'. The inside tissues are less dense and juicy in 'Dimyanka' and 'Baracke'. Some biological and commercial properties of the cultivars are presented in Table 1.

Table 1. Properties important for commercial production in 5 local cultivars.

Cultivar	Mean weight of fruit, g	Severity of symptoms (grades)		
		Venturia inaequalis		*Podosphaera leucotricha*
		leaves	fruits	leaves
'Johnatan' (control)	121	2.6	1.8	2.8
'Zimnica'	125	0.2	0.2	0
'Kamyanka'	98	0.4	0.5	0.2
'Baracke'	76	0.5	0.5	0.1
'Dimyanka'	112	0.5	0.4	0.3
'Dobrokvaska'	104	0.4	0.3	0.2

Note: Grading system for reading of symptoms was according to Ryabow 1969 :

0 - no symptoms;
1 - few very small lesions;
2, 3, 4 - no more than 5, 25, 50% of leaf or fruit surface, respectively, is damaged;
5 - more than 50% of leaf or fruit surface showed infection symptoms.

Experiments showed that 55-63% of young plants bred from free pollination of these rural cultivars were resistant to apple scab and powdery mildew. As important genetic material, they should be used in breeding programs as well as their ancestors.

Plant resources onto south-western slopes of Carpathians should be studied more intensively with the aim of their preservation and utilization of promising genotypes.

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I like to express my gratitude to the rural people who helped during expeditions to collect plant material.

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Gene transfer in apple and pear : new strategies for control of fungal and bacterial diseases

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Abstract : Gene transfer offers an interesting alternative breeding method for pome fruits, as it permits a one-step integration of a gene conferring increased resistance into commercial varieties of known agronomical value. Today, both apple and pear are amenable to Agrobacterium-mediated gene transfer, and the number of transformed varieties and rootstocks is regularly increasing. The choice of appropriate transgenes and promoters is a key point for the success of this method. Already several teams have engaged gene transfer programs aiming at increasing scab or fire blight resistance in apple and pear. So far, the strategies under study have been mostly the use of lytic peptide genes (attacin, cecropin, lysozyme) to control fire blight, and antifungal proteins or chitinases to control scab. Transgenic plants are evaluated in greenhouse in several european countries, and field trials are already established in USA. These preliminary results are promising, but they also raise various types of questions. What will be the stability of transgene expression along the tree life ? Will the combined expression of several defense/resistance mechanisms prevent the capacity of adaptation of the pathogens ? Will the risk of adverse secondary effects (on human health or environment) be low enough to permit public acceptance of transgenic fruit varieties ? Adapted methods will have to be developed to answer these questions fully.

Key words : gene transfer, disease resistance, apple, pear

Introduction

Disease resistance is one of the main objectives of apple and pear breeding. Conventional breeding already released successful scab resistant apple varieties and multi-resistant varieties are in preparation. However, the recent discovery of new strains of scab overcoming the *Vf* gene highlighted the importance of combining several mechanisms to obtain durable resistance. Precise molecular markers will be necessary to achieve this complex objective. The developement of biotechnology methods for apple and pear started in the 1970's, with pioneer work about micropropagation. In the 1980's, several regeneration techniques from mature tissues were available, making possible the development of breeding oriented applications in the 1990's. Gene transfer is the more recent of these tools. It was achieved first on apple (James *et al.*, 1989) then in pear (Mourgues *et al.* 1996). It is a powerful method to study the effects of a precise gene sequence on plant-pathogen interactions. Furthermore, it offers an interesting alternative breeding method for pome fruits, as it permits a one-step integration of a gene conferring increased resistance into commercial varieties of known agronomical value.

Pome fruit gene transfer : state of the art

Apple and pear are natural hosts of *Agrobacterium tumefaciens*, causal agent of crown gall and natural vector of gene transfer. Thus, transformation of these two species is based on the co-culture of in vitro leaves with a disarmed *A. tumefaciens* strain carrying the genes of interest on a binary vector. Adventitious buds are then regenerated in presence of a selective

agent (kanamycin) in order to select for transformed cells carrying a kanamycine resistance gene. The process takes about one year, from the start of the experiment to the stage of acclimatized transgenic plants available for testing in greenhouse. The rate of transformation varies from 1 to 40 % according to the genotype. The number of transformed varieties and rootstocks is regularly increasing, for both apple and pear. However, improvement of the efficiency of transformation is still needed for some genotypes.

Genetic engineering strategies to improve resistance to fungal or bacterial diseases

Several types of approaches have been proposed for the rational creation of plant resistant to microbial diseases through genetic engineering (Lawton, 1997; Mourgues *et al.*, 1998; Salmeron & Vernooij, 1998). A direct lytic action on the pathogen can be achieved with transgenes encoding lytic peptides from insect origin (attacin, cecropin) or lysozyme from various origins. These broad-spectrum strategies can be particularly efficient when several antimicrobial genes are expressed in synergy. Inactivation of a toxin or synthesis of an insensitive target have already been used to produce transgenic plants resistant to bacterial diseases. Other strategies to inhibit microbial pathogenicity or virulence factors include the competition for nutrients essential for virulence, or the inhibition of products of avirulence genes by plantibodies. Enhancement of plant defense genes can be achieved with the overexpression of PR proteins (chitinases, glucanases) or phytolaexins (resveratrol). Modification of systemic acquired resistance signaling by overexpression of regulatory genes or enhanced production of reactive oxygen species can also lead to increased disease resistance. Finally, artificially programmed cell death can be based on the use of specific inducible promoters to control a localized hypersensitive reaction.

Already several teams have engaged gene transfer programs aiming at increasing scab or fire blight resistance in apple and pear. So far, the strategies under study to control fire blight have been mostly the use of lytic peptide genes such as attacin, cecropin and lysozyme (Norelli *et al.* 1998, Hanke *et al.*, 1998, Reynoird *et al.* 1999). Future approaches include the use of the harpin gene hrpN (Abdul-Kader *et al.*, 1998) and lactoferrin gene (Chevreau, pers.com.). Genetic engineering to control scab is based on the use of antifungal proteins (De Bondt *et al.*, 1998) or chitinases (Wong *et al.*, 1998). Transgenic plants are evaluated in greenhouse in several european countries, and field trials are already established in USA and have given promising results. In several cases, demonstration of a correlation between the increased resistance and the expression of the transgene is underway.

Unanswered questions about gene transfer for pome fruit breeding

Genetic engineering is still a very recent tool in the hands of pome fruit breeders, and various questions have to be answered before improved plant material can be proposed to growers. Durability of the resistance is a very important concern for long-lived crops such as fruit trees. Assessment of transgene expression patterns in apple seems to indicate a good stability until the adult stage (Yao *et al.*, 1999) but the risk of gene silencing along the tree life still has to be carefully tested. The ability of the pathogen to overcome the novel resistance varies very much with the specificity of the mechanism involved and the combined expression of different transgenes can decrease the risk of multiple mutations in the pathogen.

Biosafety assessment will be an important step for the development of any transgenic variety. Non-target effects on friendly microorganisms in the environment is an important question which has been only rarely addressed so far (Heuer & Smalla, 1999). The risk of gene flow to related species is probably quite limited in the case of apple or pear, and its possible consequences on the invasiveness of these two species does not appear as a major concern. More important is the risk of increased allergenicity or toxicity to human consumers. Eventhough some of the transgenic proteins currently studied already belong to the human

diet (lactoferrin, puroindolines), a thorough evaluation of the toxicity of proteins from insect or microorganism origin is necessary. The use of promoters driving precisely the expression of the transgene only in case of pathogen infection would also reduce the amount of transgenic protein in the consumed fruits.

Practical use of transgenic fruit varieties will not be possible without general public acceptance. A recent survey indicates that public perception of the use of modern biotechnology for food production is much more negative in Europe than in USA (Gaskell *et al.*, 1999). Clear and balanced information is necessary to explain how genetically modified varieties can help to reduce fruit production dependence on chemical inputs, without increasing environmental or health risks.

Conclusion

Much progress has been made in the field of genetic engineering of apple and pear in the last ten years, and pome fruit species can no more be considered as recalcitrant species. This new breeding method has already produced original plant genetic material which is currently beeing evaluated for disease resistance. Future requirements for practical success include : accurate choice of transgenes and promoters, use of efficient early screening tests, thorough field evaluation of the material, serious risk assessment studies and clear and objective communication.

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Increased resistance to scab (*Venturia inaequalis*) of transgenic apple plants expressing chitinases from *Trichoderma*

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Abstract : Our goal is to genetically engineer commercially important apple cultivars for resistance to apple scab (*Venturia inaequalis*). Chitinolytic enzymes from the biological control organism *Trichoderma harzianium* have in vitro activity against at least two fungi pathogenic to apple, including *V. inaequalis* and *Gymnosporangium juniperi-virginianae* (cedar apple rust).

cDNA clones of *T. harzianium* endochitinase (Ech42) and exochitinase (N-acetyl-β -D-glucosaminidase [Nag1]) genes were cloned singly and in combination into plasmid binary vectors under the control of the enhanced CaMV 35S promoter. Constructs were made of the chitinase genes with a translational enhancer sequence from alfalfa mosaic virus, their native signal peptide sequence to export the enzymes extracellularly, and nptII as selectable marker. The constructs were transferred into 'Marshall McIntosh' apple cultivar by *Agrobacterium*-mediated transformation, and transgenics were identified by PCR, ELISA for NPT II protein, and Southern analysis. Chitinase expression was quantified by a methylumbelliferone enzymatic assay and by western analysis. Own-rooted transgenic plants were inoculated with a suspension of *V. inaequalis* conidia, incubated in a mist chamber (18± 1°C and 100% relative humidity) for 48 h and later moved to a growth chamber or greenhouse. Scab resistance was evaluated based upon number of sporulating lesions per leaf, the percentage of leaf area infected, and the number of conidia rinsed off per leaf.

Level of endochitinase expression in transgenic plants was significantly correlated with scab resistance level, and also with the degree of growth reduction of transgenic lines. Level of exochitinase expression was also significantly correlated with scab resistance level, although resistance of exochitinase-transgenic lines was less than of endochitinase-transgenic lines. However exochitinase had no effect on plant growth. When both enzymes were expressed together in transgenic plants, they acted synergistically to reduce scab infection. Certain McIntosh lines transgenic for both genes that were selected for a low level of endochitinase expression and a high level of exochitinase expression were highly resistant to scab and had negligible reduction in growth in a greenhouse trial. These lines are now being grown in the field as own-rooted plants for evaluation of scab resistance, and are being propagated on M.9 rootstock for evaluation of tree performance, and fruit quality and yield.

Key words : disease resistance, genetic engineering, apple, scab

Introduction

The objective of this research is to genetically engineer commercially important apple cultivars for resistance to apple scab, which is caused by *Venturia inaequalis*. Chitinolytic enzymes from the biological control organism *Trichoderma harzianum* have *in vitro* activity against several plant pathogenic fungi [Lorito *et al.*, 1998], including *Venturia inaequalis*. This paper reports the effect of the *T. harzianum* endochitinase and exochitinase (N-acetyl-β-D-glucosaminidase) on resistance to scab when expressed in transgenic apple. A preliminary report of increased scab resistance of endochitinase-transgenic Royal Gala has been published [Wong *et al.*, 1999].

Materials and Methods

Both cDNA and genomic clones of *T. harzianum* endochitinase (*Ech42*) and exochitinase (N-acetyl-β-D-glucosaminidase [*Nag1*]) genes were cloned either singly, or in combination, into plasmid binary vectors under the control of the enhanced cauliflower mosaic virus 35S promoter. Constructs were made of the chitinase genes with a translational enhancer sequence from alfalfa mosaic virus, their native signal peptide sequence to direct the enzymes to intercellular space, and *npt*II as a selectable marker. The chitinase constructs were transferred into the apple cultivar Marshall McIntosh by *Agrobacterium*-mediated transformation [Bolar *et al.*, 1999], and transgenics were identified by PCR, ELISA for NPT II protein, and Southern analysis. The level of chitinase expression was measured by a methylumbelliferyl enzymatic assay and by western analysis. Own-rooted transgenic plants were inoculated with a conidial suspension of *V. inaequalis,* incubated in a mist chamber (18± 1°C and 100% relative humidity) for 48 h and later moved to a growth chamber or greenhouse. Scab resistance was evaluated based upon number of sporulating lesions, the percentage of leaf area infected, and the number of conidia recovered by rinsing leaves.

Results

The expression of endochitinase in transgenic apple resulted in high levels of scab resistance but also reduced plant growth. There was also a significant correlation between the level of exochitinase expression and scab resistance. The levels of resistance observed in exochitinase-transgenic lines were less than in endochitinase-transgenic lines, but exochitinase had no effect on plant growth. When both enzymes were expressed *in planta* they acted synergistically to reduce disease. Transgenic McIntosh lines were identified with a low level of endochitinase expression and a high level of exochitinase expression that were resistant to scab and had negligible reduction in growth in a greenhouse trial.

Discussion

The chitinase-transgenic Marshall McIntosh plants are currently being evaluated under field conditions for scab resistance, tree performance, and fruit quality and yield. To avoid the negative effects of endochitinase expression on apple growth we are exploring the utility of other cell wall degrading enzymes and the development of pathogen-specific expression of the endochitinase. The glucanase gene of *Trichoderma harzianum* is being cloned and will be evaluated for its effect on apple scab resistance, both singly and in combination with exochitinase. Various promoters are being evaluated in apple for induction by *V. inaequalis* to allow for expression of endochitinase only under conditions of pathogen invasion.

Acknowledgments

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Field trials of resistance of lytic protein transgenic lines of Royal Gala apple to *Erwinia amylovora* (fire blight)

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Abstract : The genes encoding the lytic proteins (LP) attacin E (att), hen eggwhite lysozyme (HEWL), and the cecropin analogs, SB-37 and Shiva-1 were transferred to 'Royal Gala' apple by *Agrobacterium*-mediated transformation. In 1998, 2-yr-old plants of 64 LP-transgenic lines grafted onto seedling rootstock growing in the field at Geneva, NY were artificially inoculated by bisecting the two youngest leaves of vigorously growing shoots with scissors that were dipped in 5 X 10^7 cfu/ml of *E. amylovora* strain Ea273. Three to five shoots were inoculated per plant on one to nine plants of each transgenic line, planted in a completely randomized design. Eight weeks after inoculation the length of the necrotic lesion was recorded and expressed as the % of the current season's shoot length (SLB) as a measure of resistance.

Twenty-one transgenic lines had significantly lower SLB than the inoculated non-transgenic 'Royal Gala' control. One transgenic line, TG138, containing the attacin E gene under the control of the proteinase inhibitor II promoter (pin), had only 5% SLB compared with 56% SLB in non-transgenic 'Royal Gala' controls, and 37% SLB in the resistant control cv 'Liberty'. Western analysis indicated that TG138 had a high constitutive level of attacin expression. Northern analysis of *in vitro* material confirmed the constitutive expression of attacin in TG138, but indicated elevated attacin expression 1 hr following leaf wounding. Another pin-att transgenic line, three 35S-att lines, and one 35S-HEWL line had significantly lower SLB than non-transgenic 'Royal Gala'.

Among 32 cecropin-transgenic lines, seven pin-SB37-trangenic lines, two 35S-SB37-transgenic lines, and one transgenic control line without any LP gene (non-LP), were significantly more resistant than non-transgenic 'Royal Gala'. The resistance of these 10 transgenic lines was statistically indistinguishable from that of resistant-control cv 'Liberty'. The fire blight resistance evaluation was repeated in 1999 with similar results.

The first flowers on LP-transgenic 'Royal Gala' plants were observed in the field in 1998, and included attacin and HEWL transgenics. A single fruit of the HEWL-transgenic line, TG211, which had shown increased fire blight resistance in a greenhouse test, appeared phenotypically indistinguishable from non-transgenic 'Royal Gala'. In 1999 several attacin lines flowered and were pollinated to produce fruit for studies of LP-gene expression, fruit quality, and cosegregation of resistance with the attacin transgene.

Key words : disease resistance, genetic engineering, apple, fire blight

Introduction

The introduction of lytic protein genes into plants by genetic engineering have been reported to enhance resistance to phytopathogenic bacteria [Düring *et al.* 1993; Jaynes *et al.*, 1987; Mourgues *et al.*, 1998; Norelli, *et al.*, 1994; Reynoird *et al.*, 1999]. However, there are few reports on the field performance of these lytic protein-transgenic plants. Here we report on the fire blight resistance of attacin E, hen egg white lysozyme, and cecropin-like transgenic Royal Gala under field conditions.

Materials and Methods

Genes encoding the lytic proteins were transferred to Royal Gala apple by *Agrobacterium*-mediated transformation (Table 1). Transgenic lines and control plants were grafted onto seedling rootstock, and planted on the Loomis Research Farm, NYSAES, Cornell University, Geneva, NY in 1996 (3-yr-old) and 1997 (2-yr-old). 5 to 10 plants of each line were planted in a completely randomized design. Plants were inoculated on 4 June 1998. Vigorously growing shoot-tips were inoculated by cutting the 2 youngest leaves of the shoot with scissors that were dipped in 5 X 10^7 cfu/ml of *E. amylovora* strain Ea273. 8 weeks after inoculation the length of the necrotic lesion was recorded and expressed as the % of the current season's shoot length as a measure of resistance. 3 to 5 shoots were inoculated per plant on 1 to 9 plants of each transgenic line, planted in a completely randomized design. Individual inoculated shoots were the unit of replication.

Table 1. Plasmids Used to Transform 'Gala' Apple for Fire Blight Resistance

Plasmid	Promoter	Signal peptide	Lytic Protein
pWIAtt	Pin	none	attacin E
pCa2Att	35S	none	attacin E
pCa2Chly	35S	native	hen egg white lysozyme
pWIC38	Pin	none	cecropin SB-37
pCa2C38	35S	none	cecropin SB-37
pBPRB37	35S	SP1	cecropin SB-37
pBCCB37	35S	SCC	cecropin SB-37
pBPRB37	35S	SP1	cecropin Shiva-1

Plasmid = *Agrobacterium tumefaciens* binary vector. Lytic protein genes were cloned into the *Hin*dIII site of pBI121 vector containing a neomycin phosphoryl transferase (*nptII*) marker gene for kanamycin resistance and a ß-glucuronidase (*uidA*) reporter gene.
Pin = wound inducible promoter of proteinase inhibitor II gene of potato.
35S = CaMV constitutive promoter with duplicated upstream sequence.
SP1 = signal peptide sequence of PR1b protein of tobacco.
SCC = signal peptide sequence of native cecropin B from *Hyalophora cecropia.*

Results and Discussion

In a field trial of 2- and 3-yr-old plants, 23 lytic protein transgenic lines were identified that were significantly more resistant than their non-transformed Royal Gala parent. These included 5 attacin E, 15 cecropin SB-37 and 3 hen egg white lysozyme transgenic lines. In addition, 3 pBI121 transgenic lines (vector controls) were significantly more resistant than Royal Gala.

TG138, an attacin transgenic line, was significantly more resistant than other lytic protein transgenics and expressed the greatest amount of attacin protein. Nevertheless, when several attacin transgenic lines were analyzed there was no statistically significant correlation between resistance and the level of attacin expression. Northern analysis indicated that one hour after wounding *in vitro* grown plants of T138, the level of attacin m-RNA increased in comparison to that of elongation factor, which is constitutively expressed. In contrast to the northern

results, western analysis of the same plant material indicated that attacin protein was expressed in non-wounded plants at a high level and an increase in protein levels in response to wounding could not be detected.

Of 13 transgenic lines that were included in both the 2-yr-old and 3-yr-old trials, 9 lines performed similarly in both trials. The performance of 1 pBI121 and 3 cecropin SB-37 transgenic lines was inconsistent between trials [Norelli *et al.*, 1999]. The same plant material was inoculated again in June 1999 with broadly similar results.

The similar level of resistance found in certain lytic protein and vector transgenic lines and the lack of significant correlation between detectable attacin and field resistance suggests that biological variation, either random or somaclonal, may contribute to the observed resistance. Now that transgenic lines have fruited, progeny analysis from crosses will allow conclusive determination of the role of these transgenes in resistance.

The gene encoding T4 lysozyme is also being evaluated, expressed either singly or in combination with attacin E, for its effect in apple on resistance to *Erwinia amylovora* [Hanke, *et al.*, 1999, Ko *et al.*, 1999]. The alfalfa mosaic virus leader sequence is being evaluated for its effect on gene expression, and a signal peptide sequence is being evaluated for its ability to target antimicrobial proteins to the intercellular space [Ko *et al.*, 1999]. The *hrpN* gene of *E. amylovora* is also being evaluated for its effect on fire blight resistance in M.26 apple rootstock [Abdul-Kader *et al.*, 1999; Borejsza-Wysocka *et al.*, 1999].

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Possible errors in genome mapping

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Abstract : For the mapping of agronomically interesting major genes or quantitative trait loci (QTLs), well saturated genetic maps are required. Because backcrossing is not feasible with apples, due to the long juvenile period and the self incompatibility, genetic analysis is performed in a full-sib progeny of a single cross and results in two parental maps. As markers Isozymes, AFLPs, RFLPs, RAPDs and Microsatellites can be used. To construct maps with accurate marker location and consistent marker sequence for the alignment of corresponding chromosomes, it is important to use a sufficiently large mapping population, to use markers of good quality and to understand the mapping software (*i.e* JoinMap ™). All individuals must be screened with easy to score and highly reproducible markers. Their segregation must follow a mendelian pattern and the recombination frequencies between the markers must be in accordance with all other markers. Because it is not easy to detect markers of low quality it is necessary to understand the mapping procedure so the results can be interpreted and the low quality markers identified. In this paper, the most common problems will be discussed.

Key words : mapping, apple, JoinMap™

Introduction

Genetic analysis and genome mapping of animals and plants are promising and popular research areas (Park and Lewin 1997; Paterson 1996). In addition to the desire to understand the function and the expression of genetic information it is a goal to evaluate traits of economic importance.

In the field of plant breeding the object is mainly to select plants with desired properties. This was and is done, using the traditional breeding and selection processes, based on phenotypical characteristics. However, the use of genomic maps and molecular markers has certain advantages over the methods of traditional breeding: a) Markers associated with genes, coding for agronomically and economically interesting characters allow an early selection for these traits. This is potentially useful in perennial plants with a long juvenile phase and characters expressed only after years like mildew resistance or fruit quality in apple. Years of costly field maintenance can be avoided; b) These markers deliver clear qualitative answers and are not subject to environmental variability. c) Pyramiding of resistance genes is only possible with the aid of markers associated with the genes to be combined. d) For the detection and the analysis of QTLs we have to rely also on genetic maps, molecular markers and their qualitative answers since the absence or presence of one single factor of the quantitative trait cannot be determined phenotypically.

Furthermore the localization of genes for map based cloning and transgenic plants is possible and we gain general knowledge about genome structures and evolution.

Genome mapping is possible because of crossing over events during meiosis, where recombinations between homologous chromosomes occur. This leads to the dissociation of markers in the progeny which were associated in the parents. The number of recombinations serves as a measure for the distance between two markers on the parental chromosome. This *genetic distance* is measured in centiMorgan (1cM = 1 recombinant in 100 individuals) and

represents the probability of a crossing over between the markers rather than a physical distance in base pairs.

Because backcross is not feasible in apple due to the long juvenile period and the self incompatibility, genetic analysis is generally performed in a full sib progeny of a single cross. Various types of markers are being used in studying apple genetics and constructing genetic maps: Isozyme markers (Manganaris *et al.* 1987), RFLP markers (Maliepaard *et al.* 1998) RAPD markers (Koller *et al.* 1994, Conner *et al.* 1997), AFLP markers (Maliepaard *et al.* 1998), SSR markers (microsatellites) (Gianfranceschi *et al.* 1998, Guilford *et al.* 1997).

The advantages of genetic maps and molecular markers over the traditional selection methods can only be utilized when reliable maps with accurate marker and gene locations are at hand. On reliable maps, the sequence of the markers on the chromosomes and the distances between them are stable. If more markers are added or some are taken away, no major changes occur (Fig.1). Additionally, markers on homologous chromosomes in different varieties have the same order and the chromosomes can be aligned easily (Fig. 2).

To increase the reliability of the maps, sources of errors must be known and the problems should be eliminated. In this paper we report possible errors that occur and may remain unnoticed, and we discuss the possibilities to eliminate them.

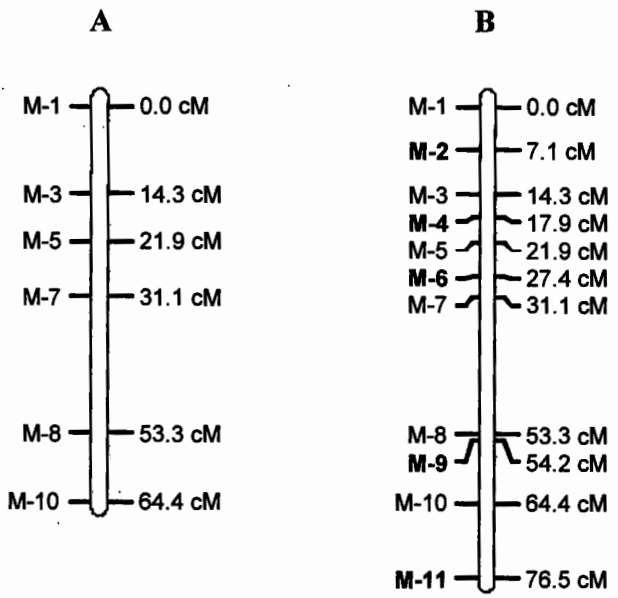

Figure 1. A : One linkage group (chromosome) of a reliable map consisting of 6 markers B : The same linkage group, recalculated with 5 additional markers. No changes in distance and marker sequence occurred. M-# represent the marker locations, distances are noted in centiMorgan.

Materials and Methods

The progeny of a cross between the two cultivars Fiesta and Discovery was used to construct a genetic map. This population was created for the detection and analysis of QTL resistance against scab and mildew.

220 dominant RAPD markers and 66 codominant microsatellites were tested so far on 120 individuals (out of 330) of this progeny. DNA extraction and marker reactions were performed as described by (Gianfranceschi *et al.*, 1996, 1998). Dominant markers were only used when they were heterozygous present in one parent and homozygous absent in the other parent as it would be the case in a backcross.

For each marker the genotype of every individual was determined and the markers were split into two groups each containing only markers which are informative for the respective parent. The lists of markers and genotypes were fed to the computer program JoinMap ™ (Stam and Van Oojen 1995) which performed all data analysis according to the thresholds set by the user. Markers segregating together were considered to be linked and were assigned to linkage groups (grouping). These groups should correspond to the chromosomes. Within every linkage group the recombination frequencies between all marker pairs were calculated (rec calculation) which lead to the genetic distances between the markers on the map. In the last step of the mapping process, the markers were arranged in the linkage groups according to their recombination frequencies (map assembly). This data analysis resulted in two parental maps.

In order to identify elements affecting the output maps quality, maps were constructed consisting of 40, 80 and 100 individuals. The reproducibility and the scorability of the markers was determined as well as their segregation pattern and their recombination frequencies with other markers. Using the software - JoinMap ™- for the map calculation, a wide range of threshold values was utilized and the different output maps were compared.

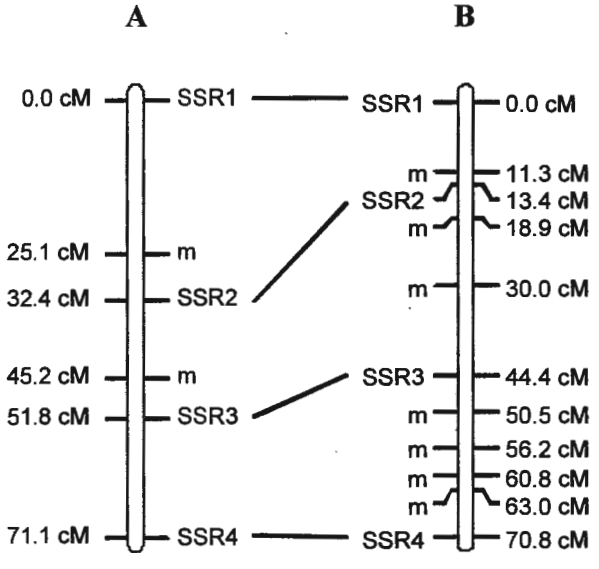

Figure 2. Properly aligned homologous chromosomes of the varieties Fiesta (A) and Discovery (B). SSR# represent microsatellite markers being present in both cultivars, m representing dominant RAPD markers, distances are noted in centiMorgan.

Results and Discussion

Output maps were considered to be reliable and accepted only when adding or removing markers did not result in changes of marker sequences or the distances between them, when markers on homologous chromosomes in different varieties had the same order and the chromosomes could be aligned easily (Fig. 2 and Fig. 6).

Problems affecting the output maps quality occurred on different levels in the mapping process. The problems were not necessarily obvious but the resulting maps showed the possible errors.

Population

On the level of the mapping population, errors arose from the number of individuals. If the mapping population was too small, the calculated statistical values were not reliable which resulted in a low quality map. With subsets of 40 individuals, all originating from the same population, maps were constructed. Some chromosomes of the maps showed completely different marker sequences, indicating the low map quality (Fig. 3). This explains to some extent the different findings of other authors, using various numbers of individuals for the mapping of the same genomic region (Gessler *et al.* 1995, Hemmat *et al.* 1998, Gardiner *et al.* 1996, Tartarini 1996). A minimal population size could not yet be determined, since only 120 individuals were tested.

A large number of outcrosses in the population might also cause problems. But they were easily detected with microsatellite markers and could be excluded from the analysis (Fig. 4).

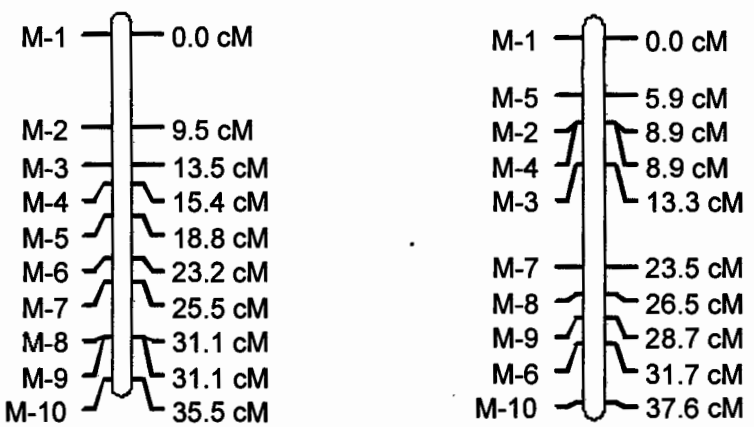

Figure 3. Using subsets of only 40, randomly picked individuals from the same population, resulted in complete different marker sequences. M-# represent the marker locations, distances are noted in centiMorgan.

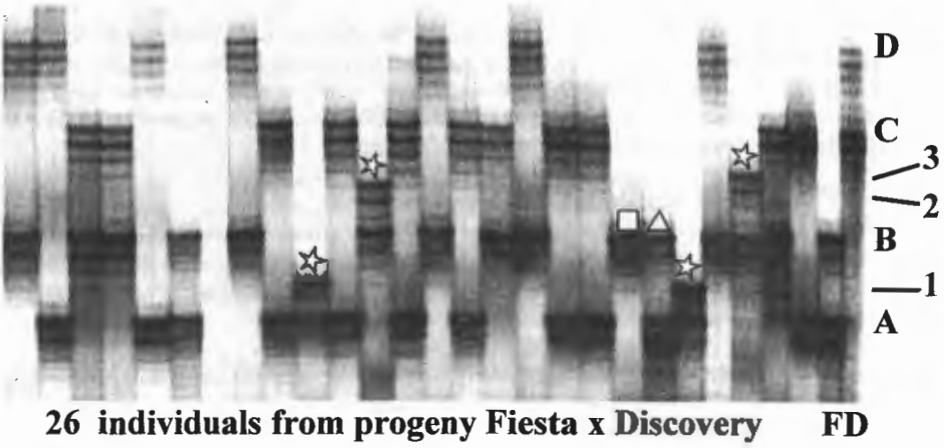

Figure 4. Microsatellite marker reaction on 26 individuals of a progeny and the parents (Fiesta, Discovery). A, B C, D representing the parental alleles, inherited to the progeny. 1, 2, 3 representing non parental alleles in the individuals marked with ☆ (outcrosses). Other irregular genotypes which do not originate from a cross between F and D are marked with △ (parental genotype, self) and □ (homozygous for one parental allele)

Markers

Far more problems occurred on the level of markers. Almost all inconsistencies could be tracked back to single problematic markers.

To construct maps with accurate marker locations and consistent marker sequences a certain number of markers with good quality is required. The number of markers only affects the map density and does not lead to serious errors.

The intrinsic quality of the markers is dependent on their reproducibility and their scorability. Markers based on specific primers like SSRs are generally more reliable and easier to score than those originating from unspecific primers like RAPDs. Therefore the chances of false scorings were smaller with microsatellites.

Moreover there are other, less evident factors of quality like the segregation ratio of the markers. Certain markers deviated strongly from the expected 1:1 or 1:1:1:1 segregation ratio. These distortions often lead to problems with the marker arrangement on the chromosome. However, this was only the case if only one marker was distorted. If an entire set of linked markers showed corresponding distortion, no problems occurred.

Reproducibility, scorability and distortion were checked for every single marker without big efforts. For example in the linkage group of Fig. 5, the single markers analysis showed a heavy distortion of marker M-5. It was not discarded because it might have been the only present marker of a distorted set not causing any problems.

Nevertheless there are criteria where the quality can be determined only in conjunction with other markers. Some markers showed recombination frequencies which were not in accordance with the other markers in the group. Some markers were only linked to few others in the group while those few others were tightly linked to the rest. Such markers could only be identified by detailed analysis of the results produced by the mapping software.

Most problems were detected during or after the map assembly, the core task of the program, where the markers were arranged on the chromosome. Starting with the statistically best assured marker pair of a linkage group, the program assembles a map by adding one by one marker. After each marker a goodness-of-fit measure is calculated upon which the decision is made whether the marker fits or not (Stam and Van Ooijen 1995). As a result of this strategy, three mapping cycles may be performed :

1st cycle Only well fitting markers are positioned.
2nd cycle Markers put aside in the 1st cycle are given a second chance. They might fit well after a number of other markers have been placed on the map.
3rd cycle All markers are forced on the map, even the ones that do not fit, neglecting the user supplied thresholds.

While the maps after the 1st and the 2nd round were often the same and met the criteria for good maps, the 3rd round maps needed some attention. Here the problematic markers caused considerable differences between recombination frequencies and map distances, as well as major rearrangements in the marker sequences on the linkage groups. If the non fitting markers were identified and eliminated, stable maps resulted (Fig. 5). Although the markers put on the map only in the 3rd cycle were not always the problematic ones. Sometimes low quality markers were already placed in the 2nd round and prevented the positioning of the remaining markers.

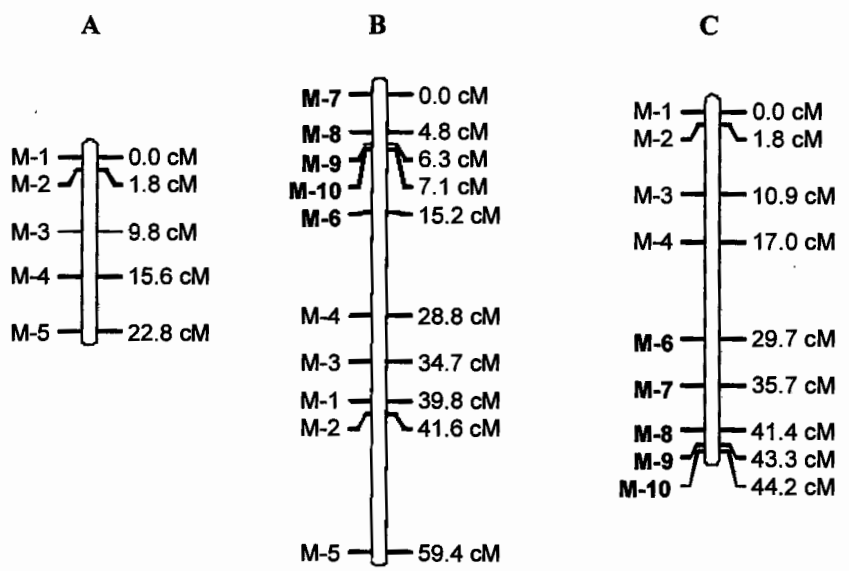

Figure 5. A : Linkage group after the 2nd mapping cycle. B : Same linkage group after the 3rd mapping cycle, with all markers forced on the map. C : Linkage group recalculated after Marker M-5 (distorted) was removed.

Markers causing any of the above problems were excluded from the analysis. Conscious of the fact that the exclusion of certain markers prevented the mapping of their genomic

region, we discarded those markers because it is far more useful to cover a possibly smaller part of the genome with the map but with higher accuracy.

Once no more bad markers could be identified, the comparison between homologous chromosomes from different varieties either proved the quality of the map or identified linkage groups which contained markers causing differences in corresponding marker sequences (Fig. 6). If the latter was the case, it was necessary to repeatedly exclude one by one single marker and recalculate the maps to identify the non fitting ones.

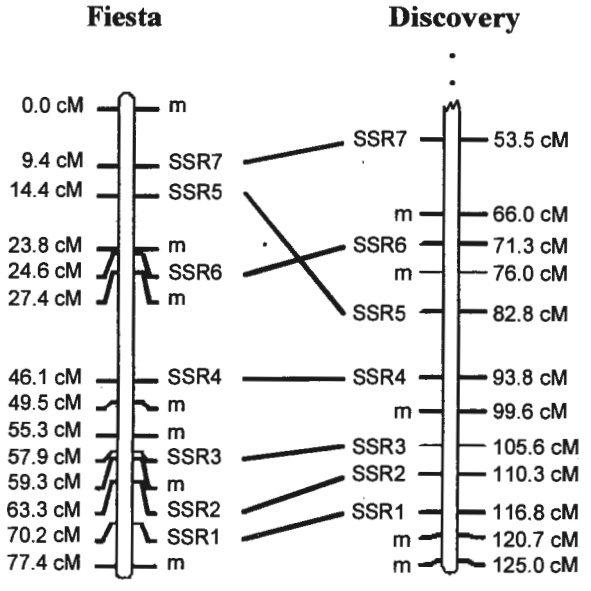

Figure 6. Homologous linkage groups from Fiesta and Discovery which cannot be aligned correctly due to inconsistent marker sequences. Marker SSR 5 showed heavy distortion and was excluded from the analysis. SSR# representing Microsatellite markers being present in both cultivars, m representing dominant RAPD markers being informative only for one parent. The reason for different linkage group lengths is most likely the incompleteness of the Fiesta map.

Thresholds

Setting the thresholds in the mapping software can be considered as a third level leading to possible errors, although this is closely connected with the marker quality. With a set of perfect markers, evenly distributed over the entire genome, the maps produced will remain the same over a wide range of settings. Since this is hardly ever the case, differences in grouping the markers (first step of the program) and rearrangements of marker sequences occur in the map assembly step when changing the thresholds for the different tasks.

Among the many statistical values and user defined thresholds used by JoinMap™, two have a major importance: The REC value which represents the recombination frequency between a pair of markers and the LOD value which serves as a statistical assurance for the REC value (Stam and Van Ooijen 1995).

Being very restrictive in the first mapping step (*i.e.* using a high LOD for the assignment to linkage groups), only tight linkages are considered whereas with a low LOD threshold the markers tend to stick together which results in too many or too few linkage groups respectively. Raising the REC threshold in the map assembly step might also lead to the consideration only of tight linkages, while certain data is neglected. Different maps are assembled when all information is used as it is the case with low REC thresholds (Fig. 7).

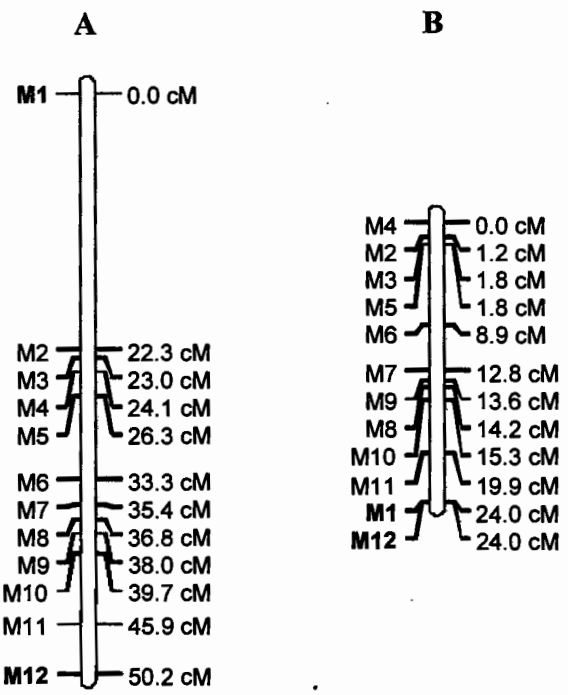

Figure 7: Two maps calculated with the same set of data. A: Low restrictions for the map assembly, all available data were used. B: High restrictions, only tight linkages were considered. Note the different sequences and the positions of M1 and M12: M1 is only linked to few other markers in the group. If the restrictions are high, the weak linkages to most of the markers are neglected and M1 is positioned according to the newly available data. Marker M1 showed heavy distortion and was excluded in the final analysis.

Conclusion

Since the errors occurring during the construction of genetic maps are not recognized at first sight, it is important to understand the tasks performed by the computer program. This enables the user to critically analyze the output results and makes aware of the possible quality of the map.

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Incidence of powdery mildew (*Podosphaera leucotricha*) on scab resistant apple cultivars over different years and places

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Abstract : Mildew incidence of several scab resistant cultivars was evaluated from 1996 to 1998 at four different sites, in plots untreated with fungicides. While in the first observation year hardly any incidence was found, it increased in the following years. With statistical analysis of variances using site, year and cultivar as influence factors, the sites could be distinguished according to their mildew pressure level in two sites each with a lower and a higher level. Significant interactions between cultivar and site as well as cultivar and year underline the importance to observe cultivars at different locations and during different years. There are clear differences of mildew incidence between the observed cultivars. The 75th percentile as a more conservative comparison criteria than the median was used for characterization of the individual mildew susceptibility. Also the maximal values are considered in the qualification as an indicator for the risk potential of each cultivar under conditions of high mildew pressure. The mildew susceptiblity of the cultivars Reanda, Retina, Ariwa, Baujade, Prima, Rewena, Rubinola, Topaz and Boskoop is low with their 75th percentile not exceeding 10% of infected leaves and maximal values under 30% and can probably be handled with an extensive plant protection schedule. Some cultivars like Resi, Angold, Saturn, Rosana with a medium susceptibility can be problematic in organic farming. Delorina and Resista are highly mildew susceptible. The high potential of cultivars with a mildew resistance (Ariwa, Reanda and Rewena) under conditions of strong mildew pressure can be shown, when even relatively "tolerant" varieties can become seriously mildew infected. Thus breeding for mildew resistance remains an important objective, as only few resistant cultivars are available yet.

Keywords : Apple, powdery mildew, Podosphaera leucotricha, scab resistant cultivars, resistance

Introduction

Many apple cultivars resistant to scab (*Venturia inaequalis*) are available nowadays. Only a few of them also carry resistance towards powdery mildew (*Podosphaera leucotricha*). Mildew control is very important on young trees, as they are hampered irreversibly in their growth by severe mildew incidence. Apart from improving the durability of scab resistance, breeding also aims to increase resistance to mildew by integrating monogenic as well as polygenic resistance sources into new cultivars (Evans, 1997). The necessity for several fungicide applications to control mildew even on scab resistant cultivars is another reason to increase the level of mildew resistance. The screening of resistance can be accelerated by using molecular markers to detect the absence or presence of resistance and to define a certain resistance source in breeding populations at an early stage (Gianfranceschi *et al.*, 1998). However, until today only major genes such as Pl_1 (Markussen *et al.*, 1995) and Pl_2 (Seglias *et al.*, 1997) are mapped and can be used in marker assisted breeding. Quantitative differences in mildew susceptibility between the various scab resistant cultivars attributed to QTL-effects (= Quantitative Trait Loci) could be exploited, first in the choice of parents and secondly to

identify QTL (Seglias *et al.*, 1997). As the mildew pathogen is highly variable under various environmental conditions and the effect of QTL can also contribute to the variability, we assessed relative mildew susceptibility of scab resistant cultivars at different sites and on different dates, in order to achieve a realistic estimation of cultivar susceptibility under Swiss growing conditions.

Material and Methods

Of a number of scab resistant or partially scab resistant cultivars the incidence of powdery mildew was evaluated at four sites during 1996, 1997 and 1998. We monitored 100 leaves of every observed tree for presence or absence of mildew symptoms. The branches were randomly chosen from different sectors of the trees. Primary as well as secondary mildew symptoms were assessed. In this study only the secondary symptoms have been evaluated as primary symptoms were assessed in different ways between the two research institutes involved. Evaluation dates varied between the years and sites (table 1). After mildew incidence was assessed, shoots with primary infection symptoms were cut.

Table 1. Location of plots and dates of annual assessments

Plot	Location	Av. of annual rain-fall and temperature	First leaf	Dates of assessment		
				1996	1997	1998
Wädenswil	beside the lake Zürich	1365mm - 9°C	1996	7.8.	9.7.	30.7.
Güttingen	beside the lake Constance	940mm - 7.7°C	1993	16.8.	10.7.	24.6.
Grabs	near Buchs, to Austrian border	1200mm	1996	17.8.	16.7.	22.7.
Oberwil	near Basel	792mm - 9°C	1995	17.9.	21.9.	19.9.

Description of the plots

The choice of cultivars planted and the number of trees per cultivar (4 and 8 trees, respectively) varied between plots. With the exception of Ariwa (Pl_1), Rewena and Reanda the cultivars do not carry a mildew resistance. In the observation years the plots were not treated with fungicides.

Güttingen

This plot contains 32 scab resistant cultivars on rootstock (M9 vf [= virus free]), planted in two blocks, one treated and the other not treated with fungicides. Only the untreated block is included in this study. The trees were planted in 1993. From 1993 to 1995 the whole plot had been treated with fungicide and had been fertilised with a relatively high level of nitrogen to overcome a certain replant depression. Eight trees per cultivar were assessed, arranged in two groups of four trees.

Wädenswil

This plot is arranged in the same way as the plot at Güttingen, except for the terminal row where we had the chance to plant the new Czech cultivars Topaz, Rajka, Rubinola and Rosana. Planting year was 1996, when these cultivars were brandnew and interested the growers very much. Unfortunately the number of trees of these cultivars is small (only four to

six) and repetitions at other places within the plot were not possible. In this plot 16 scab resistant cultivars were planted on the rootstock Lancep M9.

Table 2. Mildew incidence at the 4 sites in % infected leaves. At Güttingen and Oberwil the natural infection pressure with mildew was high, at Wädenswil and Grabs it was low

		high level of mildew pressure						low level of mildew pressure				
		Güttingen means			Oberwil means			Wädenswil means		Grabs means		
	resistances**	1996	1997	1998	1996	1997	1998	1997	1998	1996	1997	1998
Angold	VA	1.6	12.6	13.1	15.5	12.8	30.5					
Ariwa	Vf+Pl_1	0.0	6.4	5.5	9.5	5.8	7.8	0	0.6	0	1	0.3
Baujade	Vf	0.0	4.0	9.6	2.8	5.0	25.0					
Boskoop	-				4.8	14.0	19.5					
Codel	Vf	2.2	20.6	**49.0**	**18.8**	35.5	**49.0**					
Constanze	Vf							3.5	7.2			
Delgollune	-				7.5	**15.8**	20.8					
Delorina	Vf	**9.4***	**21.6**	40.0	24.3	**38.5**	43.3			**6.1**	**35.3**	**21.8**
Ecolette	Vf	0.0	11.6	14.3								
FAW 7207	Vf	0.0	4.0	8.3	1.5	25.5	15.4					
Prima	Vf				1.0	0.5	3.5	0	0.4			
Rajka	Vf							3.5	0.3			
Reanda	Vf+MR	0.0	0.5	1.5	6.5	9.3	10.5					
Renora	Vf	0.0	7.0	3.5	2.3	12.8	17.3					
Resi	Vf	0.1	2.5	3.4	2.5	5.0	37.3					
Resista	Vf	0.0	20.6	41.1								
Retina	Vf	0.0	2.8	9.1	5.0	11.5	9.5					
Rewena	Vf+MR	0.0	7.1	1.0	2.5	7.1	4.5	0.4	0.6			
Rosana	Vf				4.3	16.3	13.8	1.7	0.7			
Rubinola	Vf				3.8	1.3	5.0	**6**	0			
Saturn	Vf	0.0	9.8	11.0	3.7	11.5	16.0					
Topaz	Vf							0	1	0	0	1.3
Otava	Vf				3.0	3.0	17.8			3.7	2.8	2.5
Florina	Vf	1.3	13.5	26.4	16.0	12.0	21.8	0.1	2.3	2.5	4.5	2.8

** VA, Vf are scab resistances, Pl_1 is a mildew resistance, MR is mildew resistance of an unknown source

* bold numbers represent the highest annual values

Oberwil

This plot is managed by the Research Institute of Organic Agriculture (FiBL) under the guidelines of certified Organic Farming. Thirty two new scab resistant cultivars or selections were planted. Boskoop and Glockenapfel serve as comparison standards. Rootstock is EMLA (M9 vf). Per cultivar 8 trees are randomly distributed over two blocks and 16 rows, the treatments are randomly split over the rows.

Grabs
This is a small plot with one row of 14 different cultivars. It was included because Otava and Topaz are present only here and in one other plot. Four trees of every cultivar on rootstock T338 (M9 vf) were planted in 1996. It is a mixture of scab susceptible and resistant cultivars. In our study the cultivars Topaz, Florina, Ariwa, Delorina and Otava were included.

Evaluation method and statistical analysis
For evaluation the cultivars were assigned to three groups according to the number of sites at which they were evaluated and the total number of observed trees. The statistical analysis was performed with "JMP" software (SAS Institute, Carry (NY)). With the data of Florina and Ariwa a complete three factor analysis of variance could be performed including all four plots and two observation years. The cultivars, which were not present at all four sites, were distributed in two comparison groups. In one group all the cultivars planted in at least two plots are gathered. The other group contains the cultivars standing in only one plot or, although being present in two plots, having only a few trees. Analysis of variance (ANOVA) and tests for normal distribution of the residuals were carried out for each group separately. When the factor "cultivar" was significant, a Tukey test for means comparison was run. The results with Florina and Ariwa were used as references in the other two groups.

Results and discussion

Development of the pathogen population
It could be observed, that mildew incidence of most of the cultivars increased with every additional year of observation (table 2). In the first observation year very low incidence was found in all sites, less pronounced at Oberwil, where the first year of assessment in 1995 was not integrated in this evaluation.

Analyses of variance
Analysis of variance with the two cultivars Florina and Ariwa
A complete three factor ANOVA could only be performed with the cultivars Ariwa and Florina, being the only ones present in all four plots. Only two years, 1997 and 1998 were included, as in 1996 none or very little mildew infections appeared at Wädenswil, Grabs and Güttingen.

Table 3. Tree factor ANOVA with the cultivars Ariwa and Florina

Factors and Interactions	F Ratio	Probability H° is true	Significance	Figure
year	6.0	0.01	s.	
cultivar	34.5	< 0.0001	s.	2
site	40.6	< 0.0001	s.	1
[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
year * cultivar	6.3	0.01	s.	3
site * cultivar	9.8	< 0.0001	s.	4

The factor site was highly significant (table 3). At Grabs and Wädenswil the mean mildew pressure level with approximately 2% was considerably lower than at Oberwil and Güttingen with 12% (fig. 1). Also the year and cultivar effects were highly significant (fig. 2 and 3). In 1997 the level of mildew incidence was significantly lower than in 1998 (fig. 3). However,

this effect was only due to the behaviour of Florina showing approximately two times higher incidence in 1998 (significant interaction year • cultivar, fig. 3).

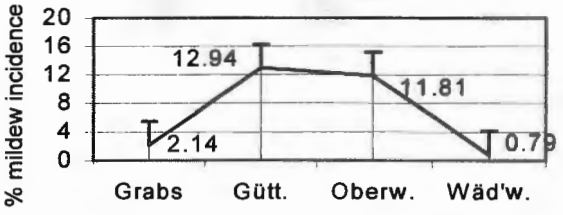

Figure 1. Av. % mildew incidence per site

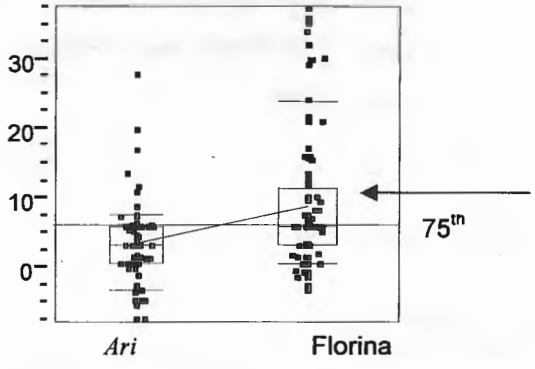

Figure 2. Mildew incidence of the cultivars Ariwa and Florina. Data from four sites and two years, corrected for the significant influences of site and year. Boxes represent the 50% quantile (50% of all data points lie within the box). The box shows the group median as a horizontal line accross the middle and the quartiles (25th and 75th percentile) as its ends. The 10 th and 90th percentiles are shown as short lines above and below the box. The two group means are connected by a (diagonal) line. The horizontal line accross the whole figure is the over all mean.

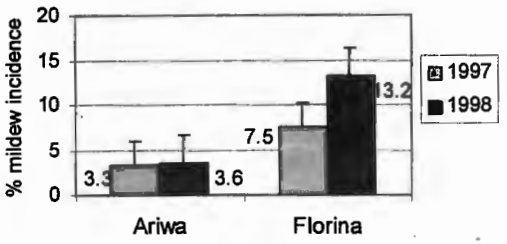

Figure 3. Av. % mildew incidence per year and cultivar

The significant interaction between site and cultivar underlines, that Florina reacts more sensitively to conditions of high mildew pressure than Ariwa (fig. 4).

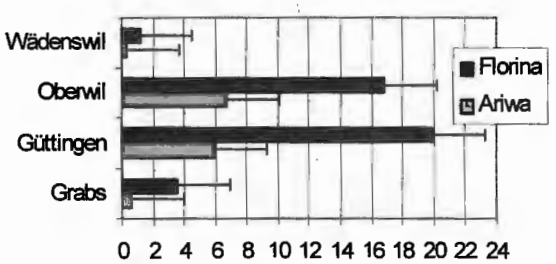

Figure 4. Av. % mildew incidence per site and cultivar

Cultivars present in at least 2 plots

In the group of cultivars that were present in at least two plots we found four subgroups with significantly different mildew susceptibility (fig. 5). But clearly Delorina and the selection 'Code 1' expressed the highest susceptibility.

In addition to the Tukey test and to increase the confidence for interpretation of the cultivar susceptibility, we use the 75th percentile of mildew incidence data and the maximal value. The maximal values indicate the cultivars susceptibility under the most extreme condition observed.

When comparing the mildew incidence of the cultivars present in at least two plots, the different levels of mildew pressure in the four plots (table 2) should be taken into account. The most striking cultivar is Prima with the maximum value of 11% infected leaves falling together with the 75th percentile (fig. 5). Reanda, Retina and Renora have the same 75th percentile of 7, but differ in their maximal variance: Reanda possessing a mildew resistance, remains below the 20% level, whereas Renora is near the 40% level. The three mildew resistant cultivars Ariwa, Rewena and Reanda show together with the susceptible cultivars Prima, Retina, Baujade and FAW 7207 tolerably low incidence with approximately 10% of infected leaves. Florina (12%) and Angold (19%) exceed the 10% level. Only the cultivars

Delorina (36%) and Code1 (47%) have essentially higher mildew incidences. These last two cultivars also have large maximal values reaching 60% and over 70% of mildew incidence.

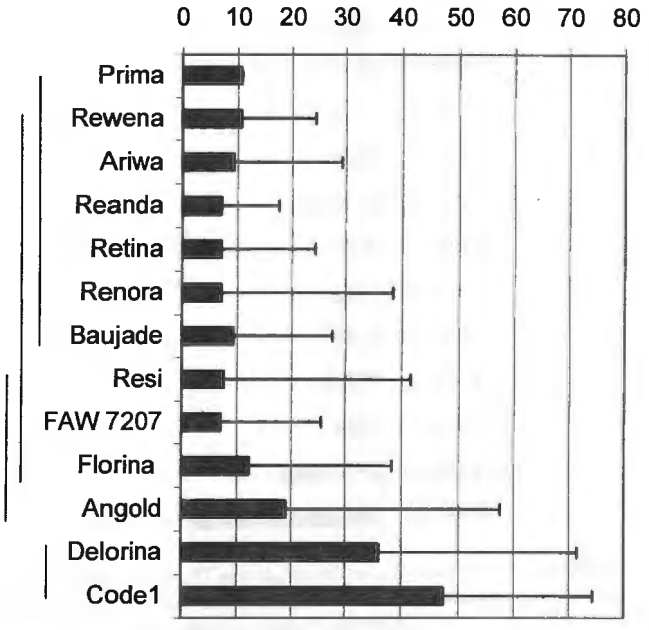

Figure 5. Mildew incidence in % infected leaves of cultivars planted at 2 or 3 sites (1996-1998)

Cultivars planted only in one plot or with very few trees (fig. 6)

The cultivars in only one plot or with few trees can be divided in three subgroups with distinctly different incidence levels and some intermediate groups.

Due to the smaller database the results of these cultivars are less confident (table 2). Rubinola shows the lowest mildew incidence at the 75 th percentile, but this is combined with a rather high variance indicated by the maximal value. Topaz has low values and a very small variation, with the maximal value hardly exceeding the 75th percentile. The data of Topaz however, originate from the two plots with low mildew pressure. Rosana and Saturn have an especially high variance with maximal values climbing up to 60-70 % of incidence. Boskoop, the standard cultivar for organic farming, remains at the 10% limit with a maximal value below 30%. Florina, reference cultivar, is in the middle range. Only Delgollune and Resista have considerably higher mildew incidence.

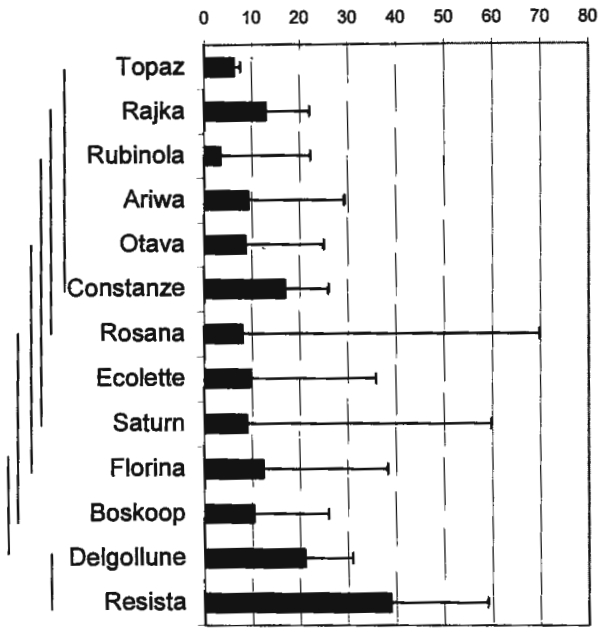

Figure 6. Mildew incidence in % infected leaves of cultivars standing at only 1 site or having very few trees (1996-98)

Conclusions

We characterize the susceptibility of the observed cultivars using three criteria, (i) mildew incidence of the 75th percentile, (ii) maximum value and (iii) statistically significant differences. Scales for mildew incidence on productive trees were defined by several authors, among them Borecki (1987). The scale which we use here (table 6) is somewhat more severe than Borecki's as the mildew level at the 75 th percentile is considered and not the mean's level. Pitera (1994) used Borecki's scale in a similar assessment of mildew incidence of 140 cultivars and selections in 1990 and 91, but assessed only leaves on the youngest shoots. Although the above suggested method is to some extent empirical, it reflects the susceptibility of a cultivar to a highly reliable and a fairly comparable extent where statistical mean comparison tests reach their limits. This is important also when practical cultivar recommendations are required.

Cultivars with a low susceptibility are Reanda, FAW 7207, Retina, Ariwa, Baujade, Prima and Rewena (table 6). In the group with less confident data Rubinola, Topaz, Otava and

Boskoop show rather low susceptibility. The qualification of Florina as only medium susceptible to mildew is confirmed by our data. It is joined by the cultivars Resi, Renora and less certainly Ecolette and Delgollune. The means of Florina and Angold for example do not differ significantly (fig. 5). But the susceptibility of Angold with a far higher maximal value is much more variable than Florina's. The smaller variance of data with Florina means that this cultivar is most probably easier to handle than Angold under practical conditions. This can be crucial for the growing success with a certain cultivar, especially in extensive management systems as e.g. in organic production.

The fact that site, year and interactions between those and the cultivars influenced the mildew incidence significantly, underlines the necessity to examine cultivars with a commercial potential in different growing regions over several years. The assessment in low level mildew regions may appear negligible. But in practice it is also useful to know the entire possible range of incidence of the same cultivar at a low as well as a high mildew pressure level. The presented cultivars should be assessed in the same way for at least one or two more years, in order to diminish the influence of the very low incidence of the first year. Additional assessments could be focused on the two plots with the higher level of mildew pressure.

Table 6. Mildew susceptibility of the observed cultivars

low susceptibility	**medium susceptibility**		**high susceptibility**	
75th P.* < 10%, max. < 30%	75th P.< 20% and max.< 30%	75th P.< 20% max. < 50%	75th P. < 20% and max. >50%	75th P.> 20% and max.> 50%
cultivars present at 2 or 3 sites				
Reanda (7/18)		Resi (7/42)	Angold (19/58)	Delorina (36/71)
FAW 7207 (7/26)		Renora (7/38)		Codel (47/74)
Retina (7/24)		Florina (12.4/38)		
Ariwa (9/29)				
Baujade (9/28)				
Prima (11/11)				
Rewena (11/24)				
cultivars present at 1 site or at 2 sites with a few trees				
Rubinola (3/22)	Rajka (13/22)	Ecolette (9/36)	Rosana (8/70)	Resista (39/59)
Topaz (6/7)	Constanze (17/26)	Delgollune (21/31)	Saturn (9/60)	
Otava (9/25)				
Boskoop (10/26)				

The numbers in brackets indicate: (75th percentile/maximum value)
* 75th P. = 75th percentile, ** max. = maximal value

For breeding as well as for growing a number of cultivars with low susceptibility to powdery mildew were detected. The small number of scab resistant cultivars with low mildew susceptibility stresses the need for breeding further mildew resistant cultivars.

The frequent observation of slight mildew symptoms on mildew resistant cultivars asks for further research and could point to the important role of QTL in the resistance mechanism (Evans, 1997).

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Post-harvest diseases of apples : chemical and biological control, new data

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Abstract : Post-harvest diseases and physiological disorders during apple cold storage are economically very significant. Up to now, various fungicides of the group of benzimidazoles were largely used in post-harvest. However today, many resistant strains (*Phlyctaena vagabunda, Penicillium spp.*) are present in several producing areas. Moreover no triazole or imidazol seem to be effective against lenticel spot disease (*Phlyctaena* sp.) principal cause of decay in Europe. On the other hand, various fungicides, are effective in post-harvest : anilinopyrimidines (pyriméthanil), phenylpyrrols (fludioxonil), strobilurines. In fact, as the legislation of the post-harvest processing is very constraining, fungicides could be used in the orchard before cropping, and have thus a possibility of reducing the losses. Alternative methods could be implemented. Biological control using the application of natural compounds extracted from edible plants (carvone from mint, eugenol from clove) is currently very promising. However, the extracts are not very effective at room temperature and they must be combined with a thermal processing (hot water 48 – 50°C during 3 minutes). Under these conditions a remarkable increase in efficacy was obtained in particular against *P. vagabunda.* With regard to physiological disorders, the same approach was applied. For example, to control scald, spinach extracts (phenolic compounds) or soya bean extracts (tocopherols) in combination with a thermal processing could replace the use of synthetic antioxydants.

The use of these biological treatments in packinghouses is discussed.

Key words : post harvest diseases, chemical control, biological control

Introduction

Up to now most post-harvest diseases on pome fruits could be considered as problems of past, being completely controlled by the extensive use of pre- and post- harvest treatments with chemicals (Eckert and Ogawa, 1988). Such a processing was admitted by the consumers.

Lenticel spot disease caused by *Phlyctaena vagabunda* (syn. « *Gloeosporium* » teleomorph : *Pezicula alba)*, is a latent parasite (Bompeix, 1988). At harvest, it can be present as mycelium into lenticels and spores on the surface of the fruit. This parasite is prevalent by far in Europe. Wound parasites are mainly *Penicillium* spp., (one of them, *P. expansum* releases patulin, a carcinogenic toxin) but *Botrytis cinerea* and *Rhizopus stolinifer* can also be found on decaying fruits.

Classical fungicides such as phthalimides and dithiocarbamates partially control these diseases. Others compounds such as thiabendazole (Bondoux and Bompeix, 1969) benomyl (Bompeix and Morgat, 1969) were applied successfully in France, Italy, Great Britain against *Phlyctaena.* Their efficacy was almost 100%. This remarkable situation in the field of plant protection was not sustained in recent years. First of all because consumers are worried about postharvest treatments with synthetics chemicals. A powerful movement of opposition developed among consumers against this type of processing. In parallel, during this period, organic agriculture was considerably reinforced. Another problem appeared gradually : the

selection of strains resistant to benzimidazole compounds (Seng and Bompeix, 1983). Nevertheless, these compounds are very effective as well in orchard treatments and the argument based on commercial assessment of "untreated after harvest" is sometimes used. However, then, an improved protection by the use of chemicals in the orchards becomes necessary.

Orchard treatments, for example with carbendazime and thiophanates, may cause the selection of resistant strains. Even if there is no risk of introducing resistant strains of the parasite into packinghouses, because pycnidia do not release spores in this environment, the efficacy in this case could be considerably reduced. Resistance level could be as high as 1000 or 5000 $\mu g.ml^{-1}$. In the complete absence of treatment with benzimidazoles compounds in an orchard localised in the center of France, the frequency of resistant strains and the level of resistance remained constant for 4 years. In an organic farm after seven years, 27 % of the strains were resistant to benzimidazoles sprays that had been applied before this period. This could be due to the biological characteristics of this parasite which causes cankers on the branches of apple trees once installed and then continues its growth.

1 Efficacy of the new fungicides on post-harvest diseases

First of all, triazoles and imidazoles are not very effective against *Phlyctaena vagabunda.* Among these last fungicides imazalil is the unique fungicide permitted in post harvest, but only against *Penicillium* spp. Others fungicides groups are more effective : anilinopyrimidines (pyrimethanil), phenylpyrrol (fludioxonil) strobilurines. As far as organic culture is concerned, sulfur and copper salts are quite ineffective against post harvest parasites. Consequently post harvest losses during the storage are of very high incidence. Because of the legislation on the processing of post harvest, it seems practically impossible to release a new fungicide for post harvest sprays. Therefore these chemicals are aimed at being used as orchards applications.

2 Efficacy of biological control

2.1 Fungal diseases

According to El Ghaouth (1997) microorganisms and natural antifungal compounds are alternatives to synthetic fungicides for the control of post harvest diseases.

As far as others alternative methods are concerned, we tried in the past to use hot water treatments. However, times of application were much too long and such a strategy was not industrially feasible. Maximum acceptable duration for the technical services in packinghouses is about 2 to 3 minutes only. Two objectives need to be reached :

1 – Selectivity (no phytotoxicity)

2 – Efficacy against the parasites.

With regard to the first point, it can been seen on table 1 that the impact on the fruit of thermal treatment differs according to varieties – Golden Delicious, Belchard are more susceptible to 50°C during 3 minutes, (which provokes a slight change in coloration). Some varieties like Kent, Pinova, Falstaaf are very resistant. The efficacy can usually be excellent on a short term basis, that is during the first months of storage.

Hot water treatment appears to delay considerably the evolution of the disease, with variations according to the fruit varieties (Bompeix and Cholodowski-Faivre, 1997). It could be possible to apply higher or lower temperatures. We noticed a maximum for temperature and time of about 50°C lasting 2 to 3 minutes. Below 50°C the efficacy dropped rapidly.

Table 1. Hot water risk injury

Cv.	45°C			50°C			55°C			
	2 min.	3 min.	21 min.	2 min.	3 min.	6 min.	1 min.	2 min.	3 min.	6 min.
Belchard[2]	0	0	0	0	h	-	0	-	-	-
Canada gris[2]	0	0	0	0	0	0	0	0	-	-
Cox's orange pippin*[1]	0	-	0	0	h	H	h	h	-	-
Elstar[2]	0	0	0	0	0	H	0	0	h	H
Falstaaf[2]	0	0	0	0	0	-	-	-	-	-
Golden delicious[2]	0	0	0	0	h	H	0	h	H	H
Granny smith[2]	0	0	0	0	0	0	0	0	0	h
Idared[2]	0	0	-	0	0	-	0	H	-	-
Kent[2]	0	0	0	0	0	0	0	0	0	h
Pinova[2]	0	0	0	0	0	-	0	0	h	h
Red chief[2]	0	0	0	0	0	0	0	h	h	h
Conférence[2]	0	0	0	0	h	-	0	H	H	-

0 : no symptom
h: moderate heat injury (skin)
H : severe heat injury (skin)
[1]Burchill, 1964
[2] Bompeix, Cholodowski-Faivre, 1997/1999

In the case of microbiol antagonists such as bacteria or yeasts (Janisiewicz, 1998) a negative result is generally obtained. This is not surprising with latent parasites because they are already in contact with living cells of their hosts, and thereore protected from attack or competition of microorganisms used for biological control. Consequently, we made the choice of the use of natural compounds. We first examined the result obtained by medical doctors and pharmacists with regard to human mycoses. It clearly comes through from several publications that the antifungal effect of mint extract and clove is widely recognized.We tested therefore the terpenic compounds extracted from these plants, carvone and eugenol, but their effect at room temperature was found to be rather modest. We then thought of a combination with thermal treatment. In preliminary tests *in vitro* with carvone a good fungicidal efficacy could be observed on spores and mycelium for *Botrytis cinerea* and *Phlyctaena vagabunda* as well. Conversely the effect against *Penicillium* was disappointing. Applied to the fruits with storage time up to 8 months, carvone increased the effect of the thermal processing (Bompeix and Cholodowski-Faivre, 1997). With eugenol a prolonged antifungal effect was also obtained (Patent Bompeix and Xeda Int., 1995/1996).

For example, in march, on Falstaaf apples harvested in september the previous year in an organic farm and stored at 2°C, losses caused by *Phlyctaena* were as high as 47 %. The efficiency of the hot water treatment alone was 78.7 % and when combined with eugenol (2.0 g/l) it was 87.2 %. In may, losses were 77 % and the efficacy of hot water treatment was 51.9 % and 75.3 % when combined with eugenol.

2.2 Physiological disorders : scald

In the case of scald ethoxyquine and diphenylamine are extensively used as antioxydants, they are far more toxic than fungicides. Therefore we considered their replacement by antioxydant of natural origin, such as phenolic compounds (spinach extract), rosmarinic acid (Rosemary) and tocopherols from soya. Here again, when applying these compounds at room temperature, the efficacy was very low. But, when combined with hot water treatment, the efficacy increased and it was possible to use these treatments in packinghouses.

Conclusion

New synthetic fungicides could be used against post harvest diseases of pome fruits but they must be considered only as usable in orchard sprays. We noticed that their efficacy is not as good as for benzimidazole compounds. In addition, sooner or later, resistant strains could appear. For biological control a high number of natural substances are either not very effective or too expensive. Some of them, usable on an industrial basis, like eugenol, give results comparable to synthetic pesticides. The alternative or biological control methods could thus be used not only in the case of the organic agriculture but also in the case of loss of efficacy of the synthetic compounds.

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Assessment of *Penicillium* risk on pome fruit in storage

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Abstract : *Penicillium* rot is one of the most common postharvest diseases of apple, mainly caused by *Penicillium expansum* Link.. It usually occurs on wounded fruits, by inoculum naturally present in cold storage rooms. Because of resistance to benzimidazole fungicides, control consists in preventive action using disinfectants and reducing risks of injury. Assessment of *Penicillium* risk is possible with Airtest, an "aerobiocollector" specially adapted to collect airborne spores of fungi. Since 1997, a general survey is being carried out in French warehouses. The level of airborne *Penicillium* in cold rooms varies from less than 10 to more than 5000 spores/m^3. Collected *Penicillium* isolates belong to the *P. expansum* and *P. brevicompactum* series. Many other fungi species have been found, such as *Botrytis cinerea, Alternaria alternata* or *Rhizopus stolonifer*. Pathogenicity tests on collected isolates show that *P. expansum* is often highly pathogenic on apples, unlike *P. brevicompactum*, often slightly or non pathogenic on apples. Half of the *P. expansum* isolates are resistant to benzimidazole fungicides.

Key words : postharvest diseases, *Penicillium*, pome fruit, risk assessment

Introduction

Blue mold is one of the most common postharvest diseases of apple, but is also important on pears, which are however more susceptible to *Botrytis cinerea* Pers.. The losses in cold stores and warehouses due to rots vary from one year to another but can reach 5 to 15% (Bernard *et al.*, 1992). The causal organisms belong to the genus *Penicillium* but *P. expansum* Link. is known as the most common and economically important species (Jones & Aldwinckle, 1990). Infection in the orchard is rare and *Penicillium* rot usually occurs on wounded fruits through conidia naturally present in warehouses (Bondoux, 1992, Giraud *et al*, 1994) and in flume water of packing-houses (Spotts & Cervantes, 1993).

In France, chemical treatments before harvesting (tolylfluanid, benomyl, thiophanate-methyl) are mainly focused on latent pathogens such as *Pezicula alba* Guthrie. Postharvest treatments with fungicides are often used to control *P. alba* and other pathogens such as *Penicillium, Botrytis, Alternaria*. Thiabendazole, presently the only registered fungicide in France on apple for this application, and benomyl (authorized until a few years ago) are now less effective against *P. expansum,* because of a partial resistance to benzimidazole fungicides. With IPM development, it will become necessary to limit preharvest treatments and suppress unnecessary postharvest spraying.

In that case, control of *P. expansum* consists in (Giraud *et al.*, 1998) :

1) Reducing risks of injury and bruise susceptibility on fruits by careful picking and packing, respect of harvest dates, adequat calcium nutrition, reducing temperature stress and setting the sizers.
2) Reducing infestation risks by cleaning and disinfecting cold rooms, bins and sizers, and by changing water of packing lines.

Assessment of *Penicillium* risk is actually a mean to decide about disinfecting and to compare the efficiency of disinfectants. It is based on measurement of the airborne spores level in cold rooms, before storage.

Materials and methods

Description of methods
Two methods can be used :

1) "Petri dishes": five 90mm plates with standard agar medium are placed opened on the floor of the cold room for 24 hours. After this period, they are closed, and collected for a 3-days incubation period at room temperature; then, developed colonies are identified and counted. Very easy to carry, this method was used up to now to compare efficiency of disinfectants.

2) "Airtest" is an "aerobiocollector" specially adapted to collect airborne spores of fungi, but also bacteria. This tool (marketed by LCB) was initially developed to check air quality in the food industry and in laboratories; we started using it in pome fruit storage in 1995. Air volume is selected by pressing keys on the side of the tool, and a sample of atmosphere of the cold room can be sucked through a perforated cap (220 holes). Spores are collected on a 65mm plate inside the cap. After 4 days of incubation, colonies can be identified and counted. The most probable number of colonies in the Petri dish is obtained by using Feller's Distribution:

For n counted colonies, the most probable number is N:

$$N = 220 \cdot \sum_{i=1}^{n} 1/(221-i)$$

Knowing the discharge of the tool (100 L/mn), the population level in the sampled atmosphere can be calculated (number of spores per m^3) .

Species identification
The fungi genera are identified by colour on PDA medium (Potato Dextrose Agar, Difco) and morphology of colonies, conidiophores and conidia. As it is very difficult to recognize *Penicillium* species, isolates have been identified by F. Jailloux (INRA Bordeaux) and using descriptions in literature (Pitt, 1979, Raper & Thom 1968).

Pathogenicity of Penicillium isolates
In a first experiment, 2-week old PDA cultures of 4 isolates were flooded with sterile water and suspensions adjusted to 10^3, 10^4, 10^5 and 10^6 conidia/ml. In a second experiment all the isolates in collection were prepared in suspensions, adjusted to 10^5 conidia/ml. "Golden Delicious" apples were surface-sterilized with alcohol and wounded at 5 locations per fruit (2mm diameter and 3mm depth). Each wound had received 10µl of conidial suspensions, and the fruits were placed at room temperature for 7 days. Lesion diameters were measured.

Resistance to benzimidazole fungicides of Penicillium isolates
Each pathogenic isolate was grown on PDA amended with 1, 5, 10, 50, 100, 500, 1000 ppm of benomyl (Benlate, Dupont de Nemours) and of thiabendazole (Decco 20S, Elf Atochem).

Colony diameters were measured after 7 days, compared to control, and 50% effective dose (ED_{50}) calculated.

Results and discussion

Method used for measuring the Penicillium level in cold stores

As the "Petri dishes" method is based on the faculty of airborne spores to fall into the plate, there is no relation between the number of colonies in the plate and an aerial conidial population. For that reason, comparisons between cold rooms and assessment of a risk threshold are difficult. After the preliminary tests, comparing the two methods, Airtest was chosen to be the most representative system to study the airborne fungi level in a cold room. In addition, the time necessary for sampling is shorter (a few minutes).

Survey of the Penicillium level in French warehouses using Airtest

Since 1997, we have visited with Airtest 18 warehouses, located mainly in the Southwest but also in the Val de Loire and in the Southeast regions, totalling more than 200 analysed cold rooms. The samplings show a variable level of *Penicillium* in cold rooms, with an overall homogeneity in a same warehouse. The average level varies from less than 10 to more than 6000 spores/m^3.

When the population in cold rooms exceeds 2000 spores/m^3, managers admit to having rot problems, and under 150 to 500 spores/m^3, we consider the store as quite clean.

In order to have a first idea of a potential risk, we have defined 4 classes of airborne *Penicillium* populations: less than 150, 150 to 499, 500 to 2000 and over 2000 spores/m^3. Figure 1 shows different levels of *Penicillium* population in some warehouses, measured before disinfecting.

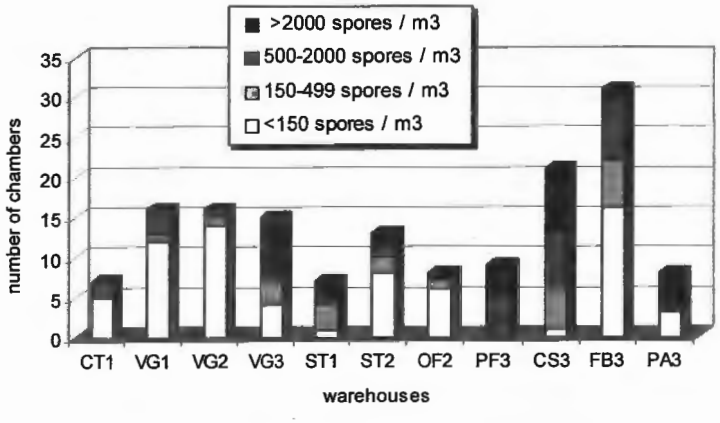

Figure 1. *Penicillium* level in cold rooms of 8 warehouses (2 letters coding the name) before disinfecting (code number squares with year of sampling: 1=1997, 2=1998, 3=1999) according to the population class.

Airborne fungi

In pome fruit storage, the most common airborne fungi, aspired by Airtest, are *Penicillium sp.* and *Cladosporium herbarum* (Pers.) Link., but many other fungi species were found, such as

Botrytis cinerea Pers., *Alternaria alternata* (Fr.) Keissler, *Fusarium sp.*, *Epicoccum nigrum* Lk. or *Rhizopus stolonifer* (Ehrenb.) Vuill.. As for *Penicillium*, 3 species were identified by F. Jailloux during an investigation in French warehouses: *P. expansum* Link., *P. stoloniferum* Thom (*P. brevicompactum* Dierckx series (Raper & Thom, 1968)) and *P. verrucosum* Dierckx.

Pathogenicity tests of Penicillium isolates

A first experiment was carried with reference isolates of *P. expansum*, *P. stoloniferum* and *P. verrucosum*. These results (Table 1) show that our isolate of *P. verrucosum* is not pathogenic on apple, *P. stoloniferum* is weakly pathogenic and *P. expansum* highly pathogenic.

Table 1. Necrosis diameters (average of 4 replicates) on apples after 15 days of incubation (mm)

		Conidia / ml			
Species	isolates	10^3	10^4	10^5	10^6
P. expansum	PxA_2	6	38	50	60
P. stoloniferum	Pslf	0	0	13	15
P. verrucosum	Pver	0	0	0	0

These results were confirmed by a second experiment, applied on 22 isolates collected by sampling with Airtest (Table 2). 10 out of 12 isolates from the *P. expansum* series are highly pathogenic on apples. Among 10 isolates from the *P. brevicompactum* series, 6 are only slightly pathogenic and 4 non pathogenic on apples. From now on, in a sample of atmosphere, we will try to take into account only colonies from the *P. expansum* series.

Table 2. Necrosis diameters (average of 10 replicates) on apples inoculated with 10µl per wound of 10^5conidia/ml suspensions, after 15 days of incubation (mm).

Isolates of *P. expansum* series		Isolates of *P. brevicompactum* series	
PxA_2	29.7 ± 0.9	Pslf	2.3 ± 0.6
PxA_1	29.1 ± 0.9	P1b	1.5 ± 0.8
P109	29.5 ± 1.1	P1c	5.7 ± 0.9
P114	32.6 ± 0.6	P111a	7.3 ± 0.9
P115	30.6 ± 0.8	Porgr	5.4 ± 2.1
P117a	30.4 ± 0.7	*Pv1*	1.2 ± 0.6
P117b	28.3 ± 1.1	P1136	0
P1183	29.4 ± 0.6	*P1182*	0
Pcef	29.0 ± 0.9	P1184	0
P1x	28.7 ± 0.8	P119	0
Pv	10.9 ± 2.8		
P1181	0		

Susceptibility of Penicillium isolates to benzimidazole fungicides

Our pathogenic isolates from the *P. expansum* series are often resistant *in vitro* to benomyl but seem to be relatively more sensitive to thiabendazole. Our isolates of the *P. brevicompactum* series are generally sensitive to these fungicides (Table 3) except one isolate. These results confirm that a lot of isolates of *Penicillium* causing decays in storage are resistant to benzimidazole fungicides.

Table 3: Benomyl and thiabendazole ED_{50} (50% effective dose) and necessary dose for a total inhibition (ED_{100}) in an *in vitro* test of pathogenic collected *Penicillium* isolates in cold stores (ppm).

		benomyl		thiabendazole	
	Isolates	ED_{50}	ED_{100}	ED_{50}	ED_{100}
P. expansum series	P109	>1000	>1000	20	50
	P115	820	>1000	2	50
	P117a	460	>1000	5	500
	P1x	460	>1000	3	100
	PxA_2	560	>1000	14	50
	PxA_1	80	>1000	10	50
	P117b	3.5	10	<1	<1
	P1183	<1	<1	2	5
	P114	<1	<1	<1	<1
	Pcef	<1	<1	<1	<1
	Pv	<1	<1	<1	<1
P. brevicompactum series	P111a	630	>1000	25	500
	P1b	200	>1000	<1	5
	Porgr	1	5	<1	<1
	Pslf	<1	5	<1	<1
	P1c	<1	<1	<1	<1
	Pv1	<1	<1	<1	<1

For two years now, Airtest has been a service available from Ctifl, aimed at improving the quality of fruits after storage, and it is now considered as a tool for Integrated Management of postharvest diseases of apple and pear.

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Pre-harvest assessment of the risk of storage rots in Cox apples

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Abstract : Over seven years (1991-1997), the incidence of rotting in stored Cox apples from 20-25 orchards was related to various factors in order to identify likely determinants of rotting that could be used pre-harvest to assess the risk of rotting in store. The factors assessed included orchard factors (% bare ground, % low hanging fruit, fungal inoculum and fruit quality such as russet and cracking), previous orchard rot history, fruit mineral composition and rainfall. The best variables identified to assess rot risk were as follows: *Botrytis* rot – rot history, rainfall from June to harvest; *Monilinia* – orchard incidence of *Monilinia*; *Nectria* – orchard incidence of canker; *Gloeosporium* spp – rot history, crop load, calcium concentration in the fruit, rainfall during month before harvest; *Phytophthora* rot - % bare ground and % crop < 0.5m above ground, rain from 15 days prior to harvest. No useful predictive variables were identified for *Penicillium* or *Mucor*. The parameters identified were used to predict pre-harvest the likely rotting in Cox from six orchards. The actual rotting in fruit samples from the six orchards stored until February/March 1998 agreed closely with that predicted.

Key words : rot risk, post-harvest fungicide.

Introduction

The storage of Cox apples until March/April is essential in order that the UK industry can regulate the supply of fruit onto the market to compete with imported fruit. Until recently all fruit destined for storage beyond Christmas was treated routinely post-harvest with fungicide to control storage rots regardless of the need for treatment. Recent surveys of rotting in untreated stored Cox apples have indicated that significant losses occur in most seasons (Berrie, 1997) but that, in each year of the surveys, fruit from up to 40% of orchards stored until March had < 2% losses and would not have required treatment. The ability to predict pre-harvest which orchards have a low risk of rotting and thus which fruit could be stored without fungicide treatment would avoid unnecessary use of fungicides and minimise losses due to rots. Several fungal rots are important in Cox – *Phytophthora syringae, Botrytis cinerea, Nectria galligena, Monilinia fructigena, Gloeosporium* spp and *Pencillium expansum* and rotting is related to orchard site (Berrie, 1989; 1997). Attempts have been made elsewhere to predict rotting in store. Techniques have included the use of paraquat (Biggs, 1995) or immunoassay or PCR methods on samples of apples collected pre-harvest to detect latent infections of fungal rots. However, while such methods may detect the pathogen, they are difficult to implement in practice and give no guidance on likely rot development, which is dependent on other factors such as fruit composition and storage conditions. The overall objective of the work described below was to identify the factors affecting rotting in store and use them to develop a system for assessing pre-harvest the risk of rotting in store. Such a system could be used to decide on the need for treatment or the storage potential of the fruit if no treatments were available or permitted.

Materials and methods

Criteria for assessment of rot risk

Over the period 1991-1997, studies were conducted on fruit from 20-25 Cox orchards per season. The orchards used in the study were selected to include different orchard types (multi-row beds, intensive plantings on M9 rootstock, semi-intensive on MM106 rootstock), with a variety of disease problems, and located at sites in Kent with different rainfall patterns. Each year two weeks prior to harvest, orchard assessments were carried out of factors likely to affect the incidence of fungal rotting in store (Table 1). Additional information on likely fungal inocula in the orchard was taken from packhouse records on the incidence of rots in fruit previously stored from that orchard. Data on rainfall for each orchard were collected from either an on-site automatic weather station (METOS, Pessl, Weiz, Austria) or from Meteorological Office Synoptic weather stations or from Meteorological Office Rainfall Recording Centres located within 5 km of the orchard. At harvest, a representative sample of 500 fruits was picked from each orchard, ensuring that apples were removed from each part of the fruit canopy. An additional 20-30 fruit were also collected for mineral analysis. The 500 fruits were stored in controlled atmosphere storage (3.5°C, <1% CO_2, 1.25% O_2) until March, when the fruit was removed from store, the losses assessed and the rots identified either visually or by culturing on Potato Dextrose Agar (PDA). The data collected were analysed statistically. Because of the large number of potential variables, knowledge of the biology and epidemiology of the fungal rots was used to identify variables likely to be most influential in predicting rotting. Stepwise and backward elimination regression procedures (Draper & Smith, 1981) were then used to identify variables affecting the incidence of each of the major rots and devise regression models.

Testing rot risk assessment models

In August 1998, six orchards of cv Cox were selected to test the models derived for each of the main storage rots of Cox. Orchards were selected on the basis of previous rot history to provide a range of potential rot problems. In late August or early September, each orchard was visited and assessed for orchard factors identified as influencing rotting in store. These, together with the assessment procedures and criteria, are listed in Table 2. At harvest, either six bulk bins of fruit selected at random or a random sample of 500 fruits picked into boxes were obtained from each of the orchards. These were picked as near as possible to the optimum picking dates for Cox for long term storage and stored in a commercial controlled atmosphere store in 1.25%O_2, <1% CO_2 at 3.5 °C until February or March 1999. An additional sample of 20 fruits was collected at harvest and analysed for minerals (Ca, K, P, N, Mg as mg/100g fruit). In addition relevant daily rainfall (mm) data from blossom to harvest were obtained for each orchard, with previous rot history where possible. For each orchard, the risk of rotting in store was then assessed using relevant information on orchard factors, mineral composition, rainfall data and previous rot history identified as influencing the incidence of rots in store. At the end of the storage period (February or March), the fruit from the test orchards were removed from store and the incidence of rotting and fungi responsible were determined. The results were compared with those predicted prior to storage.

Results and discussion

Criteria for assessment of rot risk

The incidence of the fungal rots varied considerably between orchards and over the seven years of the study. *Botrytis, Nectria* and *Pencillium* rots occurred in most orchards in each of

the years. The incidence of *Monilinia* was low and sporadic between 1991-1994, but increased in incidence in the later years, whereas the incidence of *Phytophthora* rot was generally low except in 1994. Rotting due to *Gloeosporium* spp. was also low and sporadic. Nevertheless it was possible to identify factors for most of these rots which could be used to assess the likely risk of rotting in store. The significant factors and the % variation accounted for are summarised in Table 3. None of the factors assessed in the orchard was identified as having a significant effect on the incidence of *Botrytis* rot in store. This result agrees with other research on the biology and epidemiology of *Botrytis* rot of apple (Harris, 1998). The criteria identified, however, do give a basis for decision making. The importance of measures of *Nectria* activity in the orchard (e.g. cankered shoots etc.) as determinants of rotting in store is obvious. However it would also be expected that rainfall would be identified as an important factor, but it was not statistically significant in any of the models. It seems likely that, in orchards with a high incidence of canker, significant fruit infection will result in most seasons, regardless of the amount of rainfall. Rainfall may only be important in determining losses in store due to *Nectria* rot in orchards where the incidence of canker is low. Infection of fruit by *N. galligena* takes place from blossom to harvest. Therefore, the total rainfall during this period is likely to be most significant.

The incidence of *Gloeosporium* rot was generally low and sporadic throughout the seven years of the study. It is therefore difficult to draw firm conclusions from these data on the important risk criteria for *Gloeosporium* rot. Previous studies (Sharples, 1980), however, have indicated that fruit low in calcium was more prone to *Gloeosporium* rot. Therefore decisions on the risk of this rot should also take account of the mineral composition of the fruit. No significant pre-harvest factors were identified for *Penicillium* rot. This is not surprising, as this rot is rarely seen in the orchard and it is assumed that infection occurs at harvest, particularly during post-harvest fungicide drenching. The incidence of Mucor rot, *Botryosphaeria* rot, *Fusarium* rot and *Diaporthe* rot was too low for accurate models to be constructed.

Apart from *Nectria* rot where the selected model accounted for more that 90% of the variation, the best models accounted for only 34-52% of variation. The incidence of the rots in the study orchards was dependent on seasonal weather and consequently rot incidence was sporadic. This is the most likely explanation for the low percentage variation accounted for in the models. The results of this study, however, do provide information on variables that can be assessed pre-harvest to give an indication of likely rotting in store. This system will not give, and was never intended to give, a prediction of actual numerical losses; it aims to provide sufficient information on which a practical decision on the likely risk of rotting in store and the required action can be made. An example of the decision process for *Phytophthora* rot and *Monilinia* rot is given in Fig 1 and 2.

Testing rot risk assessment models

Based on the mineral composition of the fruit, fruit from all the test orchards was suitable for medium (January/February) or long term storage (March) (Luton, 1987). The risk of rotting for each orchard, using the assessment system, is summarised in Table 4. Rainfall in 1998 was exceptionally high, especially in June and September, when twice the monthly average rainfall was recorded. The lowest rot risk was recorded for Gypsy orchard. Fruit from this orchard could be stored without treatment. In contrast, orchards Molland, Reservoir and 56 Acres were predicted to have high incidence of *Nectria* rot and were therefore recommended for early marketing.

In all orchards, the actual incidence of rotting was as predicted (Table 5). The highest incidences of rotting were recorded in Molland and Reservoir orchard, where *Nectria* was the main cause of losses. Losses due to *Monilinia* were slightly higher than expected in 56 Acres.

This may have been due either to the time at which the pre-harvest orchard assessment was conducted or to the sample size of 20 trees being inadequate for the size of orchard. Fruit susceptibility to *Monilinia* increases as the fruit ripens (Xu & Robinson, in press), and therefore the orchard incidence increases as the fruit nears harvest. Thus, if the pre-harvest orchard assessment is done too early, it may not accurately reflect the orchard incidence of *Monilinia*. Additional assessments of *Monilinia* at harvest would overcome this problem. The orchard 56 Acres is an exceptionally large orchard and it may have been more appropriate to divide this orchard into smaller sections for the purposes of orchard assessment.

Conclusions

1. Models derived from data collected over seven years from 20-25 Cox orchards were constructed for each of the main storage rots and could be used to make reasonably accurate pre-harvest predictions on the likely rotting in store.
2. Where orchard assessments on fungal inoculum, especially *Monilinia*, are conducted 2-3 weeks prior to harvest, it may be necessary to re-assess the incidence at or nearer harvest.
3. The numbers of trees used in the orchard for assessment must be representative of the orchard size.

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Table 1. Variables assessed pre-harvest as possible determinants of rotting

Main Item	Factors	Assessment
Orchard type	Tree rows	Single or multi-row bed
	Rootstock	M9 or MM106
	Pollinator	Cultivar
	Bare ground	% bare ground
	Grass/herbicide strip	1 = No, 2 = Yes
	Mulch	1 = No, 2 = Yes
	Weeds	% cover
		% alive
Fruit quality	Mineral composition	Analysis for N P K Mg Ca K/Ca ratio Mn B (mg/100g)
	Sugar content	% sugar
	Russet	% fruit with light or rough russet
	Cracking	% fruit with cracking on stalk end or cheek
	Hail	% fruit with recent hail damage
	Crop load	Score 1 = very low, 2 = low, 3 = moderate, 4 = high, 5 = very high.
Inoculum	Petal debris	% fruit with petals/fruitlet remains
	Dry eye rot (*Botrytis*)	% fruit infected
	Phytophthora	% infected fruit
	Low hanging fruit	% crop < 0.5 metre from ground
	Canker	% trees, % shoots
	Wet eye rot (*Nectria*)	% infected fruit
	Brown rot	% fruit (tree + ground)
Weather	Rainfall	Total rain (mm) 20/4 – harvest
		Total rain (mm) 20/4 – 31/5
		Total monthly rain (mm) June, July, August, September (-harvest)
		Total numbers of rain days per month
		Numbers of days per month where rainfall is > 10 mm per day
		Cumulative rainfall (mm) 5, 10, 15 and 20 days before harvest.
Rot history	Previous years' rots from store	Rot incidence in previous year (not available for 1991)

Table 2. Procedures for assessment of orchard factors affecting rotting in store.

Fungal Rot	Factor	Assessment Procedure	Criteria for risk
Brown rot	Incidence of brown rot in orchard	Select 20 trees at random and assess incidence of brown rot to obtain % fruit with brown rot. Assess as near harvest as possible.	Brown rot incidence of > 1% = high risk
Nectria rot	Incidence of canker and wet eye rot	Select 20 trees at random and assess incidence of cankered trees or shoots with canker.	> 40% trees with canker = high risk 15-40% = moderate risk <15% = low risk 0 = no risk >1.0% shoot canker/tree = high risk
Gloeosporium rot	Crop load	Assess crop load as light, moderate or heavy.	Light crop = risk
Phytophthora rot	(1) % bare ground	Inspect orchard. Estimate bare ground under trees taking into account mulch and weed cover.	(1) 100% bare ground (overall herbicide) = high risk (2) Overall grass, or mulch or weed cover (0% bare ground) = low risk (3) Herbicide strip (20% or> bare ground) = risk
	(2) % crop < ½ metre from ground	Select 20 trees at random and assess % crop < ½ metre from ground as near to harvest as possible.	15% or > = risk

Table 3. Significant variables identified as influencing rot incidence in store.

Fungal rot	Significant variable	% Variation accounted for
Botrytis rot	rot history rainfall (mm) June – harvest	) 34 – 46)
Monilinia fructigena	orchard incidence of *Monilinia*	51
Nectria rot	% trees with canker % shoots with canker % wet eye rot (rainfall (mm) blossom – harvest)	)) 91)
Gloeosporium rot	rot history crop load rainfall (mm) month before harvest	)) 45))
Phytophthora rot	% bare ground· % crop <0.5 m above ground Cumulative rainfall (mm) from 15 days before harvest	))) 45 – 52))

Table 4. Risk of likely rotting (shown as L= low, M= Moderate, H= High) due to main storage rots for six Cox orchards 1998

Assessment parameters	CW108/ 109	TL109	Gypsy	Molland	Reservoir	56 Acres
Total Rotting						
History	L	H	L	H	H	H
K/Ca ratio	L	L	L	M	H	L
Rain (Aug-harvest)	H	H	H	H	H	H
Overall	L-M	M	L-M	H	H	H
Botrytis						
History	M	M	L	L	L	M
Rain (Jun – harvest)	H	H	H	H	H	H
Overall	M	M	L-M	L	L	M-H
Brown rot						
Orchard Brown rot	M	H	L	M	L	M
Overall	M	H	L	M	L	M
Nectria						
Orchard canker	M	M	L	H	H	H
Rain (Bloom-harvest)	H	H	H	H	H	H
Overall	M-H	M-H	M	H	H	H
Gloeosporium						
History	L	H	L	H	L	L
K/Ca	L	L	M	M	H	L
Crop load	L	L	L	L	L	H
Rain (20 days-harvest)	H	H	H	H	H	H
Overall	L	M	L-M	M-H	M	M
Phytophthora						
Bare ground	M	M	L	L	H	L
Low fruit	M	M	M	L	L	L
Rain (15 days-harvest)	H	H	H	H	H	H
Overall	M	M	L-M	L	L	L
Overall assessment	L-M	M	L-M	H	H	M-H
Action						
Store Jan/Feb	No treatment	Treat	No treatment	Market pre-Christmas	Market pre-Christmas	Market pre-Christmas
Store March +	Treat	Treat	No treatment			

Table 5. Losses due to rots and fungi in six Cox orchards assessed between March and April 1999

		% losses							
Orchard (date assessed)	**Total rotting**	***Botrytis* rot**	**Brown rot**	***Nectria* rot**	***Gloeosporium* rot**	***Phytophthora* rot**	***Pencillium* rot**	***Mucor* rot**	**Other rot**
CW108/109 (3-3-99)	6.0	1.1	1.2	2.8 *		0.4	0.4	0	0.1
TL109 (3-3-99)	7.3	0.8	1.5	4.6*		0.1	0.2	0	0.1
Gypsy (27-4-99)	3.6	0.7	0.5	0.5	0.5	0	0.7	0	0.7
Molland (27-4-99)	31.8	0.4	6.2	15.4	3.3	0.5	3.6	0	2.4
Reservoir (10-3-99)	38.9	0.9	0	35.5	0.9	0	0.9	0	0.7
56 Acres (10-3-99)	8.9	0.6	2.2	3.7	2.2	0	0.2	0	0

* Combined data for *Nectria* and *Gloeosporium* rots.

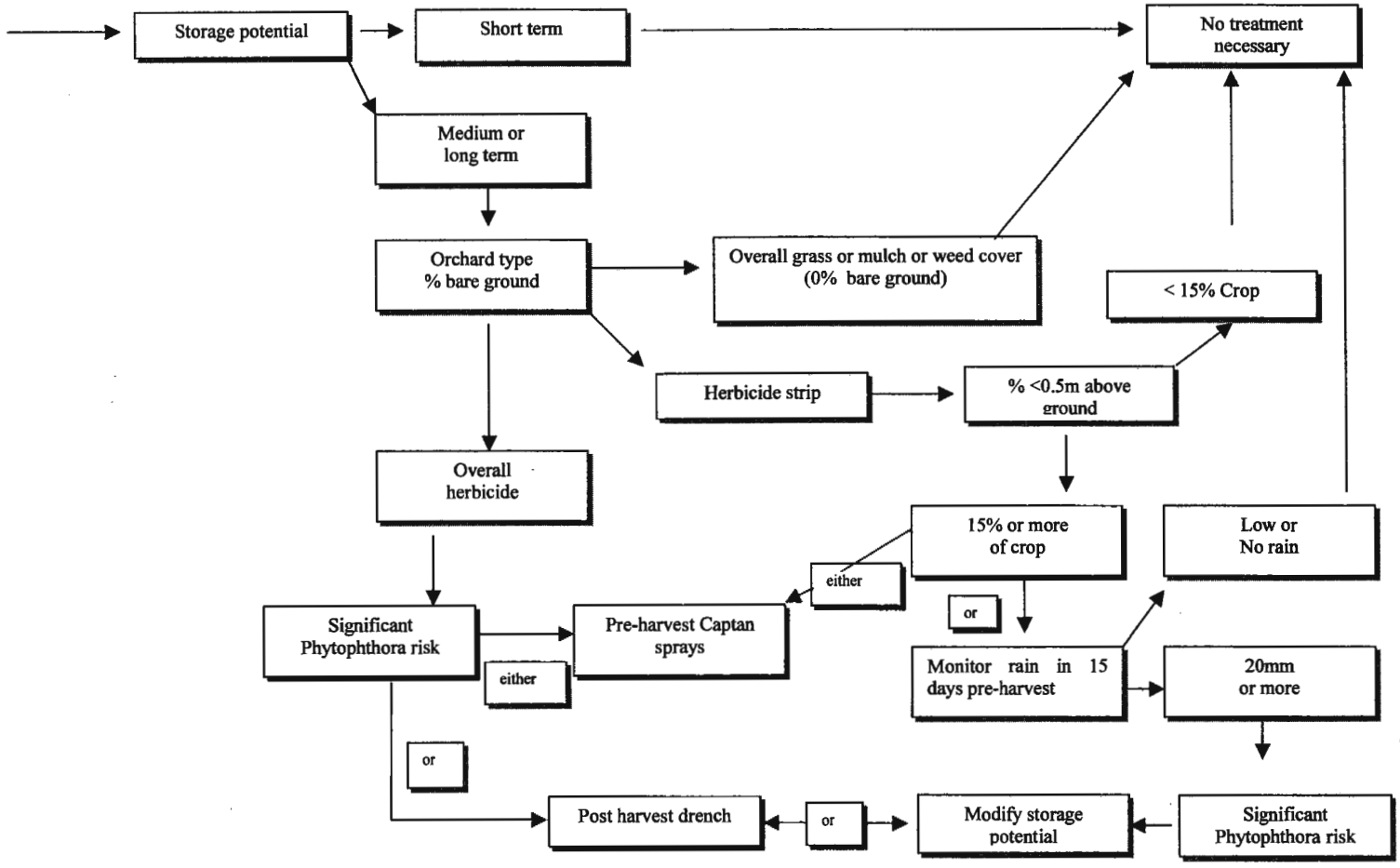

Figure 1. Rot risk assessment for *Phytophthora* fruit rot

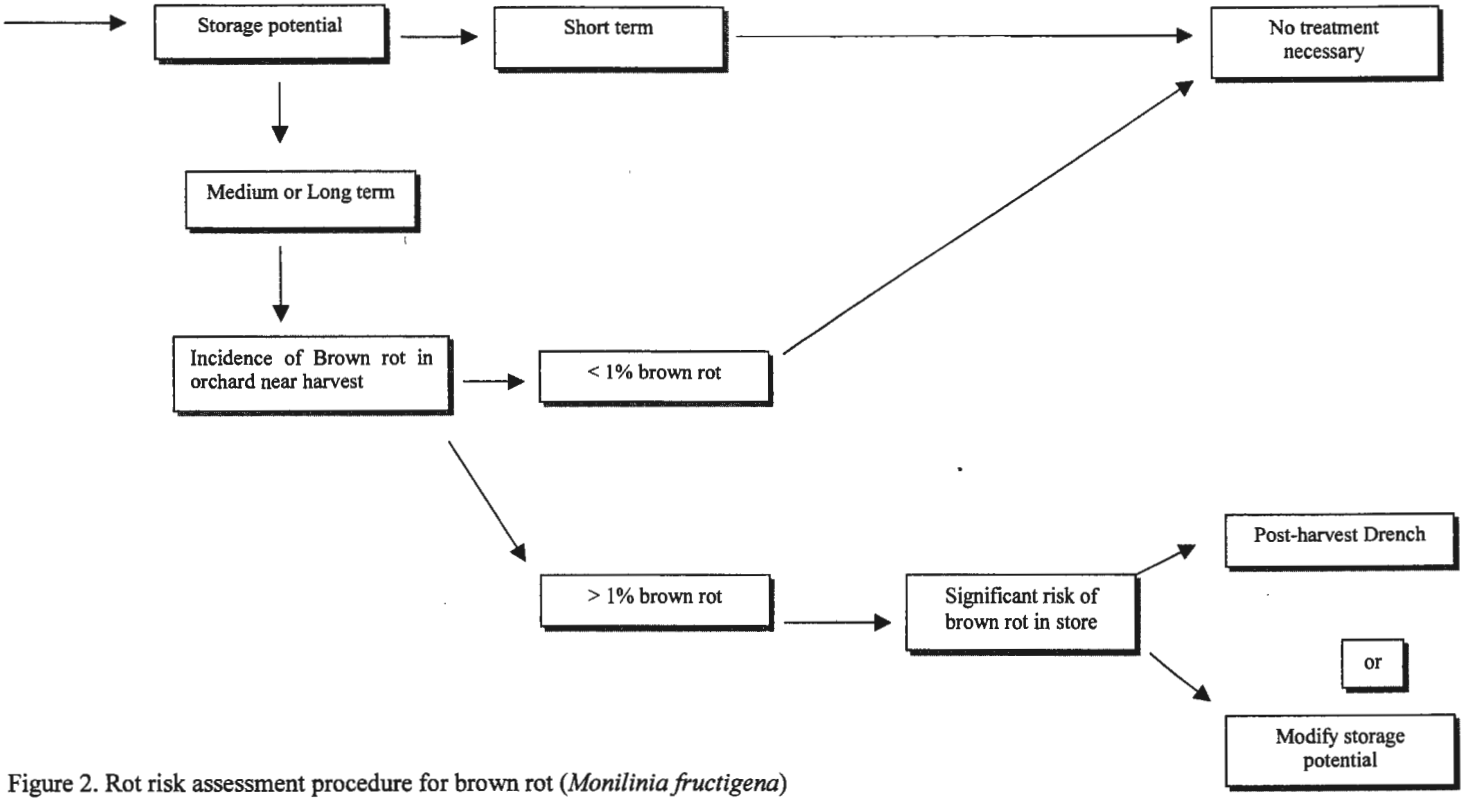

Figure 2. Rot risk assessment procedure for brown rot (*Monilinia fructigena*)

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A review of apple scab research presented at IOBC "Integrated Control of Pome Fruit Diseases" workshops, 1987-1999

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Abstract : Presentations at the IOBC "Integrated Control of Pome Fruit Diseases" Workshops in Lana, Italy (1987), Brissago, Switzerland (1988), Godollo, Poland (1990), Lofthus, Norway (1994), Croydon, England (1996), and Fontevraud-l'Abbaye, France (1999) are reviewed. In 1987, the "typical" IPM program was centered on a warning system consisting of weather equipment that monitored and recorded the weather variables used to predict Mills' infection periods, software to predict Mills' infection periods, strategies to apply fungicide based on the distribution and predicted "severity" of the infection periods, and several means to notify the grower of infection conditions. Its purpose was not to replace fungicides with other control practices; rather, it was to improve the scheduling of fungicides. In 1999, this warning system remains the central, dominant feature of the "typical" IPM program, and improved efficiency in scheduling fungicides during the primary scab season, continues to be the main achievement. Research since 1987 has continued to focus on a defensive (protectant fungicide) approach to scab management, with several results that have increased fungicide efficiency: (a) refinement of Mills' infection criteria, (b) a model that predicts the progress of ascospore maturation, (c) a procedure to predict an orchard's potential ascospore dose, (d) a "scab-risk" action threshold based on an autumn assessment of foliar scab, and (e) a model that relates tree growth stage (target size) and target susceptibility to risk of infection. Offensive approaches aimed at reducing the initial inoculum (resistant cultivars, cultivar mixtures with partial resistance to scab, sanitation, and biological control agents) have also been investigated. Breeding scab-resistant cultivars has received the most attention, and new, promising scab-resistant cultivars have been released. In other studies on offensive approaches, (a) sanitation research has resulted in a "sanitation" action threshold that can be used in conjunction with a "scab-risk" action threshold to reduce the fungicide dose in a "moderate-risk" orchard, (b) biological control agents aimed at stopping the sexual stage have been reported, and (c) cultivar mixtures have been demonstrated to have potential to slow scab buildup. Other research has provided new knowledge about the pathogen, pathogenesis, and the epidemiology of the disease that has relevance to scab management. As a result of this research, procedures and practices are now available that allow a broadened approach to scab management that integrates offensive and defensive tactics, and this appears to be the most promising approach for substantial further reductions of seasonal fungicide dose in orchards planted with susceptible cultivars. The economics of scab management programs is an area that has received little attention, but it will undoubtedly become more important when trying to convince growers to accept new practices and procedures they perceive as increasing risk to the crop and may require new equipment, more labor, more of the grower's time, and increased expertise. Integrating scab management with the management of other diseases and arthropod pests, incorporation of apple IPM into ICP programs are other areas that will likely need more attention as scab IPM programs continue to evolve.

Key Words : *Venturia inaequalis*, sanitation, biological control, integrated pest management, IPM

Introduction

The subgroup "Orchard Diseases" of the International Organization for Biological and Integrated Control of Noxious Animals and Plants (West Palaeartic Regional Section)

(IOBC/WPRS) has organized five Workshops on Integrated Control of Pome Fruit Diseases : Lana, South Tyrol, Italy (1987) ; Brissago, Switzerland (1988) ; Lofthus, Norway (1993) ; Croydon, England (1996) and the present Workshop in Fontevraud, France. Papers presented at the first four Workshops have been published : Obstbau Weinbau 1987, WPPS Bulletin 1989/XII/6, Nor. J. Agric. Sci. 1994, and Ann. Appl. Biol. 1997. The Workshops were attended by University and government researchers, apple consultants, advisory/Extension personnel, and other specialists from within the West Palaeartic Region and by guests, mainly researchers, from countries outside the region. The subgroup also met at Gödöllo, Hungary in 1990 at the international symposium on integrated plant protection in orchards, organized jointly by the West- and East-Palaeartic Regional Section of IOBC, and presentations on scab at that meeting are also included in this review.

Verheyden (Verheyden 1995) has discussed important recommendations for integrated control agreed upon by the subgroup at the Lana, Brissago, and Lofthus Workshops. At Lana, the subgroup agreed on the following objectives (Butt 1994; Verheyden 1995) :

1. to reduce the usage of fungicides before and after harvest
2. to search for biological control agents
3. to promote the breeding and introduction of varieties with durable resistance against multiple diseases
4. to make chemical and non-chemical control methods compatible
5. to avoid harmful side-effects of fungicides on the environment, crop and beneficial organisms used in Integrated Pest Management (IPM) programs
6. to develop and implement disease forecasting and infection warning systems
7. to develop and use pathogen and disease assessment methods
8. to determine disease damage thresholds and adopt action thresholds
9. to facilitate the exchange and dissemination of information, collaborative studies and standardization of methods.

It should be pointed out that although objectives of the subgroup and the most critical research needs for the region were agreed upon in the early Workshops (Verheyden 1995), there has not been a concerted effort to develop collaborative research projects among researchers with common interests within the Palaeartic Region. One notable exception is in the area of breeding for scab resistance. It should also be noted that nearly all of the apple scab research worldwide reported in 1987-1999 that has had a significant influence on apple scab IPM programs was presented at these Workshops.

One other conference organized by IOBC/WPRS since 1987 should be mentioned, as there were 11 presentations on apple scab. The International Conference on Integrated Fruit Production in Cedzyna, Poland in 1995, organized as a joint meeting of IOBC/WPRS, working group "Integrated Plant Protection in Orchards" and working group "Stone Fruit," and ISHS, working group "Integrated Fruit Production " (IOBC wprs Bull. 19, 1996). Seven presentations were on defensive scab management practices (warning and predictive systems related to applying fungicides and fungicide application and performance), three presentations were on offensive scab management practices (cultural practices for scab-resistant cultivars, urea application to reduce the ascosporic inoculum, and antagonists of *Venturia inaequalis*), and one paper analyzed the virulence of *V. inaequalis* populations.

It has been twelve years since the first Workshop, and 156 papers and posters have been presented on apple scab at the six Workshops. This paper reviews these presentations in relation to the objectives agreed on in Lana and, in the process, addresses the following questions. What changes have occurred in apple scab management over the twelve years? What insights are revealed for advancements in apple scab management in the early part of

the 21st Century? The focus will be on managing primary scab caused by the primary (ascosporic) inoculum, because preventing primary infections has been considered the key to managing scab and, consequently, has been the emphasis in all scab management programs developed to date.

How management practices control primary scab (Figure 1)

The most basic model explaining disease occurrence is the disease triangle taught in introductory plant pathology courses. It illustrates that disease occurs when a pathogen and susceptible host come in contact and interact under environmental conditions favorable for infection. The less favorable one of these components is for disease development, the shorter that side of the triangle and the less disease that develops. If one side is eliminated, e.g., the host is resistant, there will be no triangle and no disease will occur. How does apple scab and apple scab management relate to this model ?

Each apple cultivar has a level of susceptibility to the pathogen population in an orchard, so for each cultivar, the amount of susceptible tissue and target size are critical determinants of lesion density. Ascospore dose, i.e., the density of ascospores discharged into the orchard air, is the main pathogen component that determines lesion density. Key environmental variables favoring scab development are moisture on the leaf and fruit surface and temperature, and the longer the plant surface remains wet at a given temperature, the higher the proportion of spores deposited on the surface that will infect. Key biological components in the environment are microorganisms and animals that attack the pathogen directly or destroy the source of inoculum.

Each management practice is aimed primarily at one of the three components. Practices aimed at the pathogen reduce the ascosporic inoculum at the source (e.g., sanitation and biological control) or reduce the ascosporic inoculum deposited on susceptible tissue (e.g., protectant fungicide). Practices aimed at the cultivar eliminate infection (e.g., cultivar major gene resistance) or reduce the number of infections (e.g., cultivar with partial innate resistance). Practices aimed at the environment create conditions less favorable for the production of pseudothecia or availability of ascospores at the source, e.g., developing a ground cover that traps some of the discharged ascospores, or less favorable for infection, e.g., pruning practices that enhance drying of leaf and fruit surfaces.

Defensive vs offensive strategies

In 1936, Keitt (1936) argued eloquently that we need ..."a sounder scientific foundation for our orchard disease control programs," and that we need to supplement ..."the limited and temporary effectiveness of our protectant [fungicide] measures with the more enduring values that may be derived from increased application of the principles of eradication [sanitation] and immunization [resistant cultivars]." His concern was that the protectant chemical program, which was based on a *philosophy of defense* aimed at preventing scab on the fruit, allowed the buildup of a high overwintering population of the fungus on the leaves. Keitt was apparently the first person to recognize that reducing the *quantitative* level of the ascosporic inoculum was the key to improving scab control programs, and his remedy for the problem was to redirect the fungicide program to attack the pathogen at the source of the ascosporic inoculum, thus shifting the emphasis of control programs to a *philosophy of offense*.

Defensive management approaches include the application of protectant, curative, and eradicant fungicides to protect the plant from primary infections and slow the buildup of secondary scab and cultural practices that lessen environmental conditions favorable for infection. Offensive management approaches include any practice that reduces the ascosporic inoculum at the source or makes use of apple's natural resistance to scab: sanitation,

biological control, scab-resistant cultivars, and plantings of cultivar mixtures with partial resistance.

Apple scab IPM programs in 1987 (Figure 2)

The initial scab programs designated as IPM that were introduced in the early 1980's were centered on a warning system consisting of weather equipment that monitored and recorded the weather variables used to predict Mills' infection periods, a table, graph, or programming built into electronic monitoring equipment to predict Mills' infection periods, strategies to apply fungicide based on the distribution and predicted "severity" of the infection periods, and several means to notify the grower as quickly as possible (e.g., telephone hot line and radio announcements) of current infection conditions (MacHardy 1996). This program was not designed to replace fungicides with other control practices; rather, it was designed to improve the efficiency of using fungicides. The biological basis for this warning system was first reported in 1944 (Mills 1944).

Scab management programs in 1987 were still based on a philosophy of defense, i.e., applying protectant fungicides, and this was true with programs labeled "IPM." The fungicide schedule had improved considerably since 1936, but the strategy to protect the plant with fungicide continued to be defensive. There had also been significant advances in sprayer technology, (e.g., development of the air-blast sprayer), in fungicides (e.g., the discovery and development of organic fungicides), and in horticultural practices (e.g., planting cultivars on semi-dwarfing or dwarfing rootstock) that collectively increased the efficacy of a fungicide application in controlling scab.

Research in the intervening years, 1987-1999 (Table 1)

Contributions to defensive practices

Apple scab management programs in 1987 did provide acceptable control of scab, and orchards following an "IPM" program of scab management did often use less fungicide compared with orchards that scheduled fungicides on a "calendar" or tree fruit-bud development stage with little or no consideration of infection conditions. Nevertheless, research continued to seek improvements in the warning system approach to scab management, as evidenced by the 63 presentations relating to defensive scab management practices. As a result of this research, there were several important advances in scab management programs, notably: (a) electronic weather monitoring instruments and sensors were improved, (b) agrometeorological networks within regions were developed, (c) scab simulation software (e.g., BIOMAT, RIMpro, and VENTUM^{M}) to optimize scab management decision-making was developed, and (d) an action threshold to aid in scheduling fungicides based on a prediction of ascospore dose was developed. There were also studies related directly to fungicides: studies that evaluated the performance of new fungicides and determined their role in scab management programs, evaluated spray application equipment and techniques, examined anti-resistance strategies, and considered the side effects of fungicides on beneficial organisms.

Research results that have had the greatest impact (or have the greatest potential to impact) on defensive strategies present in 1987, mainly through further reduction in the seasonal fungicide dose, are (a) a model that predicts the daily progress of ascospore maturation based on degree-day cumulation from a biofix (bud-break), (b) a procedure to predict ascospore dose in an orchard, and (c) a "reduce-fungicide" action threshold based on

an autumn assessment of leaf scab that identifies a "low-risk" orchard in which several tactics that reduce the recommended seasonal fungicide dose can be employed.

Contributions to offensive practices

Forty-eight presentations were related to offensive approaches to scab management, mostly involving scab-resistant cultivars (37 presentations), with breeding for scab-resistant cultivars the dominant topic (17 presentations). Another 18 related presentations discussed the performance and horticultural characteristics of resistant cultivars, overcoming resistance, resistant pathogen races, resistance gene mapping, and technical impacts of transgenic disease resistant apples. The aim of this research is to eliminate the ascosporic inoculum through the release of cultivars with major gene resistance.

Four presentations considered the potential of planting cultivars with different levels of scab resistance as an approach to reducing the fungicide dose recommended for orchards planted with highly susceptible cultivars. This research is aimed at reducing the ascosporic inoculum by eliminating the proportion of ascosporic inoculum in an orchard that is unable to attack one or more of the cultivars other than the "source" cultivar (or has a lower "infection efficiency" on the other cultivars) and by slowing subsequent disease buildup by increasing the distance conidia must travel between two susceptible hosts. Two presentations reported on differences in susceptibility of apple cultivars and ontogenic resistance.

Seven presentations were on two other offensive approaches aimed at reducing the ascosporic inoculum at the source: sanitation practices (treating the leaf litter with urea or mulching the leaf litter) and biological control (treating the leaf litter with antagonists of the pathogen).

Contributions to our knowledge of the pathogen, pathogenesis, and epidemiology

Thirty presentations were on research that provided new knowledge of the pathogen, pathogenesis, and disease epidemiology. As a result of that research, we now have a better understanding of (a) pathogen virulence and population (race) structure in an orchard, (b) physiological and structural changes that occur during pathogenesis, (c) the relationship of the genetics of the host and pathogen to the interactions that occur during pathogenesis, (d) the production, discharge, and dispersal of ascospores, (e) the ascospore dose in an orchard, and (f) the moisture and temperature conditions required for infection.

Apple scab IPM programs in 1999 (Figure 2)

In 1999, the scab warning system present in 1987 is still the central focus of scab IPM programs, but with a more accurate table to predict infection periods, improved electronic weather monitoring instruments with more accurate sensors, new scab simulation software programs (e.g., BIOMAT, RIMpro, and VENTUM^{M}) and improved delivery systems designed to optimize decision-making and inform the grower. New knowledge of the pathogen has also been incorporated into some programs: a model that tracks ascospore maturation daily through the primary scab season and an action threshold that aids in scheduling fungicides practices in an orchard based on a prediction of ascospore dose.

The impact of apple scab research since the mid-1980's on scab management programs (Figure 3)

Shift in emphasis from a "regional" to "individual-orchard" scab management program

In 1987, apple scab management recommendations were regional: all orchards in a region were assumed to have the same high "scab pressure," i.e., high inoculum dose, and, thus,

should follow the recommended scab management program that had been developed in orchards with high "scab pressure." *Perhaps the most significant impact that research since the mid-1980's has had on scab management programs has been to shift attention to the differences in "scab pressure," i.e., ascospore dose, that exist in individual orchards within a region.* This shift has been accomplished in part through development of a procedure to predict an orchard's level of "scab-risk" (ascospore dose) and a "scab-risk" action threshold for fungicide decision-making based on that prediction. Accompanying these developments was the development of scab simulation software designed to optimize decision-making within an orchard. The software makes use of infection conditions monitored by a weather station in the orchard and other pertinent inputs, and has the potential to factor the level of "scab-risk" and other inputs such as the ascospore maturity model into the decision-making process. Also, it was demonstrated that an orchard with a low "scab-risk" can be managed successfully with a seasonal fungicide dose less than recommended in the regional recommendation. In addition, a "sanitation" action threshold was developed that justifies the use of sanitation practices in a moderate "scab-risk" orchard. Studies that quantified differences in cultivar susceptibility to scab and differences in race composition of the pathogen population within orchards planted with cultivar mixtures also helped to delineate differences in orchards and, in the process, strengthen the biological data base needed to evaluate the potential of using mixed-cultivar plantings as an additional practice to reduce "scab-pressure" in an orchard.

Shift in emphasis from a "defensive" to an "offensive" approach to scab management
In 1987, apple scab IPM programs focused on applying fungicides to protect the trees from infection by ascospores that were being discharged into the orchard air. There was no attempt to reduce the supply of ascospores at the source. This approach is still the focus of nearly all scab IPM programs, but the most-advanced programs in 1999 have shifted the focus to sanitation practices that reduce the supply of ascospores. This has been accomplished by determining the percentage reduction in ascosporic inoculum resulting from mulching or treating the leaf litter with urea and using that percentage reduction in inoculum to establish a "sanitation" action threshold that justifies the use of sanitation practices in orchards identified as "moderate scab-risk." Renewed interest in using biological control agents to attack the overwintering stage of the pathogen (e.g., employ antagonists of *V. inaequalis*) or reduce the source of ascosporic inoculum (e.g., enhance leaf litter removal by earthworms) and in making use of cultivar mixtures to reduce the number of primary infections are also evidence of increased interest in shifting the management focus to *offensive* practices. Collectively, these research programs have laid the foundation for the development of scab management programs well into the 21st Century.

How have the objectives of the IOBC subgroup "Orchard diseases" agreed upon in Lana been addressed with respect to apple scab?

All nine objectives agreed upon at the Lana Workshop in 1987 have been addressed, but not with equal intensely or success, as seen in the following summary statements relating to each objective :

Obj. 1 : Fungicide usage has been researched intensely, and as a result has become more efficient since 1987. The number of fungicide applications and the seasonal fungicide dose have often been reduced, but not greatly, since 1987, and it appears to have reached a plateau.

Obj. 2 : A search for biological control agents has been limited to two research units, and not until the mid-1990's, so there is much remaining to do with this objective.

Obj. 3 : Breeding for durable resistance has been intensive against scab but limited against multiple diseases. New scab-resistant varieties have been released, but they have not been readily accepted into commercial apple production.

Obj. 4 : Efforts to make chemical and non-chemical methods compatible have been limited to one research program that integrated sanitation practices and fungicide applications in a way that supplemented one or more fungicide applications with sanitation.

Obj. 5 : The side-effects of fungicides on beneficial organisms is another area that has received little attention, but undoubtedly this area will receive greater attention as adherence to ICP Guidelines becomes more critical and beneficials harmful to arthropod pests, but harmed by fungicides, assume a greater role in insect pest management.

Obj. 6 : The development and implementation of disease forecasting and infection warning systems has been one of the most intensely investigated objectives, and the investigations are largely responsible for the changes that have occurred in scab IPM programs since 1987.

Obj. 7 : The development and use of pathogen and disease assessment methods has been investigated intensely by one research program, and has resulted in an assessment method to predict ascospore dose in an orchard.

Obj. 8 : One research program has developed action thresholds for applying sanitation practices and scheduling fungicide applications, but to date the thresholds have not been adopted outside the region in which the thresholds were developed and are unlikely to be adopted in other regions until the thresholds have been tested in each region and validated or revised.

Obj. 9 : The exchange and dissemination of information has occurred mainly through the IOBC Workshops and their published Proceedings. In addition, a group of apple consultants and researchers from seven countries within the region have formed a "grass roots" organization that meets annually to discuss a wide range of topics including warning systems, scab simulation programs, weather monitoring equipment and associated scab prediction software, scab management strategies, demonstration plots, and fungicide efficacy trials. Also, several research units are active in D.A.R.E. (Durable Apple Resistance in Europe), a European project to develop durable resistance of apple to scab and mildew.

Insights into the next "level" of apple scab IPM programs

Apple scab IPM programs have improved steadily over the approximately 20 years since apple scab IPM programs were first introduced, but this review has identified several shortcomings in current scab IPM programs and research programs that, if ignored, may hinder their continued development over the next 20 years:

(i) the defensive philosophy of control remains the dominant approach, with reliance on a single measure (fungicide application) rather than an integration of management practices (e.g., sanitation and scab resistance complementing fungicide usage),

(ii) improvements in scab warning systems during the past 12 year have not resulted in a significant reduction in the number of fungicide applications or the seasonal fungicide dose,

(iii) efforts to integrate scab management practices and decision-making with that of other diseases, with insect pest management programs, and with integrated fruit production (IFP) guidelines has been minimal, and
(iv) the economics of scab management programs has been largely ignored.

There are several reasons besides environmental and human health concerns that justify increased efforts to reduce the seasonal fungicide dose in an orchard:

(I) pathogen resistance to several important fungicides is becoming more of a problem, and implementing fungicide-resistance strategies is becoming more complicated as insect and disease IPM programs become more complex,
(ii) 8-10 applications of fungicide throughout the season to control scab are not unusual, even in programs labeled "IPM," and this is counteractive to arthropod management tactics that are becoming increasingly more "biologically-based," as several fungicides are harmful to beneficials introduced to manage important insect and mite pests, (iii) combinations of fungicides are applied more frequently, and this is increasing the seasonal fungicide dose and pest management costs, and
(iv) fungicide is accounting for a much greater proportion of the yearly pesticide dose in many apple IPM programs in recent years because of the success of entomologists in developing economic thresholds for making spray decisions and replacing insecticides and miticides with beneficials, and this is placing a greater burden on plant pathologists to improve disease management programs for compliance with IFP and ICP guidelines.

Reducing fungicide to control scab does, however, have one major drawback that must not be overlooked: diseases considered "minor" because they were being kept in check by fungicides applied to control scab may increase to a level that will require one or more fungicide applications. There is one other consideration that limits the extent to which the seasonal fungicide dose can be reduced : the disease complex in an orchard may require a fungicide to control a disease other than scab even though a fungicide to control scab could be eliminated.

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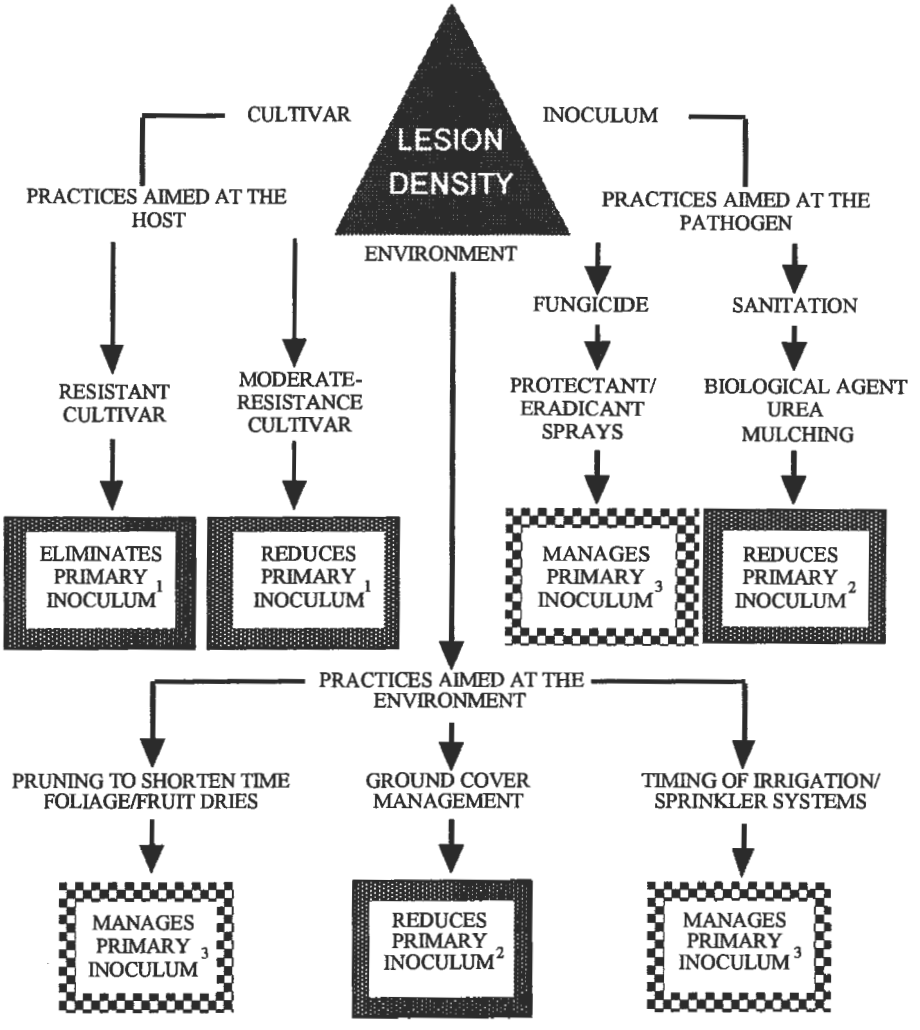


Figure 1. How management practices aimed at the host, the pathogen, and the environment control of primary apple

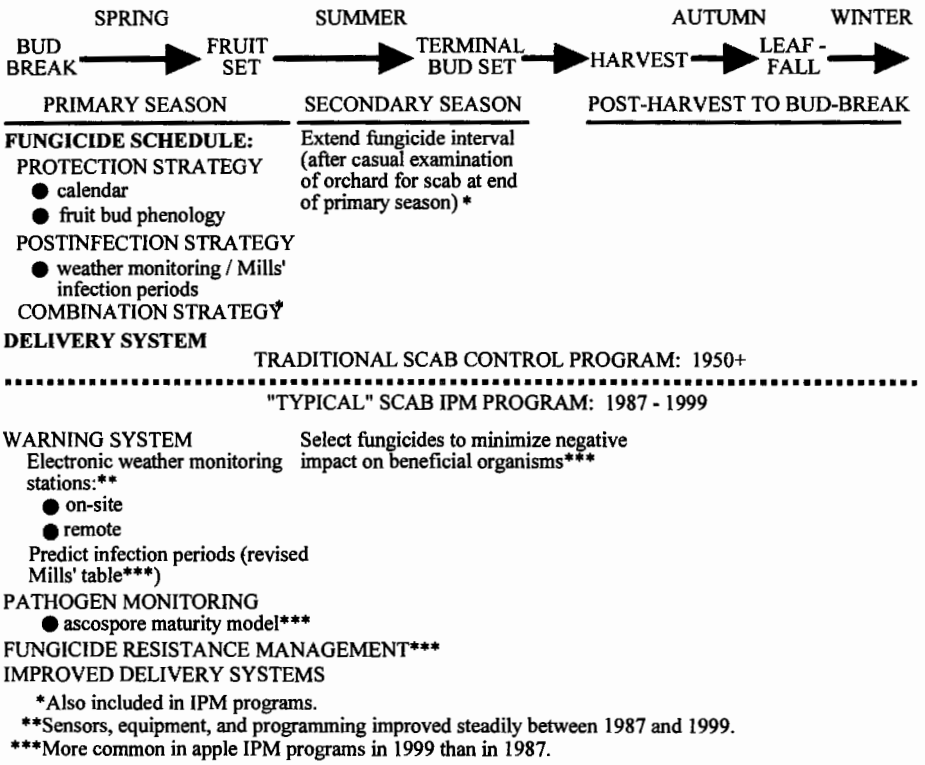
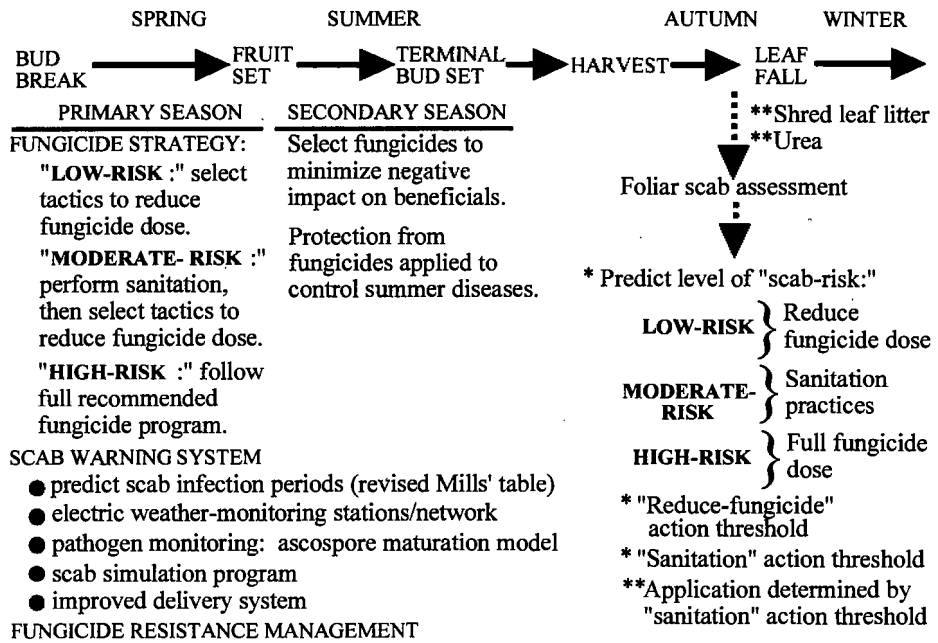


Figure 2. Traditional and IPM programs to control apple scab. The IPM program places greater emphasis on a warning system to aid in decision-making to schedule fungicide sprays.

Table 1. Presentations on apple scab at IOBC "Integrated Control of Pome Fruit Diseases" Workshops in Lana, Italy (1987), Brissago, Switzerland (1988), Gödöllo, Hungary (1990), Lofthus, Norway (1994), Croydon, England (1996), and Fontevraud-l'Abbaye, France (1999).

PRESENTATIONS RELATING TO :	Lana 1987	Bris-sago 1988	God-ollo 1990	Lof-thus 1993	Croy-don 1996	Fonte-vraud 1999	TOTAL
DEFENSIVE SCAB MANAGEMENT PRACTICES							63
Weather monitoring	1	4	0	3	1	0	9
Warning systems / scab prediction / disease simulation	4	0	4	9	5	1	23
Scab management programs	4	2	3	2	0	0	11
Fungicides/strategies / performance / mode of action	1	1	1	5	4	1	13
Fungicide resistance / resistance management	0	0	0	2	1	1	4
Action thresholds	0	0	0	2	1	0	3
OFFENSIVE SCAB MANAGEMENT PRACTICES							48
Eliminating ascosporic inoculum:							
scab-resistant cultivars	5	8	2	4	6	12	37
Reducing ascosporic inoculum:							
partial resistance - mixed plantings	0	0	0	2	1	1	4
sanitation	0	0	0	1	1	0	2
biological control	1	0	0	0	1	3	5
OBTAINING NEW KNOWLEDGE ON							33
The Pathogen: virulence / taxonomy / population genetics	0	0	0	1	4	0	5
Pathogenesis: resistant/susceptible interactions / physiology / wall-degrading enzymes	1	2	1	2	2	2	10
Epidemiology: pathogen development / life cycle / infection conditions / spore trapping	1	0	2	5	4	6	18
THE BROADER PICTURE							12
Side-effects	2	3		1	0	1	7
Relationship to IPM, ICM, IFP, and quality assurance programs	0	0		4	1	0	5



IPM PRACTICES/PROCEDURES AVAILABLE BUT NOT WIDELY USED OR ARE BEING RESEARCHED

PRIMARY SEASON	SECONDARY SEASON	POST-HARVEST TO BUD-BREAK
Pathogen monitoring: pseudlthecial squash mounts.	Lesion threshold for fungicide program in summer.	Biological agent antagonistic to *V. inaequalis*.
Scab-resistant cultivars.		Microorganism decomposition of leaf litter.
Mixed plantings of cultivars with partial resitance to scab.		Earthworm removal of leaf litter.

Figure 3. Features of the most advanced apple scab IPM programs in 1999.

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Acremonium strictum: a potential antagonist of *Venturia inaequalis*

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Abstract : In an Orchard conducted after the biological production guidelines in Austria, a whitish cover was found on apple scab lesions. From such lesions a fungus was isolated and identified as *Acremonium strictum.* Spores of this isolate of *A. strictum* when applied on apple scab lesions led to a whitish mycelium covering entirely the lesion and inhibiting sporulation of *V. inaequalis.* Apple scab lesion development was inhibited in glasshouse tests by the fungus if spores were applied prior or contemporaneously to those of *V. inaequalis.*

Introduction

A whitish deposit on old apple scab lesions was observed by K. Schloffer in an Austrian orchard near Gleisdorf (47°5' N, 15°42'E) in September 1995. The orchard was conducted after the biological guidelines. On a nearby conventional orchard secondary scab was much more frequent without however this whitish deposit was observed. This gave the impression that the agent causing the whitish cover suppressed the development of secondary scab. As scab control is a problem in biological conducted orchards and in future even more if copper fungicides will be prohibited, we were interested to test the potential of this agent as biocontrol of scab.

Material and Methods

Leaves with scab lesions covered by a whitish deposit were furnished by K. Schloffer.

For isolation PDA, V8, Malt extract, King B and K_2HPO_4 agar at the indicated rates were used. On these agars leaf segments where distributed, after 72 h at 20 °C single fungal colonies were transferred to new petridishes until a single type of fungus/petridish could be observed. Of all types of fungi isolated a spore suspension was made and applied directly on 2 week old scab lesions on Boskoop, James Grieve and Spartan. From all lesion on which the whitish cover was again observed the causing agent was reisolated and compared to the original isolate.

To determine the potential to reduce scab, spores of the isolate determined as the causing agent were applied as water suspension ($5^* 10^7$ conidia/ml) at six times points, 9, 2 days prior, contemporaneously and 2, 7 and 13 days after inoculation with *V. inaequalis* of young leaves of potted one year scions of the rootstock M26. After inoculations the trees were kept for 48 h at 100% rH at 18 °C and at ca 80% afterwards.

All trees were drop-inoculated with *V. inaequalis* (250'000 c/ml) at a particular date and kept again for 48 h at 100% rH. Scab lesions development was observed at different times.

Results and Discussion

From the several fungi which were isolated from the apple leaves only the isolates determined as *Acremonium strictum* Gams (syn *Cephalosporium acremonium*) *Moniliales* were able to reproduce the same symptoms on scab lesions. This fungus, already known to colonise other fungi (McGee *et al*, 1991), can be cultivated on Maltagar and sporulates abunantly with single celled tiny hyaline conidia (2 µm). The conidia are formed endogenously in tubes, which are not clearly as such differentiated and are dispersed on a thin mycelium departing at a right angle from the carrying hyphas.

After 5-7 days the scab lesions were covered with a whitish deposit from which *A. strictum* could be again isolated or identified. No development was ever noticed outside of the scab lesions.

From these results we estimated that this fungus had the potential to control scab development. A field assay in Austria with an artificial inoculation of scabbed trees however was unsuccessful. A second set of experiments was made to determine in controlled conditions the optimum application time in relation to the age of the scab lesions.

Trees treated with *A. strictum* at the two dates (-9 and -2 days) before the scab inoculation showed significantly less and less severe lesions than untreated trees (table 1). In trees treated at the scab inoculation time or shortly afterward (+2 days) scab lesion number was similar as the control however with a reduced number of sporulating lesion. Later *A. strictum* (+7 and +13) treatment had no clear effect on lesion severity. As soon as the trees came out of the high humidity to the normal condition of the greenhouse (60% rH) the just visible whitish cover of the already present lesions disappeared or did not increase.

The results from the treatment with *A. strictum* 9 days prior to the scab infection, are most probably biased as the leaves were older than the ones inoculated in the other treatments and in the control. Ontogenic resistance was most probably already limiting scab infections.

From this preliminary experiments we conclude that a) the used isolate of *A. strictum* has the potential to colonise specifically scab lesions at continuously high humidity; b) it is capable of surviving a limited period on leaves without scab; c) it may impede scab conidia to infect the leaves.

Recently we were able to identify the same fungus on leaves collected in scab screening test of apple seedlings in a green house (material furnished by M. Goerre and M. Kellerhals).

Table 1. Effect of *Acremonium strictum* on scab severity on M26 drop inoculated with *V. inaequalis*. Severity was evaluated on 5-8 inoculation sites and 4 to 8 trees per treatment with 0 = no symptom 1 = chlorotic or small necrotic flecks, 2 = clear chlorotic and necrotic lesions, 3 = with barely detectable sporulation and 4 = clear sporulation. Statistical data was calculated with one tree as unit. Evaluations were made after 13, 15, 19 and 26 days after the inoculation with *V. inaequalis* and scores averaged.

Inoculation date with *A. strictum*, 0 = inoculation date of *V. inaequalis*	Severity (scale 0-4)	Standard Deviation	1-p (probability to be different from Control)
-9	0.64	0.34	0.995
-2	1.36	0.55	0.945
0	1.91	0.56	0.635
+2	1.97	0.75	0.52
+7	2.35	0.87	0.039
+13	2.51	0.86	0.27
Control	2.32	0.77	

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Prebloom fire blight symptoms on *Crataegus, Cotoneaster* and apple

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Abstract : Twig cankers have long been considered as potential sources of inoculum in spring in the USA. This has been confirmed in England by field observations on hawthorns (*Crataegus* spp.) and on apple twigs in greenhouse experiments. Ooze production and stem invasion beyond the canker margin were seen from the time of bud-swelling onwards followed by invasion of newly emerging leaves and flower bud clusters (indirect infections). Twig cankers develop following late season infections. On hawthorns, September storms seemed to have a role. With bacterial cankers of cherry and peach, autumn leaf trace infections are often associated with storms with wind blown rain which tear off leaves. Twig cankers are often difficult to detect and remove during winter pruning. Direct prebloom infections of young tissue are possible from bud burst onwards but symptoms will rarely be seen before bloom.

Key words : *Erwina amylovora*, fire blight control, storm damage, twig cankers.

Introduction

This account of prebloom disease in fire blight focuses attention on overwintering twig cankers which are often as small as 2-6mm in diameter. On pear trees in California, twig cankers may develop when there are late blossom infections between the end of August and early October (Thomas and Ark, 1934). On apple trees they are usually associated with late shoot infections. On hawthorns in England, mature terminal buds are often seen on twig cankers (Billing *et al.*, 1974; Billing, 1978). Because infections occur late in the growing season, typical symptoms may not develop. Such cankers are likely to remain unsealed (indeterminate) and many may be undetectable until after bud-break the following spring so their complete eradication is impossible.

Schroth *et al.* (1974) cite seven early USA workers who studied twig cankers and Thomas and Parker (1933) two more. Some noticed considerable between-season variation in their incidence and in the frequency with which the pathogen could be isolated during winter and spring. Their relative importance compared with larger cankers remained uncertain, but most were sure that they should not be disregarded. Given favourable conditions during bloom, only a low level of primary inoculum is needed for epidemic blight to develop. Those assessing risks from overwintering cankers often concentrate on the blossom period. Good field records for the prebloom period are rare. Where field records are lacking, greenhouse experiments can be useful for exploring possibilities. This paper gives an outline account of field observations and greenhouse experiments in England which will be published more fully elsewhere.

Materials and methods

Field observations
In England, the incidence of fire blight is usually low so opportunities for studying prebloom disease are rare, even on hawthorns, the main host observed. The period of observation was mostly March to June (post-budburst to the end of bloom). Symptoms of late twig blight were rarely evident until the following spring. In April 1999, observations were possible on a *Cotoneaster bullatus* bush.

Weather analyses
Risks of late season infections and unsealed cankers and new direct infections the following spring were assessed using BIS (Billing's Integrated System, Billing, 1999).

Greenhouse experiments
The first aim was to simulate late season shoot infections provoked by storm damage (hail or strong winds). Leaves on near mature shoots were torn off and a drop of inoculum placed on the exposed leaf trace in September. The experimental plants were M26 apple rootstocks grown to a single shoot and hawthorn seedlings. In later experiments, potted pear and apple trees were used to study infections at budburst. A drop of inoculum was placed on bursting flower buds at green tip after prior wetting to simulate spread of ooze by rain. Inoculum used was a highly virulent culture of *Erwinia amylovora* at a concentration of 10^8-10^9 cfu/ml.

Results and discussion

Field observations
Twig cankers on hawthorns were most easily detected from budburst onwards. Their incidence varied widely between seasons and between trees. Ooze was occasionally seen on the twigs but the main sign of disease was invasion of emerging leaves and flower bud clusters below the canker, sometimes with ooze production. Stem invasion had often ceased by blossom time as illustrated in papers by Billing *et al.* (1974) and Billing (1978). On the cotoneaster observed in 1999, twig cankers extended into developing leaves which often had ooze droplets on their undersides. For several years when twig cankers were common, there had been storms with wind gusts of 15m/sec or more the previous September.

On hawthorns there is considerable leaf development before bloom and evidence of direct prebloom infections of shoot tips and flower bud clusters was seen.

Greenhouse experiments
Typical twig cankers were produced on hawthorn seedlings (Billing, 1978) and on apple shoots following September inoculations.

Of 30 active apple twig cankers, 25 showed ooze, before budburst in late March, above the canker margin. During the leaf and shoot growth period ooze production was mostly below the canker margin. By May, developing leaves nearest to the canker had been invaded leading to ooze production and necrosis. By then, the appearance of these plants was comparable to photographs of natural apple tree infections published by Miller (1929), Rosen (1929) and Pierstorff (1931).

Pear and apple trees, where bursting buds had been inoculated, showed signs of ooze production and necrosis of young tissue at or before the time the first flowers opened. On pear, stipules and leaf tips were commonly affected as well as flower buds. On apples, sepal tips were commonly infected. Symptoms seen were comparable to those described by Rosen

(1929) and Miller (1929) following spray inoculations of pear flowers and apple blossom buds respectively.

Few early fire blight workers doubted the potential importance of overwintering twig cankers in some seasons and some thought they were too often disregarded (Thomas and Ark, 1934). Twig cankers are difficult to detect (Schroth *et al.,* 1974) and they are often high in the tree. Small ooze droplets are best seen before sunrise. Some believe that the cankers are not often active before bloom (Thomas and Parker, 1933) but observations described here for hawthorn and apple twig cankers warn that they may produce ooze and extend long before leaf development and bloom.

The incidence of twig cankers in any one season will depend on opportunities for infection from September to October, not at normal leaf fall time (Paulin *et al.,* 1984). A high incidence is most likely when there are late flowers on pears or late shoot growth and when there are storms which forcibly remove leaves prematurely and expose susceptible leaf traces. Culturing the pathogen from twig cankers in winter and spring has shown that survival rates can vary widely; they seem to be highest on more susceptible species and cultivars (see authors cited by Schroth *et al.,* 1974). Survival of the pathogen does not ensure canker activity in spring. It is worth comparing fire blight with other bacterial diseases such as cherry canker (*Pseudomonas syringae* pv. *morsprunorum*) and peach canker (*Xanthomonas campestris* pv. *pruni*). In both, leaf trace infections occur in autumn, prior to normal leaf fall, when rain plus strong winds spread inoculum, forcibly remove leaves and expose leaf traces. The pathogen may be sucked into xylem vessels but cankers will only form when parenchymatous tissue is invaded (Crosse, 1955, 1956; Feliciano and Daines, 1970). Autumn leaf trace infections are also important in pear and apple cankers caused by *Nectria galligena* but in this case infection may occur at normal leaf fall (Swinburne, 1975).

Few people consider risks of direct infections prebloom from budburst onwards. As with twig cankers, absence of evidence is not evidence of absence. Such infections may not be evident before bloom and they may be indistinguishable from early open flower infections.

Conclusions

Complete eradication of overwintering twig cankers is impossible. Assessment of field and weather-related risks in autumn and the following spring could focus attention on high risk seasons. Well timed copper-based spray applications post harvest and from bud swelling to bud burst in spring might help to reduce inoculum levels and infection risks.

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Study of spatial distribution of *Venturia inaequalis* ascospores in a commercial apple orchard

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Abstract : In most parts of the world, where apple scab is important, numerous fungicide applications are required for its control. These chemicals are costly and are becoming less effective due to the development of resistance in the populations of *Venturia inaequalis* (Cke) Wint. Despite the tremendous amount of research on scab management, these fungicide applications are applied on a calender basis or according to infection criteria. Very few strategies include the amount of inoculum present in the orchard as a criteria for fungicide applications. Our working hypothesis is that better timing of fungicide applications could be achieved if the airborne ascospore concentration was considered in the decision making. Volumetric spore traps can be used to measure the airborne ascospore concentration in real time, directly in the orchard. However, the level of heterogeneity of the ascospore concentration in commercial orchards is unknown. Consequently, it is currently impossible to determine the number of volumetric spore traps required per unit area of orchard to obtain a representative value of the ascospore concentration. It is therefore important to study spatial distribution of airborne ascospore concentration in order to evaluate the reliability of the spore traps. The aerobiology of *V. inaequalis* was studied in terms of a dispersal gradient from a point source, but the pattern of distribution of the ascospores is still unknown. The spatial distribution of ascospores was studied in a commercial apple orchard of Dunham, Quebec, Canada. A 0.5 ha orchard plot was split into 40 quadrates of 13.5m X 10m. In each quadrate, one volumetric spore trap was used to monitor the airborne ascospore concentration during all major rain events of spring 1999. For each quadrate, a fall scab assessment on leaves and a spring leaf litter density using the point-intercept method were also done to establish the potential ascospore dose expressed as ascospores/m^2. The correlation between the potential ascospore dose and the airborne ascospore concentration for each quadrate was established as well as the pattern of aggregation.

Key words : *Venturia inaequalis*, ascospores, spatial distribution.

Introduction

Apple scab is a major disease in most apple growing area of the world. Despite the tremendous amount of research on apple scab (MacHardy, 1996), chemicals are still the main method of control, as they are efficient and easy to use. However, there are some limitations to the extensive use of pesticides. Selection of resistant strains of *Venturia inaequalis* (Cke) Wint. (Köller *et al.*, 1991), the negative impact on the environment and an important production cost for the producers (MacHardy, 1996) are the main limitations. It is possible to reduce the number of fungicide applications by improving their timing. This could be achieved by taking into account the availability and amount of primary inoculum. However, very few strategies of apple scab management take into account the primary inoculum. One of the main methods of inoculum assessment is the potential ascospore dose technique (PAD) (MacHardy and Jeger, 1983). This method is based on a fall assessment of scab and allows the prediction of the number of ascospores that could be released per m^2 of orchard the

following spring. Experiments have shown that under low inoculum pressure, it is possible to delay the first fungicide treatment (MacHardy *et al.*, 1993). This technique provides a single prediction for the whole season, thus it is hard for a producer to know exactly when the ascospores ejection period is initiated and how many fungicide applications could be eliminated without taking unacceptable risks. This is why spore sampling could be an appropriate way of primary inoculum assessment. The advantage of this method is that it provides a measurement of airborne ascospore concentration for each rain event, in real time and specific to an orchard site. It can provide information on the onset of ascospore liberation, the amount of inoculum available and on the date of ascospore depletion. The utilisation of spore trapping in apple scab management is based on the assumption that the airborne ascospore distribution is uniform. However, very little is known about airborne ascospore distribution (Aylor, 1998) especially under commercial conditions.

The overall objective of this research was to study the spatial distribution of *Venturia inaequalis* ascospores under commercial conditions. More specifically objectives were to compare the distribution of the potential ascospore dose and the distribution of the aerial ascospore concentration.

Materials and Methods

Experimental orchard block

A commercial orchard located in Dunham, Qc., Canada was selected as representative of an average Quebec orchard. A scab evaluation was done in August to make sure that a detectable level of inoculum was present in the orchard. The orchard block was approximately 1 hectare and composed of 28 rows of about 40 semi-dwarf trees grafted on MM 106 planted in 1988. The spacing was 2 m between the trees and 4.5 m across the rows. The orchard was composed of 3 cultivars: Red Delicious, McIntosh, and Lobo. In the middle of the orchard, a block composed of 3 rows of Red Delicious, 9 rows of McIntosh and 3 rows of Lobo was divided into 40 quadrates, each containing 12 trees (4 trees on 3 rows), see Figure 1 in annexe.

Potential ascospore dose

In order to measure the potential ascospore dose (PAD), (Gadoury and MacHardy, 1986; MacHardy and Jeger, 1983) a fall scab assessment was done on October 5, 1998. In each quadrate, three preselected trees from different rows and 8 randomly chosen shoots per tree where assessed for the number of scab lesions per leaf. To obtain a good representation of the total amount of scab in the orchard, shoots from the top, bottom, inside and outside portion of the trees were assessed. Sucker shoots and the top of the tree were also assessed because scab is more likely to be present on this fast growing, susceptible tissue often with poor spray deposition. Data included the total number of lesions, the number of scabbed leaves, and the total number of leaves on every shoot. In the spring, an evaluation of the leaf litter density was done on April 20, 1999, using the point-intercept method (Mueller-Dombois and Ellenberg, 1974). Presence or absence of apple leaves was record at 100 point intercepts, corresponding to every 20 cm of 2 lines placed in diagonal in the middle of each quadrate. The scab assesment and the leaf litter density were used to calculate the potential ascospore dose for each quadrate.

Airborne ascospore concentration

To measure the concentration of airborne ascospores, one volumetric spore trap (Figure 2) was placed in the middle of each of the 40 quadrates. A CR-10 data logger (Campbell Scientific, Edmonton, Canada) was used for the activation of the spore traps at the beginning of the rain. The data logger was programmed to activate the spore traps only during daytime

(5h00 to 22h00). They turned at 2400 RPM for a maximum of 6 hours after the beginning of the rain to avoid excessive accumulation of dust on the rods.

Between the period of May 5 and June 16, 1999, the concentration of ascospores in the air was measured during 5 major rain events. The number of ascospores were counted on the plastic rods with a transmitted light microscope at a magnification of 250X.

Data Analysis

First, an analysis of variance was carried out to test the effect of location and cultivar for both the potential ascospore dose and the airborne ascospore concentration, for each rain event. Correlation between the total seasonal airborne ascospore concentration and the potential ascospore dose for each quadrate was also done to compare the spatial distribution of fall scab and the spring airborne ascospore spatial distribution. Those tests were done using the SAS software (Statistical Analysis System) version 6.12. Data were then analysed using Poisson probability distribution to evaluate randomness and negative binomial distribution to evaluate the aggregation (Campbell and Madden, 1990) of airborne ascospore concentrations. Parameters of the selected probability distribution were estimated and chi-square goodness-of-fit test was applied to determine if the expected frequency distributions significantly differed from the observed distribution (Campbell and Madden, 1990).

Results and discussion

The potential ascospore dose varied among quadrates with a minimum value in quadrate A1 of 49 and a maximum value in quadrate B2 of 17893 ascospores /m² (Figure 3). The airborne ascospore concentration varied among cultivars for each rain event (Figures 4 to 6). The minimum and maximum airborne ascospore concentration for the major ascospore ejection events are presented in Table 1.

Table 1. AAC (asc/m^3) for the major ejection event of 1999

Ejection event	Minimum	Maximum
May 5 1999	A2: 0.6	C3: 14.2
May 8 1999	E5: 2.5	B4: 20.8
May 25 1999	A3: 0.1	C5: 11.4
May 27 1999	D7: 0.4	E4: 3.1
June 8 1999	A2: 0.4	C8: 6.9

There was a significant positive correlation between the total seasonal airborne ascospore concentration (AAC) and the potential ascospore dose per quadrate, $r= 0.73$ ($P< 0.0001$).

The frequency of AAC did not follow a Poisson probability distribution indicating a non-random pattern of distribution for all rain events. Each ascospore ejection event followed a negative binomial distribution. Therefore, we concluded that the airborne ascospore concentration had an aggregated pattern. (Figures 4-6).

An example of negative binomial distribution analysis is presented in Figure 7. There was no significant difference ($P=0.85$) between the observed frequency of the airborne ascospore concentration classes on May 25 and the predicted frequency of the negative

binomial distribution, indicating that the spatial distribution of ascospore was aggregated on that rain event date.

The correlation between the potential ascospore dose and the total airborne ascospore concentration indicated that the distribution of scab incidence in the fall corresponded to the distribution of the airborne ascospore concentration in the following spring. However, the pattern of potential ascospore dose distribution was not aggregated, as was the airborne ascospore concentration for each rain event. This could be explained by the fact that the PAD represents the whole seasonal potential in comparison to each ejection event representing only part of the seasonal ascospore ejection which will vary overtime.

Significant differences in AAC were observed among the cultivars. Rows of Lobo and Red Delicious were less infected than McIntosh rows, corresponding to the area of orchard where the least concentration of spore were measured.

Further geostatistical analyses will be done to establish the number of spore traps required to obtain a representative value of the ascospore concentration in a determined orchard area.

Acknowledgement

We would like to thank Isabelle Lahaye and Martin Larivière for their technical support especially for the ascospore counting, and Claude Tougas for allowing us to use his orchard.

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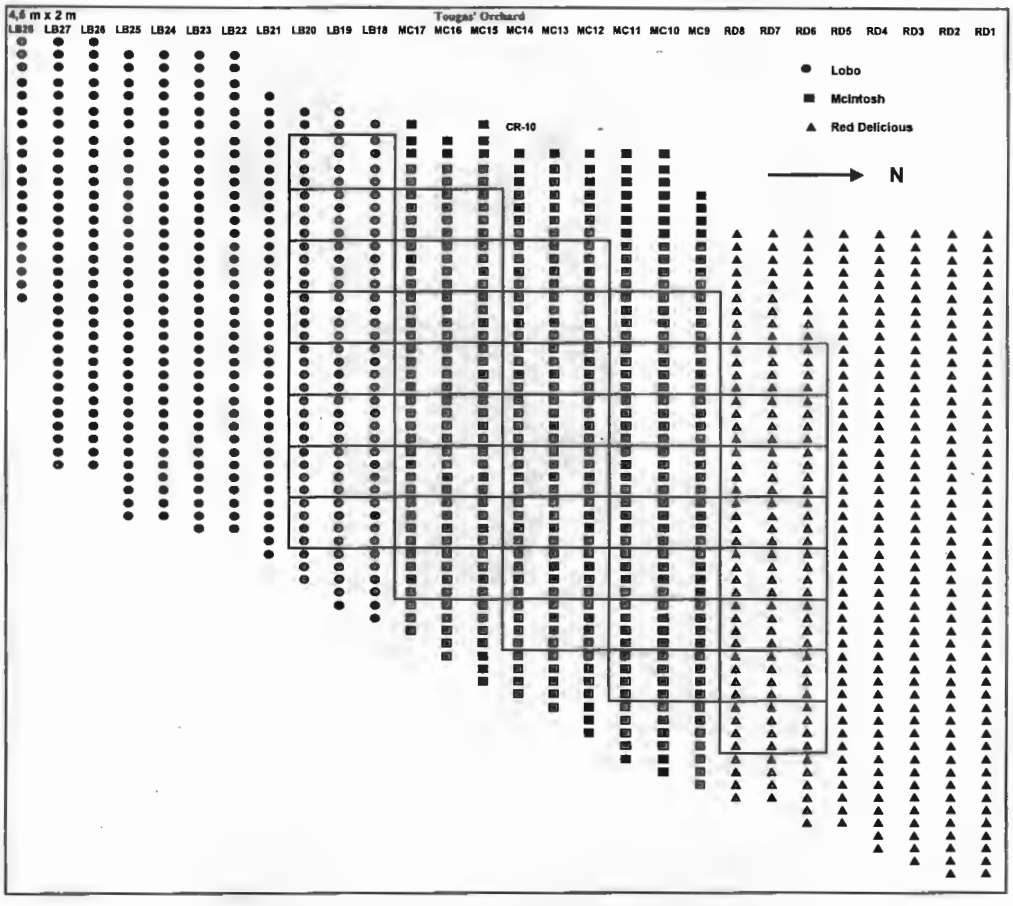

Figure 1. Experimental block. Orchard of Dunham, Quebec, Canada. Semi-dwarf trees, 3 cultivars

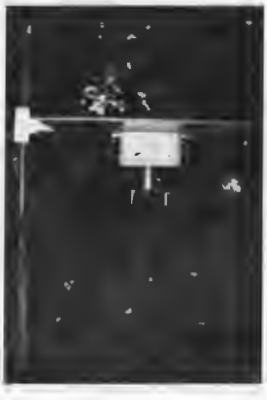

Figure 2 : Rotorod type spore sampler

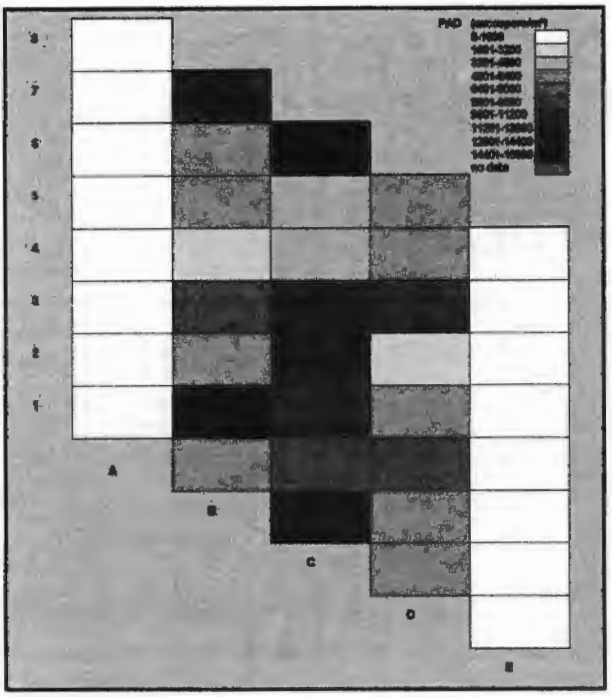

Figure 3. Potential ascospore dose distribution

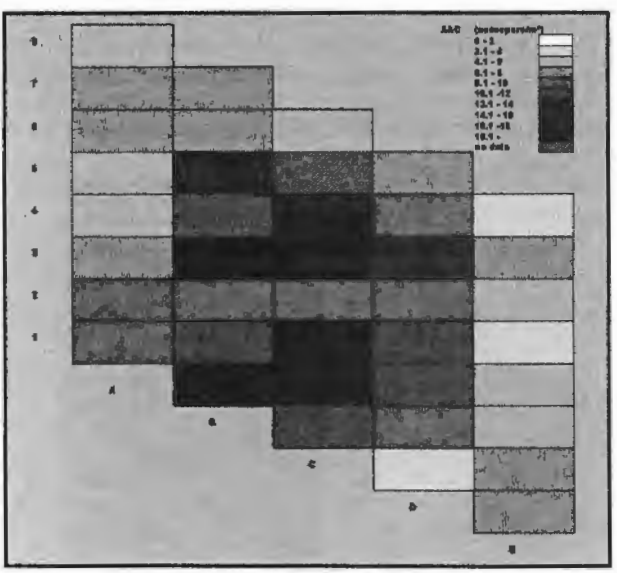

Figure 4. Airborne ascospore concentration on May 8, 1999

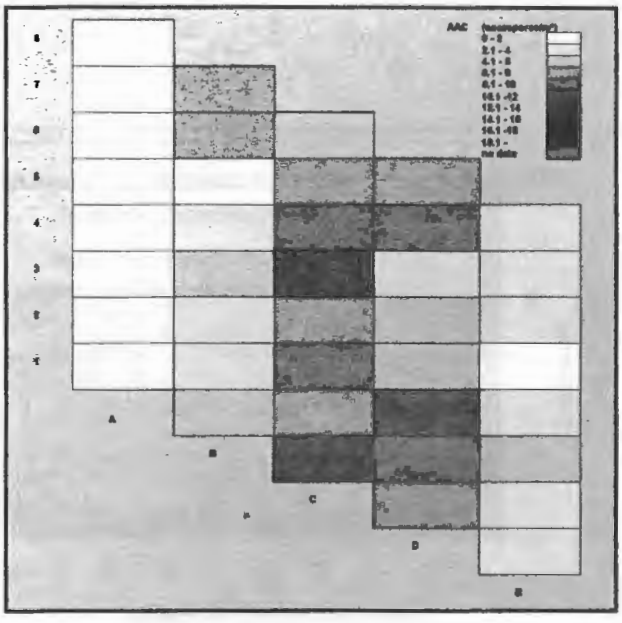

Figure 5. Airborne ascospore concentration on May 25, 1999

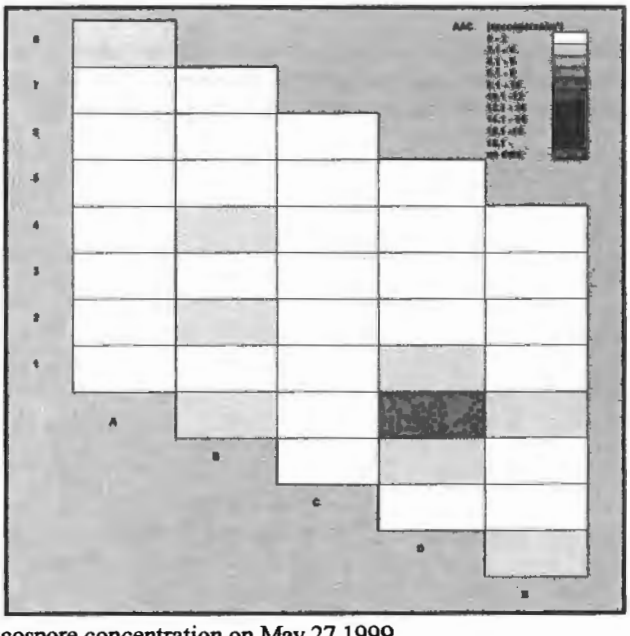

Figure 6. Airborne ascospore concentration on May 27,1999

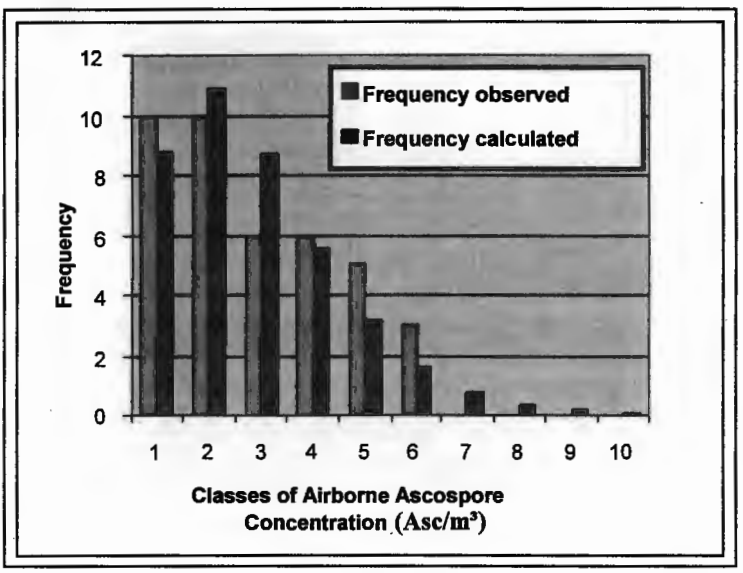

Figure 7. Frequency distribution of AAC classes. Observed value on May 25th 1999 and the predicted values of the negative binomial distribution. The AAC is divided into 10 classes ranging from 0 to 20 ascospores/m^3 with intervals of 2 asc./m^3

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Relative cultivar susceptibility to *Venturia inaequalis* ascospores under greenhouse conditions

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Abstract : In eastern North American apple culture, apple scab is the disease of greatest importance. The majority of the experiments on apple scab conducted in North America have used the highly susceptible cultivar McIntosh. Very little is known about the susceptibility of other North American commercial cultivars. It is therefore important to investigate the ability of *Venturia inaequalis* ascospores to infect these cultivars. The relative susceptibility of 21 common cultivars of central and eastern Canada to *V. inaequalis* was tested under greenhouse conditions. The cultivars used in the experiment were Cortland, Early Geneva, Empire, Golden Delicious, Golden Russet, Idared, Jersey Mac, Jonagold, Jonamac, Lobo, Lodi, McIntosh, Mutsu (Crispin), Northern Spy, Paulared, Red Cortland, Red Delicious, Royal Gala, Spartan, Sunrise and Vista Bella. The trees were inoculated with highly infected McIntosh leaves that were overwintered naturally. The leaves were placed on a screen 0.5 m from the tops of the trees and the ascospores were allowed to infect under optimal conditions. The ascospore concentration was measured with volumetric spore traps. The experiment was conducted as a completely randomised design. The factors of infection studied were disease severity, incubation time, lesion size, and conidial production. Disease severity was evaluated as the number of lesions per cm^2 of leaf. Leaf area was measured at both time of infection and lesion count. Incubation time was defined as the length of time from inoculation until the appearance of the first lesions. The average lesion size of five lesions was taken 28 days after infection, before the lesions began to coalesce. Conidial production was assessed at 28 days after inoculation by counting the number of spores produced per lesion/leaf. The difference among the cultivars was tested for each variable and multivariable grouping. A hierarchical pairwise comparison test was also carried out to rank the cultivars.

Key words : *Venturia inaequalis*, ascospores, cultivar, apple, scab

Introduction

Apple scab, one of the greatest problems in apple management through out the world, is equally the major disease in northeastern North America. Throughout the century there has been an effort to breed resistant cultivars but unfortunately none to date have been a commercial success in the North American market (MacHardy, 1996). There are several reasons why these cultivars have not been grown on a wide scale basis. Resistant cultivars have a reputation of having low fruit quality, poor storability and the most limiting of all, low yield (MacHardy, 1996). Resistance stability is another reason. Growers tend not to be willing to invest in a cultivar where the resistance is not known to be stable. There is increasing evidence that major gene resistance is being overcome by new races of *Venturia inaequalis* (Cke.) Wint.. An example of this is race 6, discovered by Parisi *et al.*, 1993a, which was able to overcome the resistance in a Prima X susceptible cross. For these reasons, producers in eastern Canada have not adopted resistant cultivars.

Despite the wide range of cultivars available to producers in North America, there have been very few studies on their relative susceptibility. A survey of specialists in apple diseases

was conducted by Aldwinkle in 1974. There were also some experiments undertaken by Szkolnik (1976) and Smith (1992) that attempted to rank cultivars but unfortunately they were never published in scientific journals.

The control recommendations used in North America are based on epidemiological studies that have used McIntosh, assuming that it is representative of most varieties (CPVQ, 1988; MacHardy and Gadoury, 1989; Mills and Laplante, 1951), but proof of this assumption is lacking. As the Canadian apple industry changes from being McIntosh based to more a diverse selection of cultivars, management strategies may vary for the cultivars depending on their relative susceptibility compared to McIntosh. The number of fungicide applications necessary for the less susceptible varieties may be reduced in the future.

For these reasons, it was proposed that commercial cultivars be ranked, based on symptom severity.

Materials and Methods

Plant production

Twenty-one cultivars, chosen for their importance in the Canadian apple industry, were used in this study. They were Cortland, Early Geneva, Empire, Golden Delicious, Golden Russet, Idared, Jersey Mac, Jonagold, Jonamac, Lobo, Lodi, McIntosh, Mutsu (Crispin), Northern Spy, Paulared, Red Cortland, Red Delicious, Royal Gala, Spartan, Sunrise, and Vista Bella. All the trees were grafted onto Malling Merton (MM) 106 rootstocks in April 1999. They were planted into 6 L pots filled with a mixture of 6:3:3 of Perlite®, peat moss and organic soil. The trees were grown outdoors until two weeks before the beginning of the experiment when they were moved into the greenhouse. One shoot was allowed to grow from each graft to form the tree. Branches and side shoots were removed from the trees.

Inoculum

Leaves from a heavily scabbed orchard in Frelighsburg Québec were gathered in November 1998. They were overwintered outside under screens until the end of January 1999. They were then placed into a cold room at 10°C and 95% relative humidity (RH) until June 15th when the experiment was conducted. Squash mounts were performed periodically to assess the maturation of the pseudothecia.

Inoculation and incubation

The trees were placed into the greenhouse in a completely randomised design with 8 replicates. The scabbed leaves were placed onto screens 0.5 m above the trees. At the beginning of the inoculation the trees and leaves were thoroughly wetted to initiate the ascospore ejection. The inoculation was allowed to continue for 6 hours. During this time two humidifiers (Watson Humidifiers, Atomising unit series AZFP, 1958) were running to keep the relative humidity at 100% and to achieve optimum levels of leaf wetness. To measure inoculum levels 10 volumetric spore traps (VST) were hung level with the top leaves of the trees. The number of ascospores were latter counted under the microscope at 250X. At 6 hours the inoculum was removed and the VSTs were stopped. The infection period was continued up to 24 hours with 100% RH.

The trees were incubated at 18°C and 70% RH until 26 days after inoculation. During the final two days the RH was raised to 100%, using the same humidifiers as before.

Data collection

The data collected in this experiment were as follows: disease severity, latent period, incubation period, lesion size, and conidial production. Disease severity was assessed as the number of lesions per leaf surface area at the time of inoculation. Severity data were taken from leaves +3 to -3 as defined by Sanogo and Aylor (1997). Leaf surface area was calculated by taking an average of the surface area of 25 leaves, without the petiole, from each leaf stage of each cultivar. The surface area was measured with a leaf area meter (Lambda Instruments, Li-cor, Li-3000 and Li-3050A). The latent and incubation periods were assessed every one to two days from the time of inoculation. Incubation period was defined as the time from inoculation to the time of the first appearance of symptoms and latent period was the time from inoculation to the appearance of conidia (Rapilly, 1991). Lesion size was taken at 28 days on leaf -1. Using a ruler, the length and width of each lesion was measured and the area calculated, using the formula for the area of an ellipse, in mm^2. An average of a maximum of 5 lesions was taken. Conidial production was evaluated at 28 days on leaf -1. The leaf was cut into 6-7 pieces. These pieces were placed into a 50 ml Falcon® tube with 20 ml of sterile distilled water. The tube was then agitated for 1 minute on a Fisher Vortex Genie® at speed 6. The leaves were then removed and 3 drops of Lugol's iodine were added. The tubes were then centrifuged at 3800 rpm (2425 *g*) for 10 minutes (Philion, 1994). The supernatant was then drawn off, leaving 5 ml of liquid. The tubes were then stored at 4°C until they could be counted on a hymacytometer (Fuchs Rosenthal, Ultra Plane, 1/166 mm^2, 2/10 mm deep).

Data Analysis

All data collected were subject to a standard ANOVA, least significant difference (LSD) test and cluster groupings. The cluster groupings were done using the linked average method. All analyses were done using SAS Systems version for Windows 6.12.

Results

As is presented in Figure 1, there were considerable differences between the cultivars in the response to infection by *V. inaequalis*. The average number of lesions per cm^2 of leaf varied from 0.349 on Red Cortland to 0.008 on Golden Russet. The differences among the cultivars were found to be significant ($P > 0.0001$) and had an LSD of 0.131. The cultivars were classed into 5 disease severity groups (Figure 1). Highlights from the groupings were that McIntosh ranked in the third grouping with 0.202 lesions/cm^2 and Spartan had a severity rating of 0.096 lesions/cm^2, which placed it in the second grouping.

The level of conidial production (Figure 2) was significantly different among the cultivars ($P > 0.0029$) with an LSD of 583. The dendrogram was sectioned into 5 levels of conidia production. Vista Bella had the highest average production at 1038 conidia/lesion cm^2 and Golden Russet with 0.00 condia/cm^2 of lesion. Cultivars of particular interest were Red Cortland and Cortland with 729 and 728 conidia/lesion cm^2, respectively. This placed them in the second grouping. McIntosh was in the third grouping with 474 conidia/cm^2 of lesion and Spartan was in the fourth grouping with 183 conidia/lesion cm^2.

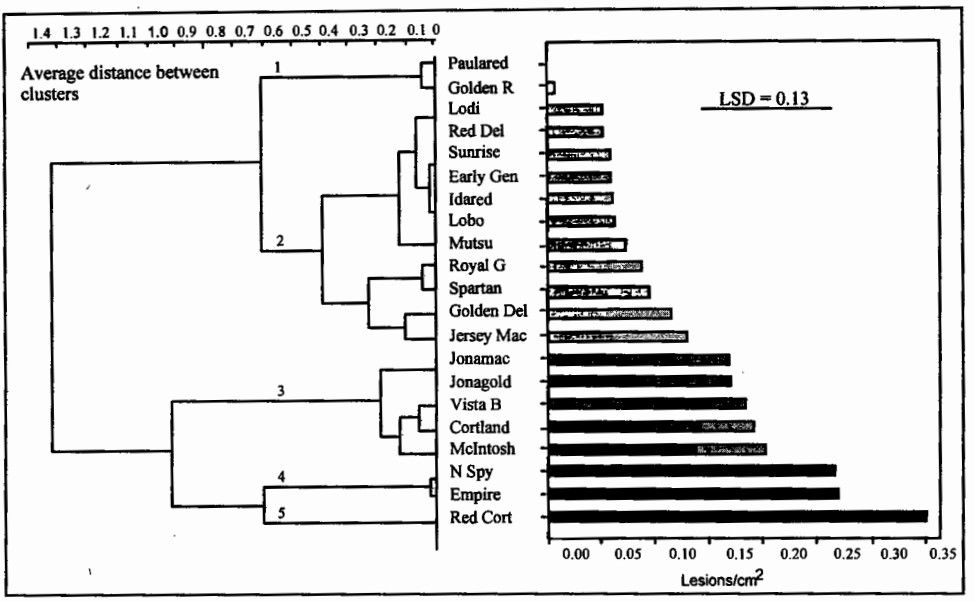

Figure 1. Disease severity presented as the average number of lesions per cm^2 of leaf for each cultivar. The numbers on the dendrogram indicate the different classes

The data for average incubation period were transformed. Zero days of incubation in Figure 3 is equivalent to 7 days so that 14 days corresponds to 21. Incubation period was significantly different ($P<0.05$) among the cultivars and an LSD of 5.4. The dendrogram was cut at 5 classifications (Figure 3). Jersey Mac had the shortest average incubation time at 3.7 days, with McIntosh as a close second at 4.3 days. Jonamac was slowest to exhibit symptoms at 12.7 days. Cortland and Red Cortland were in the second grouping at 4.8 and 6.0 days respectively. Spartan fell into the third grouping with 9.0 days.

A general index of classification is given in Table 1. This index was compiled using an average over all of the parameters measured in this experiment. Because no cultivar consistently rated a classification of 5, it does not appear in this table. Cortland and Red Cortland are the most susceptibly rated cultivars. McIntosh averaged a rating of 3 and Spartan a rating of 2. Golden Russet was categorised as the least susceptible with a rating of 1.

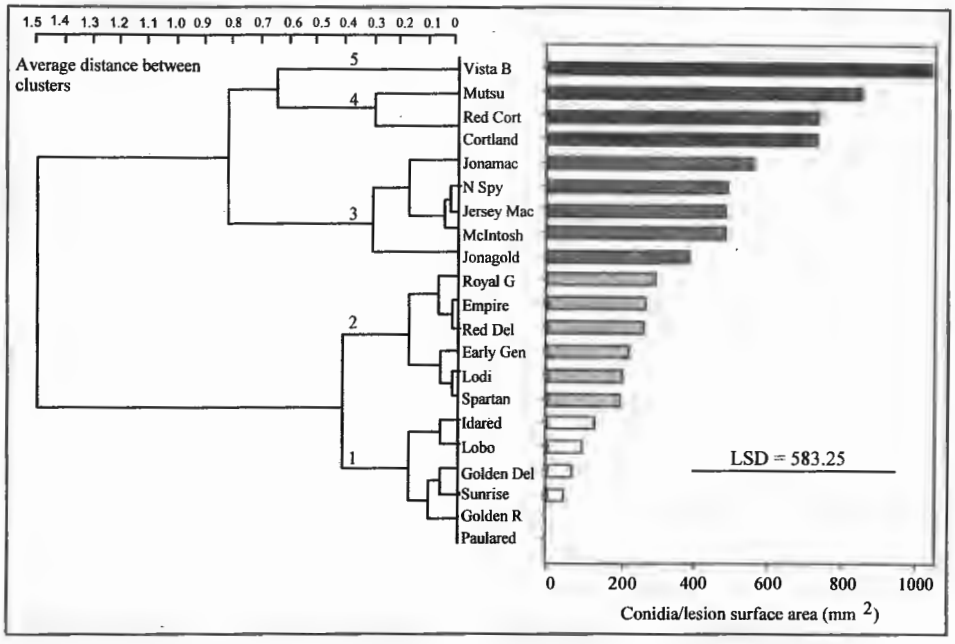

Figure 2. Conidia production for each cultivar in conidia per lesion surface area (mm^2).The numbers on the dendrogram indicate the different classes

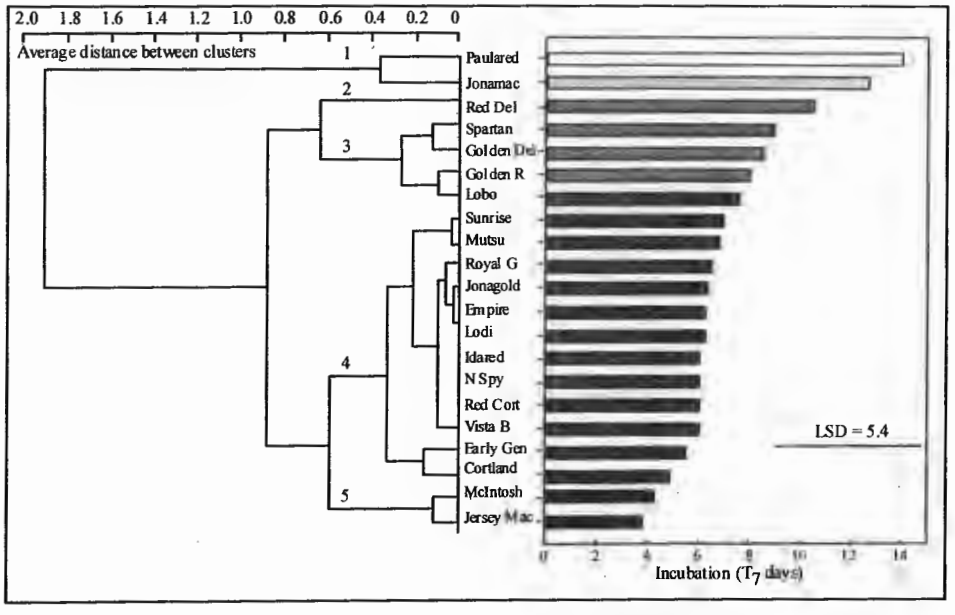

Figure 3. Incubation period in days for each cultivar. The scale for the incubation period has been transformed so that 0 is equal to 7 days. The numbers on the dendrogram indicate the different classes.

Table 1. General index of classification

Cultivar	Class	Cultivar	Class	Cultivar	Class
Cortland	4	Mutsu	3	Jonamac	2
Red Cortland	4	Northern Spy	3	Lobo	2
Empire	3	Royal Gala	3	Red Delicious	2
Jersey Mac	3	Vista Bella	3	Spartan	2
Jonagold	3	Early Geneva	2	Sunrise	2
Lodi	3	Golden Delicious	2	Golden Russet	1
McIntosh	3	Idared	2	Paulared	-[a]

[a]Paulared was not included in the index as there were not enough leaves to do a reliable assessment

Discussion

This experiment provided a first indication of the variation among susceptible cultivars. Although the order of the cultivar severity ratings differed between disease parameters, the general trend was the same. It was relatively unusual to find cultivars that had a difference of more than one category between disease parameters. Due to its general index rating of 3, McIntosh seems to be a fair representative of sensitive varieties, although perhaps caution should be used when using the results in management decisions for less sensitive varieties. It may also be possible to extrapolate a modified management system for the less sensitive varieties. It could be possible to eliminate sprays under low inoculum conditions.

Because this classification was done using inoculum from an orchard in Québec, it may not be easily applied to other geographical areas. There appears to be regional differences between isolates of *V. inaequalis*, so that cultivars that appear to be less sensitive in this experiment in other regions may be very sensitive. An example of this is the case of Red Delicious. In this experiment it was considered relatively unsusceptible, but in Europe it is found to be very susceptible (Parisi *et al.*, 1993b). This may have something to do with the planting densities. In Europe the planting density of Red Delicious is relatively high where as in Québec it is low (Childers *et al.*, 1995). To further investigate the question of inoculum, the inoculum used in this experiment is to be analysed for the identification of the races.

Acknowledgement

I would like to thank Isabelle Lahaie and Martin Larivière for their help and encouragement.

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Analysis of 1998 scab epidemic in an experimental apple orchard planted with cultivar mixtures

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Abstract : The scab epidemic was studied in an experimental orchard planted near Angers (France), in 1994. It included 2 cultivars : Smoothee, moderately susceptible to scab, and Baujade, *Vf* resistant. Each cultivar was planted in monoculture plots, and in two different mixtures (row by row and within the row), in a block design with 3 replications. No fungicide treatment against scab was applied in the orchard. Thirteen assessment of leaf scab symptoms in the orchard were done between April and August 1998, taking into account the risk periods and the ascospore ejections. The cv Baujade remained scab free. The analysis of the epidemic on the cv Smoothee showed significant differences for the severity of the disease (number of scab lesions per shoot) between the three plantation designs in June ; the epidemic was higher in the monoculture than in the mixtures, and higher in the mixture row by row than in the mixture within rows. However, the analysis of the attack on the fruits did not show significant differences between the 3 plantation designs for incidence and severity. These results are discussed taking into account the amount of inoculum in the orchard, the climatic conditions, and the ascospore and conidia infection periods.

Key words : *Venturia inaequalis*, *Malus x domestica*, epidemiology, monogenic resistance, planting strategy.

The presence in several european countries of races 6 and 7 of *Venturia inaequalis*, virulent to the *Vf* gene (Parisi *et al.*, 1999) can compromise the commercial success of the new *Vf* resistant cultivars. These new cultivars represent a great interest, especially in orchards managed by biological or integrated disease control. It seems necessary to establish strategies for the planting of the *Vf* resistant cultivars, which can preserve their interest and resistance durability. Cultivar mixtures used for several years in annual crops, can be one of these strategies. A mixture with cultivars different in their susceptibility to a pathogen, may strongly reduce the disease level, as showed with barley and powdery mildew (Wolfe, 1985). In perennial crops, few studies dealt with the interest of cultivar mixtures for disease control. In apple trees, Blaise and Gessler (1994), in a study based on computer simulation of the epidemic, showed that cultivar mixtures can reduce scab severity. To evaluate the influence of apple cultivar mixtures on *V. inaequalis* epidemic, an experimental orchard was planted near Angers, in the Loire Valley, in 1994. The scab epidemic was studied in 1996, 1997 and 1998. The 1998 results only are presented here.

Materials and methods

The orchard included 2 cultivars, Smoothee, considered as moderately susceptible to scab in France (Parisi and Trillot, 1993) and Baujade, a *Vf* resistant INRA selection (Lespinasse *et al.*, 1992). Each cultivar (900 trees) was planted in monoculture plots, and in two different mixtures : row by row (cv mixture 1) and within the row (cv mixture 2). The design was a

block with 3 replications; each block comprised 4 plots with 6 rows of 25 trees. Each plot was surrounded by a hedge planted with *V. inaequalis* non host species. The total area of the orchard was 1.2 ha. No fungicide treatment against scab was applied in the orchard, since the planting.

Thirteen assessments of scab symptoms in the orchard were done between April and August 1998, taking into account the risk periods detected by the software TAVECO and the ascospore ejections (Fig 1), both supplied by the Service Régional de la Protection des Végétaux of Angers. 162 shoots/cultivar were evaluated : 2 shoots per tree and 9 trees per plot. The severity was expressed as the mean number of lesions per shoot in each cv mixture and in the Smoothee monoculture. The AUDPC (area under disease progression curve) was calculated from the curve of evolution of leaves severity in each plot (Bousset *et al.*, 1997). The fruits of 18 trees per plot were collected and evaluated for incidence (percentage of scabbed fruits) and severity (number of scab lesions per fruit). The AUDPC and the fruits incidence and severity were tested by analysis of variance, and when the F value was significant ($P \leq 0.05$), the means of each planting system were compared with the Newman and Keuls test.

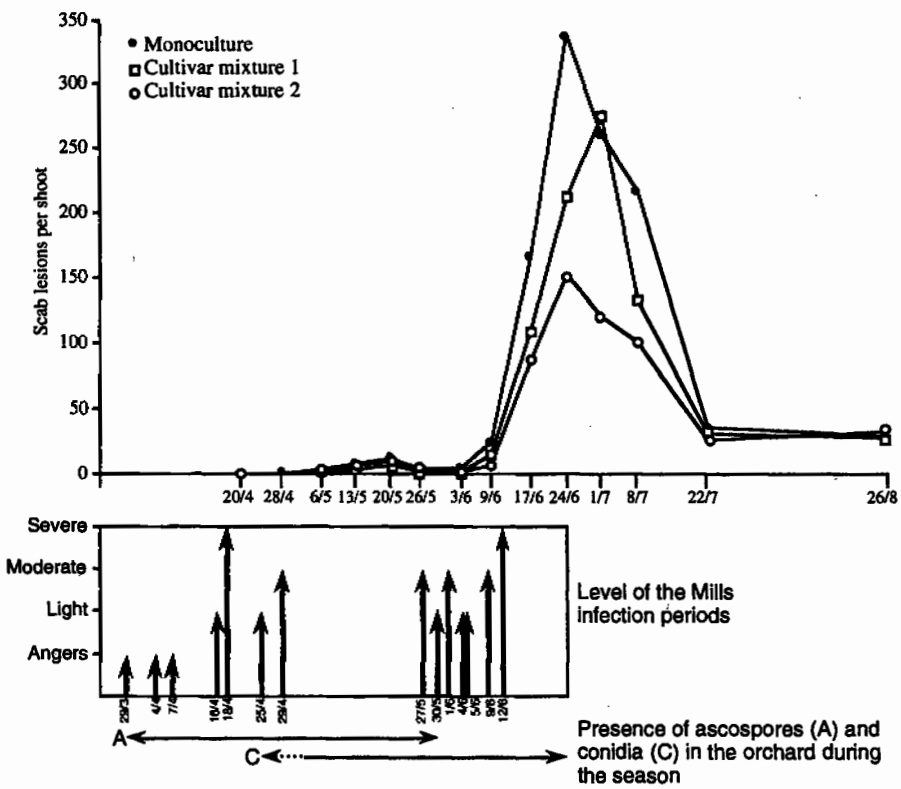

Fig. 1. Evolution of the severity on the cultivar Smoothee in monoculture and cultivar mixtures, infection periods and presence of ascospores and conidia

Results

The cv Baujade was scab free during the 4 years of the experiment. In 1998, the first ejection of ascospores combined with an infection period occurred the 29 of March and the last one, on the first of June. The first scab lesions in this orchard were noted the 28 of April. Between the 29 of April and the 27 of May, no ascospore ejection and no infection period were recorded, due to a period of dryness (Fig 1). The analysis of the epidemic on the leaves of cv Smoothee (Fig 1) showed differences for the severity of the disease between the 3 planting systems. Globally, the epidemic was significantly higher in the monoculture than in the mixtures, and higher in the cv mixture 1 than in the cv mixture 2 (Table 1). However, the analysis of the attack on the fruits did not show significant differences between the 3 planting systems for incidence and severity (Table 2).

Table 1. Area under the disease progression curve (AUDPC) of severity on Smoothee in different planting systems

Planting system	AUDPC	Newman, Keuls test (1)
Monoculture	9506	a
cv mixture 1	7052	b
cv mixture 2	4965	c

(1): values with different letters were significantly different at p = 0.05

Table 2. Incidence and severity on Smoothee apples, in the different planting systems

Planting system	Incidence (percentage of scabbed fruits)	Severity (number of scab lesions per fruit)
Monoculture	85.51 %	15.15
cv mixture	81.76 %	14.55
cv mixture 2	79.84 %	14.49

Discussion

In 1998, two different phases of the epidemic can be distinguished on the leaves. In May, the first phase was mainly caused by the ascospore ejections and the infection periods recorded in April. A slow progression of the severity and a low amount of disease were recorded, followed by a decrease of the severity. The long dry period recorded in May can explain the moderate intensity of this first phase. The second phase, in June, was principally due to conidial infections. The disease increases very quickly, and the main significant differences were recorded during this period. In July, the scabbed leaves fall, the low amount of susceptible tissues and the climatic conditions explain the decrease of the severity.

These results are consistent with the simulation of Blaise and Gessler (1994) : the mixtures within the row seems more efficient than the mixtures row by row. However, the observed reduction of the disease severity was lower than expected according to the

simulation. This can be explained by the characteristics of the orchard, different from the values chosen for the simulation. For example, the tree spacing within rows is 2 m in the simulation and 1 m in our orchard. Several others factors, difficult to estimate or to forecast, can influence this kind of study, such as the conidia and ascospores rates of release and dispersion, correlated with climatic conditions (including wind direction and speed) and conditioning the number of generations per season, or the influence of the early fall of scabbed leaves on the epidemics, different in each planting system.

On fruits, the incidence and severity were higher on the Smoothee monoculture than on cv mixtures 1 and 2, but the differences were not significant. This can be explained by the very high level of the disease (the orchard is untreated since the planting), with about 50% of scabbed leaves per shoot in August. Furthermore, between the 13 and the 27 of June (last record of meteorological data), when the differences of the leaves severity were important, no infection period leading to fruit infection was recorded.

The influence of the cultivar mixtures on the epidemic must be combined with other means of control. A low amount of fungicide treatments during the vegetative season will be applied in 2000 in the orchard, and their efficacy will be compared in the different planting systems.

Acknowledgements

We thank the Service Régional de la Protection des Végétaux, Angers (France), for the information on the periods of infection and ascospore release.

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Warning systems for the prediction of *Venturia inaequalis* infections and inoculum quantification methods

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Abstract : Potted apple trees in a high scab inoculum orchard were used to test several electronic warning devices (Mills, Ventem, MacHardy) on Metos equipment, in comparison with traditional Bazier. In addition, assessment of ascospore inoculum ejected during the rainfalls and totally present in the orchard was realized according to different spore traps and methodologies.

The new systems did not forecast a number of infections, but such failures are usually not reported in literature. The "experimental" spore trap used in these tests gave better results than Burkard sampler for practical use in the orchard and moreover permitted assessment of ascospore concentrations. The evaluation of the amount of ascospores in the orchard proved very difficult.

Key words : *Venturia inaequalis*, forecast system, alarm system, spore trap

Introduction

Warning systems for the forecasting of ascospore infections caused by *Venturia inaequalis* have been tested since 1960 to optimize treatments for "black spot" control, both in preventive and therapeutic programs (Cesari *et al.*,1975). The most famous, Mills' system with mechanical device is well adapted in high inoculum orchards (Cesari & Fiaccadori, 1992).

On the contrary the accuracy of the new electronic warning systems has not been seriously tested. It can be said only that positive results were observed for the control of scab when these systems were used (Araya *et al.*, 1996; Berrie, 1997).

The assessment of the inoculum level can improve infection forecast in the two following ways : i) quantification of ascospores concentration ejected during rainfalls in commercial orchards, using an improved spore trap, ii) quantification of total amount of inoculum in the orchard, which had never been quantified directly in the orchard, but in the laboratory only. A predictive but indirect formula was used to determine PAD (Potential Ascospore Dose) by Gadoury & MacHardy (1986).

Therefore the objectives of our research were : i) to set up a simple biological method to test the accuracy of predictions by warning systems; ii) to assess the ascospore inoculum under different situations, as emission during rainfall, that can cause infection, and as total inoculum in the orchard.

Materials and methods

Warning systems

The accuracy of four warning systems in forecasting *Venturia inaequalis* ascospore infections was tested with 10 natural rain episodes ; some of which caused infection, while the others did not. The infections were surveyed with the following epidemiological parameters : rainfall

(mm, duration, time of beginning and ending), Mills' indexes product [wetness duration(h) x mean temp.(°C)], release of ascospores. The occurrence and severity of infections were assessed with potted apple trees placed in the orchard during only one potentially infectious rainfall and subsequently the percentage of scabbed leaves and number of black spots/100 leaves were counted. The warning systems tested were : "Mills", "Ventem", "MacHardy" with Metos hardware and software ; "Mills", with traditional Bazier equipment. Prints out or graphics of these alarm systems were analyzed, together with epidemiological data, in an attempt to know if the causes of wrong forecasts depended on epidemiological parameters of alarm systems or on mechanic/electronic equipments.

Inoculum

The ascospore emissions during 10 rainfalls were assessed with two spore traps : i) Hirst Burkard, suction 10 l/min, aspirator 150 cm height, ii) "experimental", suction 1 l/min, aspirator 7 cm height. This last one is widely used in Italy especially for IFP projects (Cesari *et al.*, 1980). Scabbed leaves are placed in a wooden box on the ground and an aspirator equipment let ascospore flow from box to glass slides that can easily be removed and examined under the microscope. It is possible to assess the day and approximately the time of ascospore presence. It is called "experimental" because it does not capture from scabbed leaves randomly picked up in the orchard and placed in the box, but from the infected leaves present on 3 m^2 of ground floor, resulting from 6 samples of 0.5 m^2 each. The total ascospore inoculum in the orchard was assessed as : i) ascospores trapped with "spore tower" in the laboratory (4 extractions), ii) total ascopores trapped with "experimental" spore trap. Tests were realized in an experimental orchard, 6 year old, with an high scab inoculum (30-40% scabbed leaves in autumn).

Results

The alarm systems detected only some of the infections which occurred (table 1), especially on the first year. On the second year the results were generally better, especially for Ventem and Mills-Metos. These results are reported on table 1, where our attempts to explain the wrong forecasts of every alarm system are indicated as foot notes. The easiest failures to identify were with Mills-Bazier, which often depended on the break of paper, while the failures of electronic systems are less explainable. Their prints out put often in evidence that rains and wetnesses were correctly recorded, but simulations of infection periods did not begin.

On the contrary, all warning systems provided correct predictions (no alarms) about rains that did not cause infections (figure 1).

The "experimental" and Burkard spore trap detected a very different amount of ascospores, which were observed in higher number with "experimental" (table 1). It should be pointed out that Burkard was in a very favourable situation because it was placed in an high inoculum orchard.

The two methods ("spore tower" and "spore trap") used to assess the total inoculum in the orchard evidenced no coherent data (not shown).

Discussion

The methodology based on potted apple trees appeared simple and interesting for testing alarm systems. The preliminary results were not in favour of new warning systems and/or employed electronic equipments, in contrast with available references.

It had often proved difficult to ascertain if the failures depended on epidemiological parameters of alarm systems or on software and hardware equipments. The lack of some simulations of infections due to recorded rains and wetnesses, made the second hypothesis more likely. It should be kept in mind that the number of tests (possible infections) carried out was low, because our main aim was to point out a biological method for testing alarm systems.

The evaluation of "experimental" spore trap was positive, considering effectiveness and practical use. Moreover the modified methodology on scabbed leaves in wooden box allowed studies on inoculum concentration in the orchard, with the possibility of assessing its relationship with infection severity. Remains the problem of too numerous and different spore traps.

The assessment of the amount of inoculum in the orchard with a direct methodology does not seem to be possible and "PAD prediction" by Gadoury and MacHardy (1986) currently appears to be the only acceptable system.

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Table 1. Bio-epidemiologic data of *Venturia inaequalis* infection and warning systems forescasts

INFECTIONS							***ALARM SYSTEMS FORECASTS***			
	RAINS		ASCOSPORES spore-traps***		MILLS' INDEXES *(product)*	OCCURRENCE and SEVERITY (potted apple-trees)				
Date	mm	Beginning-ending hour	experimental ****	Burkard	h wet x mean temp	% scabbed leaves	n° lesions/ 100 leaves	***Mills (Bazier)***	**Mills**	**MacHardy**	**Ventem***
27.03.'94	7.0	02-03	491 nr		23	0	0	nr	no	nr	no
2.04.'94	12.6	01-14	1720 nr		88	4.86	9.81	no(1)	no(2)	no(2)	no(4)
5.04.'94	9,0	16-20	1367 nr		68	7.32	9.00	light	no(2)	no(2)	no
18.04.'94	4.6	16-24	3711 nr		228	6.34	6.34	no(1)	no	no	29,85**
28.03.'95	17.6	02-08	128	0	25	0	0	no	no	no	no
7.04.'95	0.2	07-08	11	0	47	0	0	no	light(3)	no	no
14.04.'95	3.6	05-10	16860	208	31	0	0	no	no	no	no
20.04.'95	6,0	21-06	9491	8	144	8.10	11.20	light	light	no(5)	1.78
22.04.'95	4.4	10-14	100494	333	561	48.23	850,00	severe	severe	yes	29.86
2.05.'95	2,0	8-11	4466	0	73	0	0	no	no	no	no

* Parameters used (Butt & Xu,1996) : rain > 0,2 mm ; max. interval between two wetnesses : 8 hours ; min. time for ground wiping : 1 h ; night delay in ascospore emission : 2 hours ; inoculum level : hight

** % scabbed leaves forecast by Ventem
*** N° ascospores/m^3 air
**** Spore trap that captures from scabbed leaves representative of 3 m^2 ground floor; data referred to 1 m^2 ground floor
***** nr : not recorded

ATTEMPTS AT EXPLANATION OF WRONG FORECASTS

1) Infection not signaled for break of wetness paper sensor due to break of wetness paper sensor
2) Infection not signaled, but rain and wetness correctly recorded on print out.
3) Wetness duration signaled by Mills-Metos was much longer as compared to Mills-Bazier
4) Infection signaled one day after the actual beginnig of infection.
5) Infection took place even if the rain occurred only during the night

Figure 1. N° occurred infections of *Venturia inaequalis* and N° alarms according to forecast systems

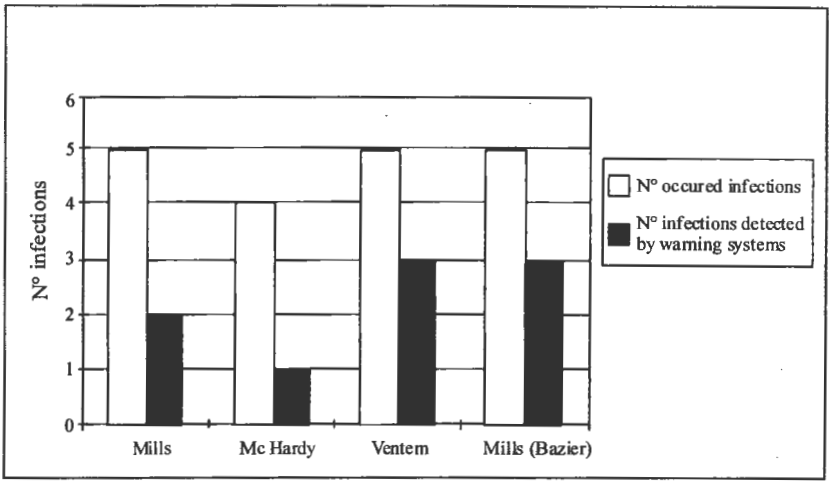
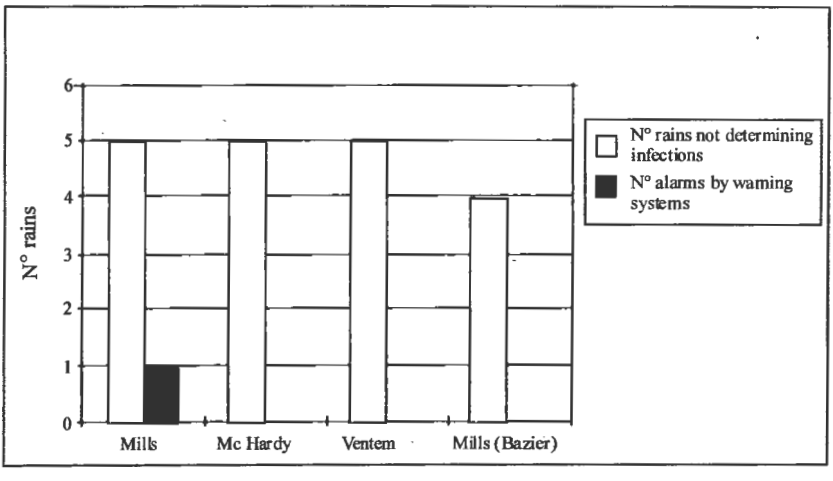


Figure 2. N° rains that have not caused infections and N° alarms according to forecast systems



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New disease outbreaks and epidemiological studies of fire blight (*Erwinia amylovora*) in Spain

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Abstract : Fire blight was first detected in Spain in cider apple in 1995. During 1996 new detections were observed in Segovia, and Navarra province, and in 1997, several focuses were detected in the North of Navarra province. In 1998, disease was detected in a nursery located in Guadalajara, in public gardens in Huesca province, and in a commercial pear orchard in Lerida province. In most cases there were difficulties to establish the origin of fire blight introduction. A weather-based fire blight risk map of the North-eastern part of Spain was made with data of 5-10 years from 80 weather stations using the Billing's Revised System (BRS) and Thomson's temperature threshold fire blight risk. The contour map clearly identified regions of high fire blight risk around the Ebro river valley in the main apple and pear growing areas of Spain. A study was performed during years 1997-1998-1999 to establish the probability of fire blight introduction with host plant material imported from 41 nurseries of different european countries including France, Italy and Belgium and accompanied with the CEE Phytosanitary Passport ZP. The work consisted of periodical visual inspections, and quality control analysis of representative plant material (including symptomless plants) with methods of detection based on enrichment-ELISA-DASI with monoclonal antibodies and PCR enrichment with specific primers, and final confirmation by strain isolation.

Keywords : fire bligh risk, plant material, CEE Phytosanitary Passport ZP.

Introduction

In Spain, fire blight was first detected in cider apple in 1995 in the Basque country (Butrón 1995). During 1996 disease continued spreading in the Basque country and new detections in different hosts were observed in a nursery of Segovia and in the Navarra province. In 1997, several focuses were detected in the North of Navarra province. In 1998, disease was detected in a nursery located in Guadalajara, in public gardens in Huesca province, and in a commercial pear orchard of 10 Ha of cultivar Blanquilla in Lerida province (Fig. 1) (Montesinos and López 1999). All detected focuses were eradicated according to official measures. However, in most cases there were difficulties to establish the origin of fire blight introduction in non-epidemic protected areas of Spain. The hypothesis of an introduction of plant material harbouring latent infections of *E. amylovora* was claimed on the basis of previous evidences, however this was never proved for the spanish outbreaks (Calzolari *et al.* 1992, Van der Zweet *et al.* 1982, Van der Zweet and Beer 1995).

Materials and methods

A weather-based fire blight risk map of the North-eastern part of Spain was made using data of 5-10 years from 80 weather stations of Catalunya, Aragón, La Rioja, Navarra and País Vasco. Disease risk was calculated from rainfall and temperature using the Billing's Revised System (BRS) and Thompson's temperature threshold (Steiner 1989, Billing 1992, Paulin *et*

al. 1994). Disease risk was established as a function of the number of potential days of infection (NPDI) from April to June, mean duration of infection periods (MDIP), and date of account of cumulated 19 °C-day above a threshold of 19 °C. A contour map was drawn to identify areas of risk.

Figure 1. Location of fire blight outbreaks in Spain since the first detection in 1995. 1, Pais Vasco; 2, Segovia; 3, and 4, Navarra; 5 and 6, Catalunya; 7, Huesca; 8, Guadalajara.

A study was performed to establish the probability of fire blight introduction with host plant material imported from nurseries of different european countries and accompanied with the CEE Phytosanitary Passport ZP. The study was performed in Catalunya during three years (1997-1998-1999) involving 41 nurseries and 200 apple and pear orchards, which imported plant material from commercial European nurseries of France, Italy and Belgium. The work consisted of periodical visual inspections, and quality control analysis of representative plant material (including symptomless plants) with methods of detection based on enrichment-ELISA-DASI with monoclonal antibodies and PCR enrichment with specific primers, and final confirmation by strain isolation (Lelliot and Stead 1987, Bereswill *et al.* 1992, 1995, Gorris *et al.* 1996).

Results and discussion

First fire blight outbreak in Spain and disease risk. The first fire blight in Spain was detected in 1995, a few km distance from the weather station of Hondarribia. From meterorological parameters, all fire blight risk indicators according to the BRS system, temperature threshold and daily infection risk, suggested that high fire blight risk started in late May, during 1993, 1994, and 1995, in coincidence with the bloom period of cider apple cultivars in the area. (Fig. 2). Therefore weather conditions were favorable to fire blight, in case of introduction of plant material with latent infections in the area.

The contour map builded up with risk data according to the models clearly identified regions of high fire blight risk around the Ebro river valley in the main apple and pear growing areas of Spain, around Zaragoza and a part of Lerida (Fig 3).

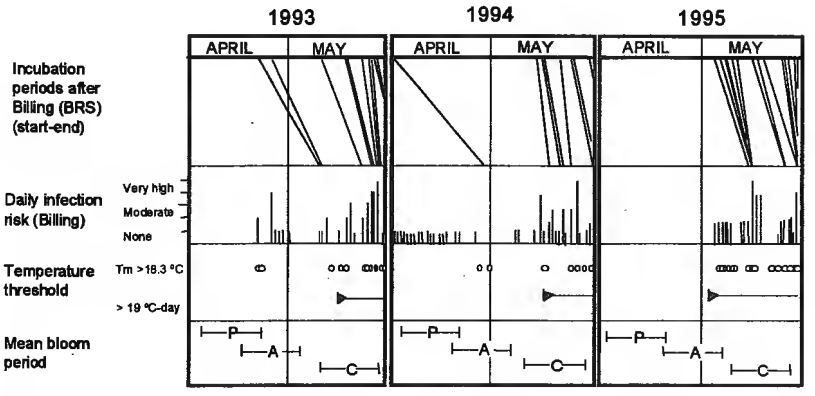

Figure 2. Fire blight risk chart according to phenoclimatic data of Hondarribia (Guipúzcoa) located near to the first disease outbreak detected in Lezo. Highly favorable conditions for disease development on cider apple (C) were observed, but not on pear (P) or apple (A).

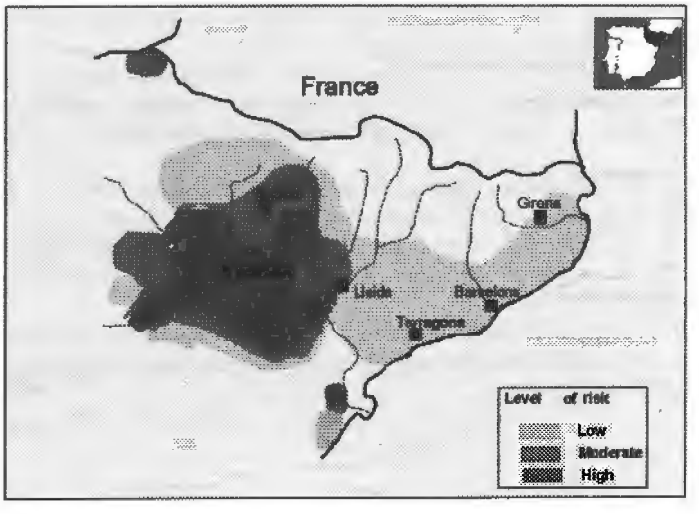

Figure 3. Contour map of fire blight risk in the Nort-eastern part of Spain according to weather data.

The analysis of the 4000 samples of imported plant material from commercial European nurseries of France, Italy and Belgium accompanied with the CEE Phytosanitary Passport ZP, using highly sensible detection methods such as the enrichment-ELISA-DASI and enrichment-PCR, did not detect *Erwinia amylovora*. Therefore, under the scope of this study, it is concluded that the ZP Passport, gives a valid assurance of *E. amylovora* absence, in spite of plant material coming from countries with fire blight and from nurseries located in non-protected fire blight areas.

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Occurrence in France of *Pseudomonas syringae* pv. *papulans*, the causal agent of blister spot on apple

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Abstract : Blister spot caused by *Pseudomonas syringae* pv. *papulans* (*PSP*) is an important disease that especially affects the apple cultivar Mutsu in eastern USA, in Canada (Ontario) and in Italy. In France, this disease has never been described. A study on epiphytic populations of *Pseudomonas syringae* isolated from apple and pear leaves in French orchards revealed a particular isolate called KA54. Its biochemical characterization showed high similarities with *PSP* strains isolated from blister spots in USA, Canada and Italy. Identical symptoms were obtained with KA54 and these *PSP* strains after inoculation of immature fruits of the cultivar Fuji, or inoculation of young leaves of the cultivars Fuji, Mutsu, Gala and Golden Delicious. In addition the Koch's postulate was verified. These results confirm the presence of *PSP* in France. In order to facilitate its diagnostic by PCR method, we developed primers by genomic comparison between *PSP* and *Pseudomonas syringae* pv. *syringae*. These primers allowed the clear-cut identification of KA54 and others *PSP* amongst a collection of *Pseudomonas syringae*.

Key words : Apple, blister spot, *Pseudomonas syringae* pv. *papulans*, PCR, primers.

Introduction

Pseudomonas syringae pv. *papulans (PSP)* is the causal agent of blister spot, an important disease of apple cv. Mutsu in Canada (Dhanvantari, 1969, 1977), the eastern USA (Burr & Hurwitz, 1979) and Italy (Bazzi & Calzolari, 1983); it has never been described in France. A survey of epiphytic populations of *Pseudomonas syringae* in French orchards of apple revealed the presence of an isolate, KA54, showing high similarities with *PSP* strains. In this study, we present the phenotypical, serological, pathological and molecular characterization of this isolate.

Materials and Methods

Strains

Isolate KA54 was compared with a collection of 10 strains of *PSP* originating from USA, Canada, Italy and diverse strains of *Pseudomonas syringae* pv. *syringae (PSS)* from the Collection Française de Bactéries Phytopathogènes (CFBP).

Phenotypical and serological characters

20 conventional biochimical tests were used according to Schaad (1988). In addition O-serogroups of the strains were assigned by Ouchterlony double diffusion (Saunier *et al.*, 1996).

Pathogenicity tests
Immature fruits cv. Mutsu and leaves of cv. Mutsu, Fuji, Golden Delicious and Gala were vacuum infiltrated by bacterial suspensions adjusted to 10^7 cfu /ml. This technique was selected because it allowed infection without mechanical injuries.

Molecular characterization
PCR method was performed using primers (named Pap) designed by genomic comparison between *PSP* and *PSS* (Kerkoud & Manceau, unpublished result).

Results

Phenotypical and serological characters
A high level of similarity was found between KA54 and others *PSP* strains except for lactate and tartrate utilisations. Two characters: production of levan from sucrose and polypectate gel pitting at pH5 (which were negative and positive respectively for *PSP)* discriminated *PSP* from *PSS*. All but one isolates (including KA54), except two rough strains (RIB), were serologically homogeneous. They belonged to a new O-serogoup (PST4) defined as giving a reaction with the antisera 196 and 287, but no reaction with 292.

Pathogenicity tests
KA54 produced typical blister spots on Fuji fruits, identical to spots caused by the *PSP* reference strain CFBP 3323. These blister spots were also clearly different from the hypersensitive necrosis induced by the *PSS* strain CFBP 3077 (2027.37). Typical *PSP* symptoms were also obtained after leaf inoculation of Mutsu, Fuji, Golden Delicious and Gala (Figure 1, 2 et 3). In addition, Koch's postulate was completed when KA54 was reisolated from the blister spot lesions of apples and leaves.

Molecular characterization
The Pap primers especially designed for this study amplified specifically a 240 pb DNA fragment of *PSP* strains (including KA54) amongst the collection of *P. syringae.* This result was confirmed with additional tests, using further isolates of *Pseudomonas* (data not shown). The same signal was obtained with strains reisolated from pathogenicity test.

Discussion

Phenotypical characters and pathogenicity tests undoubtedly showed that KA54 is identified as *PSP*. The specific Pap primers confirm theses results and could be used to facilitate the molecular diagnosis of this pathogen in further epidemiological studies. In addition, our results indicate that polypectate gel pitting at pH5 is a useful discriminating test in *PSP* identification. Finally, *PSP* appears to be present in France, although no natural symptoms have been reported in the country yet.

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Occurrence of races of *Venturia inaequalis* in an apple scab race screening orchard in Denmark

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Abstract : Scab infection on leaves and fruits on 16 differential apple varieties carrying different resistance genes against *Venturia inaequalis* was assessed in the summer 1998 on trees in an orchard at Årslev, Denmark, and the occurrence of races of the apple scab pathogen in this race screening orchard was determined. Apple scab symptoms on trees of the *Malus floribunda* clone 821 was observed for the first time in Denmark and the trees were severely infected, probably due to a new pathotype of *V.inaequalis* (race 7). On the basis of the occurrence of symptoms on the various differential varieties it seems likely that all previously described physiologic races of *V. inaequalis* (race 1-6) were present in the orchard. Differences in incidence and severity on the different varieties was observed. The susceptible varieties 'Golden Delicious' and 'Jonagold' were most infected, probably due to race 1-6. Race 2, 3 and 4 incited lesions on their differential hosts. Race 5 incited symptoms on two of three differential hosts. Race 6 probably occurred on the Vf variety 'Prima' but not on 'Florina'. The variety 'X2594' carrying Va resistance and 'Freedom' carrying polygenes and Vf resistance were both free of apple scab. Differences in presence of infections on fruits and leaves on 'Dolgo' and 'Nova Easygro' were observed indicating that organ specific resistance may occur on these varieties.

Introduction

Between apple (*Malus* spp.) and the apple scab pathogen *Venturia inaequalis* a gene-for-gene relationship exists. Resistance to *V.inaequalis* is coded by several major genes in *Malus* spp., and the genes Va,Vb, Vbj, Vf, Vm and Vr have been used for many years in breeding of apple cultivars based mostly on monogenic resistance. Several races of *V. inaequalis* can be identified and distiguished on the basis of their pathogenicity on a range of different species of *Malus* and cultivars of *Malus* x *domestica*. Race 1- 5 of *V.inaequalis* were defined several years ago (Shay & Williams, 1956; Williams & Brown, 1968) and race 6 was defined by Parisi *et al.* (1993) when scab lesion were observed for the first time in an orchard in Ahrensburg, Germany, on cultivars with the Vf gene originating from *M. floribunda* clone 821. Until then the Vf gene were considered a durable resistance gene, because Vf cultivars had been free of apple scab for more than 50 years. Race 6 does not infect the wild *M. floribunda,* which further carries the Vfh gene (Bénaouf *et al.*, 1997). Scab on a garden-grown tree of *M. floribunda* was described by Roberts & Crute in 1994 for the first time. According to Parisi (pers.comm.) this new race should now be called race 7.

In Denmark, several cultivars with Vf resistance have been grown since 1983 at The Danish Institute of Agricultural Sciences, Årslev. Scab symptoms on a few Vf cultivars were observed for the first time in 1997 at Årslev. Lespinasse (1989) proposed that every apple growing country should have an apple scab race screening orchard for determining the

occurrence and development of pathotypes of the apple scab pathogen and for exchanging information and inoculum between countries. The apple scab race screening orchard at Årslev was established in the autumn of 1996 in order to determine the occurrence of races of *V.inaequalis* under orchard conditions in Denmark.

Materials and Methods

Trees

15 apple varieties, received from the INRA collection at Angers in 1994, and the cultivar 'Jonagold' was grafted on M9 rootstocks and planted in 1996 in a field at The Danish Institute of Agricultural Sciences, Department of Fruit, Vegetable and Food Sciences, Årslev (Table1).

Table 1 - Apple varieties planted in the screening orchard at Årslev in 1996

Name	Characteristic	Resistance genes	Susceptible to race
'Jonagold'	Scab susceptible	None	1-6
'Golden Delicious'	Scab susceptible	Vg	1-6
'Akane'	Scab susceptible	Not known	2-5 (?)
'Dolgo'	Diff.host for race 2	Not named	2
h2	Diff.host for race 2	Not named	2
h3	Diff.host for race 3	Not named	3
h4	Diff.host for race 4	Not named	4
h5	Diff.host for race 5	Vm	5
h'5	Diff.host for race 5	Vm	5
h''5	Diff.host for race 5	Vm	5
'Florina'	Scab resistant	Vf and Vg	6
'Prima'	Scab resistant	Vf and Vg	6
'X2594'	Scab resistant	Va	6
'Nova Easygro'	Scab resistant	Vr	6 and 7
'*Malus floribunda* 821'	Scab resistant	Vf and Vfh	7
'Freedom'	Scab resistant	Polygenes and Vf	-

Variety characteristics, resistance and race susceptibility according to Bénaouf & Parisi (1997), Bénaouf & Parisi (1998), Bènaouf *et al.*(1997), Parisi & Lespinasse (1996), Roberts & Crute (1994)

The trees were planted in two blocks of 6 rows with 5 trees of each variety in each plot. The susceptible variety 'Jonagold' was planted between the plots to maintain a high inoculum pressure.

Symptom assessment

Assessment of scab symptoms on leaves was performed in the beginning of august 1998. All the leaves on 2 shoots per tree were assessed using a scale 1-5 derived from Anonym (1985) :

1: No spots, 2: 1 sporulating spot per leaf, 3: 2-4 sporulating spots per leaf, 4: 5-9 sporulating spots per leaf, 5: 10 or more sporulating spots per leaf. From these data the disease severity for each variety was expressed by the mean value obtained from the scores of all leaves. Incidence was expressed as the percentage of scabbed leaves for each variety.

Fruit scab was assessed at harvest using a scale 0-3, where 0: No scab, 1: 0- 0,25 cm^2 of the fruit covered by scab, 2: 0,25 - 1 cm^2 of the fruit covered by scab, 3: >1 cm^2 of the fruit covered by scab. Disease severity on the fruits for each variety was expressed by the mean obtained from the scores of all fruits and the incidence was expressed as the percentage of scabbed fruits for each variety.

Furthermore, an overall assessment of scab on the trees was made in August using a scale 1-9, where 1: No scab and 9: Serious scab.

Results

Results are presented in table 2. The presence of symptoms on fruits and/or leaves of most of the varieties with the different resistance genes indicate that all the known races of *V.inaequalis* and the *M. floribunda* 821 pathotype presumably occurred in the apple scab screening orchard at Årslev in 1998. Race 1 occurred most probably on the susceptible cultivars 'Golden Delicious' and 'Jonagold', which both were seriously attacked by apple scab. Race 2, 3 and 4 probably occurred on their respective differential hosts (h2, h3, h4), while Race 2 incited lesions only on leaves and not on fruits on 'Dolgo'. Race 5 probably occurred only on two of the three differential hosts (h5 and h'5) for this race and not on h''5. Limited symptoms on the Vf cultivar 'Prima' were observed, and these were probably caused by race 6. Neither the other Vf cultivar, 'Florina', nor 'X2594', the variety carrying the gene Va, showed symptoms. Both are susceptible to race 6. 'Freedom', carrying polygenes and Vf resistances, were also free of apple scab symptoms. The most interesting observation was the serious scab infected trees of *M.floribunda* clone 821. Both leaves and fruits were severely infected, both with respect to incidence and severity on fruits and leaves. On 'Dolgo' and 'Nova Easygro' symptoms were only recorded on leaves and not on fruits. Whole tree assessment correlated well with the data obtained from assessment of symptoms on leaves and fruits.

Discussion and conclusion

It seems likely that the seven races of the pathogen, *V. inaequalis*, occurred in this apple scab race screening orchard but in different amounts according to the incidence and severity of symptoms on leaves and fruit. Some of the varieties had no scab symptoms although they were susceptible to some of the races occurring in this orchard (h''5, 'Florina', 'X2594'). This may have been due to resistance mechanisms other than the introduced resistance genes. The observations on differences in incidence and severity between fruits and leaves on two of the varieties could be due to organ specific resistance in these varieties towards their specific races under orchard conditions. The most diseased variety besides the susceptible varieties 'Jonagold' and 'Golden Delicious' was the previously resistant *M. floribunda* 821. From personal communication with L. Parisi it seems that the pathotype on *M. floribunda* 821 (race 7) is becoming more and more widespread. Besides England it now occurs in The Netherlands, France and perhaps Germany. The resistance in Vf cultivars is beginning to become ineffective and the long standing resistance in *M. floribunda* has broken down in the apple scab race screening orchard at Årslev.

Assessement of the severity and incidence of apple scab on the varieties in this orchard will be continued in the comming seasons in order to study the development of the populations of the different races on these differential varieties.

Tabel 2 - Occurrence of scab lesions on leaves and fruits of 16 apple varieties in August 1998 and races possibly occurring on these varieties

Name	On leaves		On fruits		Tree	Races possibly occurring
	Incid.[1]	Severity[2a]	Incid.[1]	Severity[2b]	Index	
'Jonagold'	97.2	4.7	-	-	6.8	1-6
'Golden Delicious'	93.9	4.6	100	3.0	7.2	1-6
'Akane'	38.8	1.9	58.2	1.3	3.6	2-5 (?)
'Dolgo'	5.0	1.1	0	0	2.0	2
h2	9.3	1.2	16.8	0.2	3.9	2
h3	56.4	2.7	68.1	1.6	1.8	3
h4	12.6	1.3	15.1	0.2	3.2	4
h5	34.5	2.0	42.1	0.9	2.0	5
h'5	0.4	1.0	2.2	0.0	1.6	5
h''5	0	1.0	0	0	1.0	-
'Florina'	0	1.0	0	0	1.0	-
'Prima'	5.9	1.2	6.7	0.2	1.7	6
'X2594'	0	1.0	0	0	1.0	-
'Nova Easygro'	1.9	1.0	0	0	1.4	6, 7
'*M. floribunda* 821'	93.6	4.6	80.5	1.2	7.8	7
'Freedom'	0	1.0	0	0	1.0	-

1 Incidence = % of scabbed leaves or fruits.
2a Severity on leaves: score between 1 (no scab spots) to 5(>10 spots per leaf), grading scale from Anonymous (1985).
2b Severity on fruits : score between 0 (no scab spots) to 3 (>1cm^2 of the fruit covered by scab).
3 Scab index for whole tree: score between 1(scab free) and 9 (seriously scabbed).

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Evaluation of the pathogenicity of two scab isolates derived from the Vf-resistant apple cultivar 'Baujade'

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Abstract : To date, there have been three confirmed reports of apple scab lesions found on Vf-resistant apple cultivars in HortResearch orchards in New Zealand. Given the importance of the *Vf* gene in the HortResearch apple breeding programme, several monospore cultures of *Venturia inaequalis* were made from one lesion which was found on a fruit from a Vf-resistant 'Baujade' tree. Two of these isolates were compared for their pathogenicity with two isolates derived from 'Granny Smith' and a third isolate from an unknown apple host from the same orchard as the 'Baujade' isolates. The isolates were tested in replicated trials involving the pathodemes 1 to 5, the Vf-resistant accession X3189, and seedlings from 20 crosses, mostly between susceptible and Vf-resistant parents. In the seedling test, average scores were calculated for each family/isolate combination by assigning points to each seedling based on their resistance symptoms. Neither of the two 'Baujade' isolates were able to infect any of the pathodemes containing resistance genes, but did infect the susceptible host (pathodeme 1). They also did not show any shift towards virulence on the seedlings derived from Vf-resistant parents. In fact, they were less pathogenic in all crosses, except for a cross between 'Colonel Vaughan' and an open-pollinated seedling from *M. floribunda* 821. Therefore, these isolates cannot be considered as race 6. The average resistance scores and proportions of resistant seedlings varied widely among the Vf-resistant families. These findings confirm earlier observations and suggest that minor genes play an important role in the expression of the *Vf* gene.

Key words : apple scab, *Venturia inaequalis*, pathogenicity, virulence, race, differential host

Introduction

The V_f gene has played a prominent role in the development of scab resistant apple cultivars worldwide (Crosby *et al.*, 1992). Vf-resistant cultivars have frequently been reported to be infected by *Venturia inaequalis* in the orchard (Lespinasse, 1994), but the occurrence of resistance breakdown was not confirmed until 1993 (Parisi *et al.*, 1993). We see the same pattern in New Zealand where all the current advanced selections only carry the V_f gene. While Vf-resistant cultivars have been reported to be infected by *V. inaequalis* here too (Wearing *et al.*, 1995), to date there is no confirmation of *V. inaequalis* having overcome this resistance. In this paper, we report on the pathogenicity of two *V. inaequalis* isolates derived from a lesion on a fruit of the Vf containing cultivar 'Baujade' at the Nelson Research Centre in 1996. Since inoculation of the scab differential hosts in preliminary tests had not identified any virulent strains, seedlings were included in this test because they are much more sensitive, and give better symptom expression than cloned trees budded onto rootstocks.

Secondly, seedlings provide a range of resistances/susceptibilities, which allows for the shift in virulence to be measured in the ratios of seedlings in the different resistance classes. This approach was proved effective previously by Parisi *et al.* (1993).

Materials and methods

Venturia inaequalis isolates
Five *V. inaequalis* isolates were selected from the HortResearch repository :

1. 110.1 'Baujade', ex Nelson. Isolated from a fruit in January 1996.
2. 110.3 'Baujade', ex Nelson. Isolated from a fruit in January 1996.
3. 139.1 'Granny Smith', ex Auckland. Isolated from a leaf in January 1996.
4. J222 unknown, ex Nelson (near 'Baujade'). Isolated from a leaf in December 1996.
5. J243 'Granny Smith', ex Auckland. Isolated from a leaf in February 1997.

Pathodemes and seedling families
The following pathodemes were used: Royal Gala and Galaxy (h1, susceptible control), a segregate (TSR34T132) of Russian apple R12740-7A (h2, *Vh2*), 'Geneva' (h3), another segregate (TSR33T239) of Russian apple R12740-7A (h4, *Vh4*), accession 9-AR2T196 ex *Malus micromalus* (h5, V_m), and accession X3189 (a selection from Angers, France, carrying the *Vf* gene). Since X3189 has not been evaluated for its susceptibility to race 6 to date (Y. Lespinasse, pers. comm.), we would like to note that in this paper this accession represents the Vf resistant cultivars rather than host 6. The trees on M.793 rootstock in 5 L bags were 2 years old and had two or three actively growing shoots each at the time of inoculation. The inoculations were replicated three times, each replicate consisting of two trees. For the seedling tests, seed from 20 families (Table 1) were planted in root trainers (30 seeds/basket) and stratified for 7 weeks at 0C. Then the baskets were transferred to the glasshouse and the plants were grown to the six-leaf stage before inoculation with *V. inaequalis*. In both cases, the plants were kept separated in open-top chambers with plastic sides 1.2 m high.

Inoculations
Re-juvenated inoculum was produced by separately inoculating a few seedlings from a 'Golden Delicious' x 'Braeburn' cross with a suspension of conidia from each isolate grown on malt extract agar (MEA), and making new monospore cultures. Bulk inoculum was prepared in petri dishes with MEA. Inoculation techniques and incubation conditions were as described by Gardiner *et al.* (1996). The pathodemes were spray-inoculated till run-off with the individual isolates at 1.7×10^5 conidia/ml on 28 October 1998, and evaluated on 24 November 1998. The seedlings were inoculated on 29 September 1998 with 7.5×10^4 conidia/ml, and evaluated on 19-21 October 1998. Both the pathodemes and the seedlings were individually rated for disease symptoms on a scale adapted from Chevalier *et al.* (1991), where classes 0 and 1 correspond with hypersensitivity, and class 4 with susceptibility. For the seedlings, resistance scores were calculated for each family/isolate/replicate combination by assigning 5 points to seedlings showing no symptoms down to 0 point for susceptible seedlings, and dividing the sum by the number of seedlings. Such a score integrates both the classes of resistance and the proportion of resistant seedlings occurring in a family.

Results and discussion

All isolates predominantly caused symptoms typical for each pathodeme, i.e. susceptibility symptoms on h1; stellate necrotic lesions on h2 and h3; pin-point lesions on h4 and h5; and small necrotic lesions on X3189 (Table 2). As anticipated, symptom expression was not as strong on the pathodemes as on the seedlings, notably with those pathodemes containing resistances requiring long incubation periods (X3189 and to a lesser extend h3). From the

evaluation of 7,595 seedlings, it appeared that the 'Baujade' isolates on average were less pathogenic, i.e. the seedlings had higher resistance scores, than the other isolates, although the difference was significant ($P<0.05$) for isolate 110.1 only (Table 3). This difference was reflected in all the families individually, including those involving the V_f gene, with the notable exception of families 3 and 6 (Table 3). From both the pathodeme and seedlings tests, where neither 'Baujade' isolate showed virulence following re-inoculation on Vf hosts, we conclude that the 'Baujade' isolates cannot be classified as race 6 *sensu* Parisi *et al.* (1993). A plausible explanation is that under optimal conditions for the fungus and perhaps also sub-optimal conditions for the gene to express resistance, race 1 isolates can cause infection on resistant cultivars. This response is frequently observed in the HortResearch breeding programme, when seedlings scoring 3A and 3B (Chevalier *et al.*, 1991) in the greenhouse remain disease-free in the orchard.

Table 1. The families and the resistances of the parents in this study

Family	Parents	Resistance[z]
1	A20/02/273 x A40/04/119	- x Vf
2	A40/04/119 x A20/02/273	Vf x -
3	A68/03/079 x A68/03/013	Vf x Vf
4	A92-23 x Reinette Simirenko o.p.	Vf x as
5	Baujade o.p.	Vf x ?
6	Colonel Vaughan x *M. floribunda* 821 o.p.	as? x Vf/Vfh?
7	Fuji x Mildew Immune Seedling o.p.	- x -
8	Golden Delicious x Braeburn	- x -
9	Granny Smith o.p.	- x ?
10	Granny Smith x TSR33T239	- x Vh4
11	GS48 x A20/02/273	- x -
12	GS494 x A39/02/024	- x Vf
13	GS58 x A40/02/025	- x Vf
14	Horei x A24/05/038	- x Vf
15	Liberty x Geneva	Vf x Vh3?
16	Malus 6 x A40/04/119	as x Vf
17	Pacific Rose x A38/02/119	- x Vf
18	Prima x Longfield o.p.	Vf x V?
19	Royal Gala x A998/02/108	- x as
20	X3191 x Novosibirski Sweet o.p.	Vf x as?

[z] Resistance :

-	no resistance	Vh3	stellate type HR ex 'Geneva'
as	polygenic resistance to apple scab	Vh4	pit-type HR ex Russian apple 12740-7A
Vf	Vf resistance ex *M. floribunda* 821	?	resistance not confirmed or unknown
Vfh	pit-type HR ex *M. floribunda* 821		

In most families, the scores were very similar for both 'Baujade' isolates, which can be explained by the fact that both isolates came from the same (and only) lesion on the fruit. Assuming the lesion had grown from a single spore, they are expected genetically to be the same. The contrast between the 'Baujade' and the other isolates was most obvious in families

7 and 9 (Table 3), and the relatively high scores for the 'Baujade' isolates in family 7 may indicate the presence of an (ephemeral) major resistance similar to the V_g gene in 'Golden Delicious' (Bénaouf and Parisi, 1997). None of the five isolates carried the avirulence gene corresponding to this gene since all isolates grew well on the 'Golden Delicious' x 'Braeburn' seedlings for bulking up of the inoculum, while all the scores were 0 for family 8 in the comparing test (Table 3). This indicated that the isolates are well adapted to these long-established cultivars and have overcome any resistances that may have been present (Gessler *et al.*, 1993; Sierotzki *et al.*, 1994a, 1994b). A more promising candidate for a cultivar with a new major gene for scab resistance is 'Longfield' in family 18 (Table 3). Many seedlings in this family did not show any symptoms which may indicate that a putative resistance gene conferring hypersensitivity is involved. While the presence of many symptomless seedlings in family 6 agrees with the presence of hypersensitivity to apple scab in *M. floribunda* 821 (Shay and Hough, 1952), from the segregation it could not be confirmed that the V_{fh} gene (Bénaouf *et al.*, 1997) was present in the open-pollinated derivative of this clone used as a parent in this family.

Table 2. The predominant symptoms (in bold) and the range of symptoms caused by 5 isolates on 6 pathodemes. Symptoms were rated on a scale adapted from Chevalier *et al.* (1991), where classes 0 and 1 correspond with hypersensitivity, and class 4 with susceptibility.

('-' denoting weak expression; and '+' denoting strong expression of symptoms.)

Pathodeme	Isolate				
	110.1	110.3	139.1	J222	J243
h1	**4** 3B – 4	**4** 4	**4** 4- – 4	**4** 4	**4** 4- – 4
h2	**2** 1 – 2	**2** 1 – 2	**2** 1 – 2	**2** 1 – 2	**2-** 0 – 2
h3	**1** 1- – 2-	**1+** 1 – 2	**1-** 0 – 2-	**1** 1 – 2-	**1** 0 – 2+
h4	**1** 1- – 1+	**1** 1 – 1+	**1+** 1 – 1+	**1** 1 – 1+	**1** 1- – 1+
h5	**1** 1- – 1	**1** 1	**1** 1 – 1+	**1** 1	**1** 1- – 1+
X3189	**2-** 0 – 2	**2** 2- – 2	**2-** 0 – 2	**2** 2- – 2	**2-** 0 – 2

The average scores for the families where a Vf parent was crossed with a susceptible or (putatively) polygenic resistant parent ranged from 0.36 in family 17 to 2.23 in family 2 (Table 3). This is in agreement with the wide range in segregations commonly found in breeding programmes (Laurens and Lespinasse, 1996), which supports the theory of minor or modifier genes being involved in the Vf resistance complex (Rousselle *et al.*, 1974; Gessler, 1992).

The score for family 2 was unusually high. Unless an error was made at the time of pollination and pollen of a Vf containing resistant parent was applied, it would suggest that there was a strong maternal effect compared to its reciprocal cross (family 1), which showed a medium resistance score (Table 3). Family 2 had a similar score to those of families 3 and 5. In the case of family 5, the 'Baujade' mother tree was adjacent to another Vf selection (X3189) that flowered at the same time virtually providing the only pollen available for this open-pollinated parent. The relatively high scores of these families (Table 3) may in part be due to the presence of seedlings carrying the V_f gene in a homozygous state, which are known to show stronger resistance reactions than those carrying the gene in a heterozygous state (Gianfranceschi *et al.*, 1999).

Table 3. The individual and average resistance scores by family and by isolate.

Family	Resistance	Isolate					Average
		110.1	110.3	139.1	J222	J243	
18	Vf x V?	3.07	2.93	1.90	2.44	2.22	2.512
6	as? x Vf/Vfh?	1.85	1.96	2.47	3.72	2.50	2.501
5	Vf x ?	2.68	2.77	1.33	2.72	1.84	2.267
2	Vf x -	2.95	2.30	1.94	1.72	2.24	2.230
10	- x Vh4	2.94	2.50	2.06	1.86	1.59	2.190
3	Vf x Vf	1.81	2.08	2.02	1.96	2.31	2.037
20	Vf x as?	2.19	1.42	1.78	1.95	1.62	1.793
15	Vf x Vh3?	2.06	1.62	1.29	1.53	1.21	1.542
12	- x Vf	2.22	2.12	1.33	1.03	0.96	1.532
16	as x Vf	1.43	1.57	1.36	1.25	1.62	1.446
4	Vf x as	1.91	1.11	0.99	1.29	1.27	1.315
1	- x Vf	1.47	1.20	1.19	1.14	0.62	1.127
13	- x Vf	1.33	0.95	0.82	0.99	0.46	0.910
14	- x Vf	0.89	1.08	0.72	0.10	1.25	0.809
7	- x -	1.24	0.73	0.00	0.00	0.00	0.394
17	- x Vf	0.53	0.35	0.43	0.34	0.15	0.359
19	- x as	0.19	0.08	0.11	0.08	0.20	0.131
9	- x ?	0.27	0.32	0.00	0.00	0.00	0.119
11	- x -	0.12	0.09	0.00	0.00	0.07	0.056
8	- x -	0.00	0.00	0.00	0.00	0.00	0.000
Average		1.558 a[z]	1.360 ab	1.086 b	1.207 ab	1.107 b	1.263

[z] Different letters denote the isolates differ significantly in their resistance score ($P < 0.05$)

Although to date we still are in the fortunate position of not having a *V. inaequalis* race in New Zealand that can overcome the V_f gene, occurrence of resistance breakdown overseas tells us to remain vigilant in monitoring for the development of such a race as it would have a serious impact on the breeding of new cultivars currently heavily reliant on the V_f gene. Since we recognise the possibility of the future breakdown of the V_f gene, the HortResearch apple

breeding programme has a strong focus on deploying alternative sources of apple scab resistance (Bus *et al.*, 2000).

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Expression of Golden Delicious resistance to race 7 of *Venturia inaequalis*

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Abstract : The resistance of the apple cultivar Golden Delicious to a race 7 strain, avirulent to the *Vg* gene, was characterized by different observations (macroscopic, microscopic and histological). The results showed a strong necrotic reaction 72 hours after the inoculation on the young leaves. A very weak sporulation could be observed on the necrotic symptoms, scored as 2 or 3a symptoms in the scale of Chevalier *et al.* (1991). The leaves tissues underwent strong modifications. These symptoms, which could characterize the expression of the resistance of the *Vg* gene, are different from those observed in *Vf* resistant cultivars inoculated with incompatible strains.

Key words : *Malus x domestica*, monogenic resistance, apple scab, scanning electron microscopy, histology, necrotic stress.

Introduction

The cultivar Golden Delicious is one of the most cultivated throughout the world. It is considered in most of the countries as susceptible to *Venturia inaequalis*. In France, Golden Delicious is classified in an intermediate range of susceptibility (Parisi and Trillot, 1993) and 10 to 15 fungicide treatments are necessary to protect this cultivar against apple scab. However, the occurrence of *V. inaequalis* strains avirulent to this cultivar has been reported by different authors. The race 7 of *V. inaequalis*, virulent to *Malus floribunda* 821 (Roberts and Crute, 1994) is avirulent to Golden Delicious (Bénaouf and Parisi, 1997). In this study, we compared the macroscopic and microscopic expression of Golden Delicious susceptibility and resistance to virulent and avirulent *V. inaequalis* strains.

Material and methods

Young plants of Golden Delicious grafted onto MM 106 were inoculated in a growth chamber with 2 strains of *V. inaequalis* (Table 1).

Table 1. Origin and characteristics of the strains tested

Strain	Origin	Cultivar	Characteristics
104	France, 1978	Golden Delicious	Race 1 reference strain
1066	France, 1993	*Malus floribunda* 821	Race 7 reference strain[a]

a Monoconidial strain from Fl 1 isolate, found in England by Roberts and Crute (1994)

The methods for inoculation and incubation were described by Parisi *et al.* (1993). The class of the symptoms was assessed with the scale of Chevalier *et al.*(1991), which consists of 6 classes :

Class 0 : no symptom,
Class 1 : hypersensitivity (pin-point pits),
Class 2 : resistance, chlorosis and/or necrosis without sporulation,
Class 3a : weak resistance, chlorosis and/or necrosis with slight sporulation,
Class 3b : weak susceptibility, chlorosis and/or necrosis with sporulation
Class 4 : susceptibility, abundant sporulation without chlorosis and/or necrosis.

Sporulation intensity assessment was done according to the method described by Benaouf and Parisi (1998). The 4 youngest unrolled leaves of 2 to 3 shoots per sampling time and strain were collected for this study. The results are expressed as a mean number of conidia per cm^2 of leaf area.

For Scanning Electron Microscopy (SEM), the samples were collected and prepared according to Chevalier *et al.* (1991), and examined with a JSM 6301F scanning electron microscope. Samples for histological observations were fixed in 4% glutaraldehyde in 0.1M Soerensen buffer (pH 7.4) for 2 hours at 4°C under vacuum. Fixed samples were dehydrated in a graded ethanol series and embedded in technovit 7100 as discribed by Kroes *et al.* (1998). The sections of 1.5 - 2.0 µm were made with Reichert Jung rotary microtome, stained with toluidine blue O and examined with a Polyvar Reichert-Jung microscope.

Results

Macroscopic observation

Golden Delicious gave typical susceptibility symptoms (class 4 in the scale of Chevalier *et al.*, 1991) 9 days after the inoculation with the 104 strain of race 1. With the strain 1066 of race 7, we observed, 72 hours after inoculation, a very characteristic wide necrotic reaction on the infected young leaves, scored as atypical class 2 symptom.

Sporulation study

The results are given by table 2. They showed that 9 days after inoculation, conidia were produced in the case of strain 104, but not for strain 1066. The number of conidia observed with strain 104 increased between 11 and 21 days, reaching nearly 380 000 conidia/cm^2. At the same time, with strain 1066, a low amount of conidia was observed.

Table 2. Number (x 10^3) of conidia/cm^2 of leaf observed between 9 and 21 days after the inoculation of Golden Delicious with strains 104 and 1066.

Days after the inoculation	Strain 104 (race1)	Strain 1066 (race7)
9	35.7	0
11	31.7	0.4
14	169.8	2.5
17	265.3	1.5
21	379.9	2.3

SEM and histological studies

1. Compatibility (race 1) :

The SEM observations showed an abundant network of subcuticular stromatic strand. A lot of conidiophores pierced the cuticle (fig. 1) and differentiated numerous conidia. The histological studies showed no evident modification of infected leaf tissues (fig. 2).The upper epidermis remained turgescent and the density and structure of palisade tissue was not modified. There was no expression of resistance symptoms.

2. Incompatibility (race 7) :

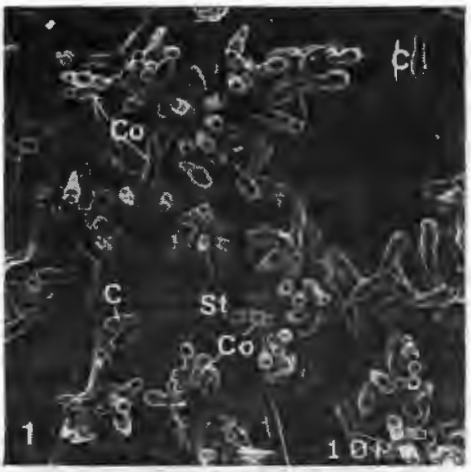
The SEM revealed on young necrosis that the fungus presence was reduced to isolated and necrotic hyphae (fig. 3). Below the isolated hyphae, a large area of collapsed upper epidermis cells were observed (fig. 4). The palisade tissue was strongly modified; the cellular density increased and the cytoplasm became necrotic.

Sometimes, after 11 days of incubation, the microscopic observations showed a very weak sporulation around the necrotic symptoms (similar to class 3a symptom). Many conidiophores were aborted (fig. 5), only a few of them produced one or two conidia. The histological study of necrotic area showed a complete destruction of foliar tissues (fig.6).

Discussion

The resistance expression of Golden Delicious to strain 1066 is a very characteristic symptom, different from the *Vf* gene resistance expression and different from the pin-point pits of *Malus floribunda* 821 inoculated with incompatible strains. This reaction is similar to a hypersensitive reaction by its rapidity and the necrotic stress induced, but the large necrotic areas observed on infected young leaves can be assimilated to an atypical class 2 symptom. After 11 days of incubation, the sporulation study revealed a very weak sporulation, confirmed by the SEM studies; this necrotic symptom with slight sporulation is a class 3a symptom according to Chevalier *et al* (1991). Bénaouf and Parisi (1997) showed that Golden Delicious resistance to strain 1066 of race 7 was due to a single gene named *Vg*. The symptoms described in this study can characterize the expression of the resistance induced by this gene.

Bénaouf et Parisi (1997) assumed that the presence of the *Vg* gene in some *Vf* resistant hybrids could explain their resistance to race 7 strains. It is one of the hypothesis to explain the resistance of Judaine in a cider apple orchard in Normandie, where the Judeline resistance was overcome (Parisi *et al*., this meeting). Both cider apple varieties carry the Vf gene. These results are consistent with the hypothesis that most of the differential interactions between apple and *V. inaequalis* have not been detected (Sierotzki *et al*., 1994), and that the genetic variability of apple, in terms of major resistance genes, is not completely explored.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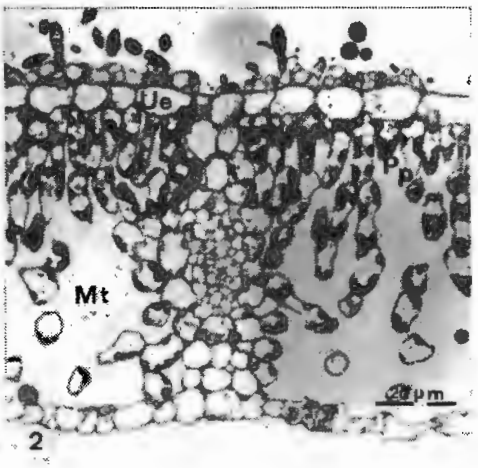
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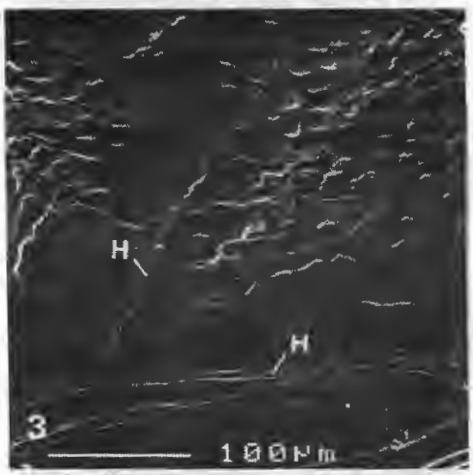
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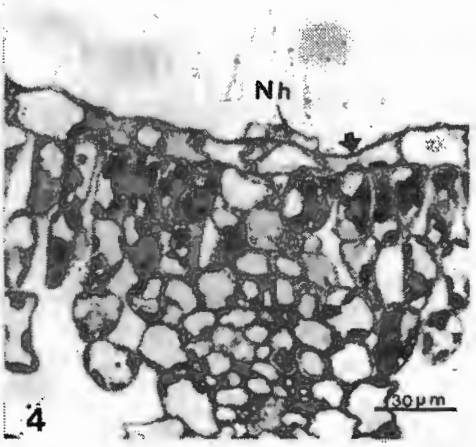
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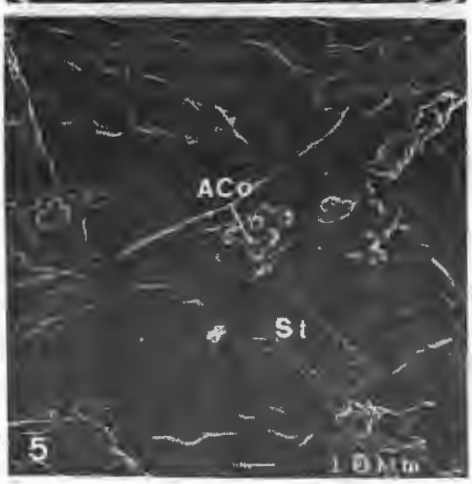
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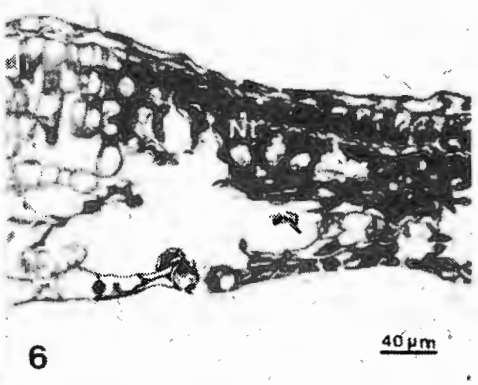
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Legends of the figures 1 to 6 :

Golden Delicious inoculated with strain 104 (race 1).

Fig.1: SEM observation of a compatible relationship; St: subcuticular stroma, Co: conidiophore, C: conidium.
Fig.2: Histological study; Ue: upper epidermis, Pp: palisade parenchyma, Mt: mesophyll tissue.

Resistance reaction of Golden Delicious to strain 1066 (race 7).

Fig.3: Isolated hyphae and leaf reaction; H: hyphae.
Fig.4: Histological study of leaf reaction; arrow: collapsed cells, Nh: necrotic hyphae.

Fig.5: On a necrotic area the stroma differentiated aborted conidiophores; ACo: aborted conidiophores; St: stroma.
Fig.6: Histological study of a necrotic symptom ; Nt: necrotic tissues.

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Localisation of a major gene for apple scab resistance on the European genetic map of the Prima x Fiesta cross.

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Abstract : A major gene conferring resistance to apple scab has been identified and mapped on linkage group 12 in the cross Prima x Fiesta. The genetic map used for the mapping of this gene is an extension of the one constructed during the European Apple Genome Mapping Project (Maliepaard *et al.*, 1998). The newly identified dominant gene is present in Prima in heterozygous condition, while Fiesta is homozygous recessive. It confers resistance towards two *Venturia inaequalis* monoconidial strains of race 7, which race is virulent to *Malus floribunda* 821 (Roberts and Crute, 1994) but avirulent to Golden Delicious (Bénaouf and Parisi, 1997). Resistance tests were performed twice on 149 individuals of the progeny in a greenhouse as well as in a climatic chamber. Resistance segregated in a 1:1 ratio ($\chi 2 = 1.96$ n.s.) towards both strains and mapped on one end of the linkage group 12 (LOD = 27) at about 3 cM from a new RFLP marker (MC105). So this major gene inherited independently from the *Vf* gene located on linkage group 1 of the same genetic map. The resistance symptoms observed on Prima and on the resistant part of the progeny were similar to those observed on Golden Delicious, which cultivar was simultaneously tested as control. Since Golden Delicious is a grandparent of Prima but absent in the pedigree of Fiesta, this major gene is likely to be the *Vg* gene identified on Golden Delicious by Bénaouf and Parisi (1997).

Key words : *Malus x domestica*, major resistance gene, *Vg*, *Venturia inaequalis*, race 7, genetic map, molecular markers

Introduction

Apple is very susceptible to scab (caused by the fungus *Venturia inaequalis*) in commercial orchards. The *Vf* gene, deriving from *Malus floribunda* clone 821, has been introgressed into domesticated apple by successive pseudo-backcrosses. It confers a resistance to *Venturia inaequalis* races 1 to 5. Many new cultivars carrying the *Vf* gene have been released (Crosby *et al.*, 1992). Most of them are susceptible to a new race of *V. inaequalis*, called race 6 (Parisi *et al.*, 1993 ; Parisi and Lespinasse, 1996). Another race of *V. inaequalis* has been characterised by Bénaouf and Parisi (1997) after isolation of monoconidial strains from the FL1 isolate collected on a scabbed *Malus floribunda* tree in England by Roberts and Crute (1994). This race 7 ('English' race) is virulent to *Malus floribunda* 821 and avirulent to Golden Delicious and to progenies descending from Golden Delicious which carries the *Vg* gene (Bénaouf and Parisi, 1997). The *Vg* gene gives typical large necrotic lesions with strong crispation of leaves about 10 days after inoculation with race-7 strains (Chevalier and Parisi, this meeting).

Here we report results on the localisation of a major resistance gene towards race-7 strains of *V. inaequalis* on a linkage group of the genetic map of the apple cultivar Prima. The whole genetic map of the cross Prima x Fiesta was initially constructed during the European Apple Genome Mapping Project (King, 1996 ; Maliepaard *et al.*, 1998) and has recently been

extended (Van de Weg *et al.*, unpublished). We show that this major resistance gene should be the *Vg* gene.

Material and Methods

Two different strains of race-7 were examined :

- strain n° 1066 deriving from the English isolate FL1 (Roberts and Crute, 1994) ; this strain was isolated and used by Bénaouf and Parisi (1997) to identify the *Vg* gene; the inoculation with this strain was repeated twice (experiments 1 and 2) ;
- strain n° 1161 deriving from an isolate sporulating on the cultivar Judeline (*Vf*-resistant) in a French apple cider orchard (same origin as the strain n° 1163 described by Parisi *et al.*, this volume) ; this strain was inoculated only once in experiment 3 on a large subset of the progeny.

147 descendants of the cross Prima x Fiesta were grafted onto rootstock MM106, potted, and grown in the greenhouse during the Spring 1997 (experiment 1) ; the same trees were grown for a second year in a climatic chamber during the Spring 1998 (experiment 2). Both these experiments were performed with strain 1066. In addition, a third experiment was performed in which 124 descendants were tested to strain 1161. This experiment was performed simultaneously with experiment 1. Most of the time, 2 trees were available for each individual in each experiment.

The protocol for the inoculation and the incubation of the plants was described by Parisi and Lespinasse (1996).

Symptoms were assessed 2 to 3 times between 10 and 25 days after inoculation. For each shoot, all the leaves were observed for the presence of symptoms, the class of the symptoms on the scale of Chevalier *et al.* (1991), and when scab lesions were visible, the percentage of leaf area with sporulating lesions. A major attention was given to the presence/absence of the necrotic resistance symptom generally accompanied by crispation of the limb of the leaf. This symptom can be assimilated to an atypical class 2 symptom in the scale of Chevalier *et al.* (1991) or to an atypical class 3a symptom since a weak sporulation could be observed in some cases. Results concerning the amount of sporulation will not yet be presented.

Genetic map distances and gene orders were computed with the program JoinMap™ (Stam, 1993) using the Kosambi function.

Results

The cultivars Prima and Golden Delicious were resistant to both strains, showing the presence of the typical necrotic resistance symptoms, although more emphasised for the second cultivar. The cultivar Fiesta was susceptible, but with low sporulation. Within the progeny, 82 descendants showed necrotic symptoms while 65 descendants showed low to high sporulation without the necrotic symptom, when tested to strain 1066. These numbers fit to the 1:1 ratio expected for the segregation of a major resistance gene ($\chi^2 = 1.96$ n.s.).

Results observed with strain 1161 were very highly consistent with those obtained with strain 1066 indicating that the same resistance gene is acting towards this French strain.

Using the marker data from the EAGMAP project (Maliepaard *et al.*, 1998) together with new AFLP and RFLP data produced by CPRO-DLO within the current DARE European project (Lespinasse *et al.*, 1999 ; http://www.inra.fr/Angers/DARE/index.htm), it was possible to localise this major gene at the end of the linkage group 12 (Figure 1), 3.4 cM apart from a new RFLP marker (MC105) with a LOD score of 27. The markers MC105, OPA01-700 and

OPAM19-1020 were interestingly slightly skewed. Since these 3 dominant markers are in a repulsion phase with *Vg*, this skewness was in the opposite direction as that for *Vg*, though within the same width as the resistance/susceptibility ratio.

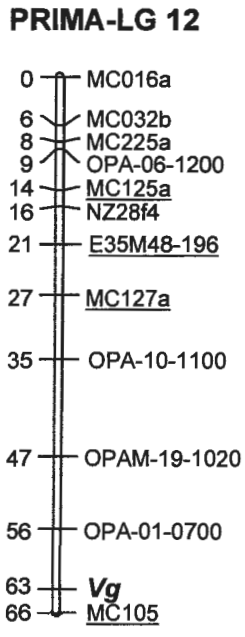

Figure 1. Prima linkage group 12 with new AFLP and RFLP markers (underlined) and the major scab resistance gene *Vg*. Distances in centiMorgans (JoinMap ; Stam, 1993) using the Kosambi mapping function.

Discussion

The necrotic resistance symptoms observed on Prima and on the resistant part of the progeny were similar to those observed on Golden Delicious. Since Golden Delicious is a grandparent of Prima but absent in the pedigree of Fiesta, the major gene segregating in the progeny Prima x Fiesta is likely to be the *Vg* gene of Golden Delicious as identified by Bénaouf and Parisi (1997).

The genetic mapping of this major gene is very interesting both for marker assisted selection aiming at major gene pyramiding (Pedersen and Leath, 1988) and for basic research on host-pathogen interactions as well as on the genetic organisation of resistance factors within the apple genome. We are currently trying to transform the RFLP marker MC105 into an easy-to-use SCAR or CAPS marker. Other closely linked markers are also searched for in another large progeny involving Golden Delicious as parent and which has already been tested to strain 1066. These markers will then be used to assess the presence of the *Vg* gene in numerous recently-created cultivars to predict or confirm their resistance status as regards to *Venturia inaequalis* race-7 strains.

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Transfer of resistances to Valsa canker and powdery mildew from wild *Malus* species to cultivated apples

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Abstract : Valsa canker caused by *Valsa ceratosperma* (Tode ex Fr.) Maire is most serious wood rot disease in Japan. Resistance of wild *Malus* species and hybrids to this disease was examined. Results of dormant excised twig assay showed that several accessions belonging to *M. sieboldii*, *M. baccata*, *M. halliana*, *M. florentina*, and *M. micromalus* were resistant to the canker. All the individual progenies derived from *M. sieboldii* showed continuous distribution of canker length, suggesting that the resistance of *M. sieboldii* to the disease is controlled by polygenes. Some accessions belonging to *M. sieboldii* were not only resistant to Valsa canker but also highly resistant to powdery mildew (*Podosphaera leucotricha*) with no visual symptoms after spore inoculations. Progenies of *M. sieboldii* ('Sanashi 63' x 'Jonathan','Jonathan' x 'Sanashi 61' and 'Jonathan' x 'MO-15') showed high resistance, indicating that *M. sieboldii* had dominant gene pair(s) for the resistance to powdery mildew. A few plants among progenies of *M. sieboldii* had an adequate level of resistance to Valsa canker with high resistance to powdery mildew.

Key words: apple, breeding, resistance, Valsa canker, powdery mildew

Introduction

When plant breeders plan breeding programs of apple, they should give priority to the breeding for resistances to economically important diseases and pests in apple. Apple breeders should aim at raising, in a systematic way, the level of disease and pest resistance in apple cultivars. This will not only significantly reduce the cost of chemical sprays but will stabilize yield in apple orchards. It will also greatly contribute to reduce the risk of environmental pollution.

Valsa canker caused by *Valsa ceratosperma* (Tode ex Fr.) Maire is the most serious wood rot disease of apple in Japan (Sakuma 1990). No commercial apple cultivars are known to be resistant to the canker. Because no curative fungicides are available for controlling the canker, it is very important to find gene sources for resistance to Valsa canker and transfer the resistance to apple cultivars.

This paper describes that several accessions belonging to wild *Malus* species such as *M. sieboldii, M. halliana, M. floribunda, M. scheideckeri, M. toringo, M. micromalus, M. florentina* and *M. baccata* were resistant to the canker. Some of the accessions, resistant to the Valsa canker, were also assessed for resistance to powdery mildew caused by *Podosphaera leucotricha.*

Material and Methods

Inoculation test for evaluation of resistance to Valsa canker

An excised twig assay developed by Fujita *et al.* (1984) was performed with some modifications to evaluate resistance to Valsa canker. Dormant shoots, removed from *Malus*

and *Pyrus* species, cultivars and their hybrids in Apple Research Center, National Institute of Fruit Tree Science, Japan, were wrapped and stored in refrigerator at 3~4°C until use.

Valsa ceratosperma isolates AVC-12 and VC96-A were cultured for 10~14 days on PDA medium at 25°C Then fungal mycelium including an agar was added into 100 ml volume of distilled water and mildly homogenized. The mixture after homogenization was used as an inoculum for the excised twig assay.

The stored dormant shoots were cut into 10cm length. Five to eight twigs per genotype were prepared. Distal cut end of twig segments was scorched with a hot iron. After scorch treatment bundles of the twigs were put vertically into a plastic box, proximal cut end of the twigs being dipped into water about 1cm depth. 10μl of the mixture including fungal mycelium of *V. ceratosperma* was dripped on the distal cut end, then the plastic box was covered with a vinyl film to keep humid condition in the box. After 10 days incubation at 25°C, the length of bark necrosis in the twig segments was measured. As for evaluation of apple hybrid plants for resistance to Valsa canker, canker length of each plant was expressed as the relative length to'Fuji', and plants with a relative length less than 50 and 50~69 were regarded as resistant and moderately resistant, respectively. Plants with the length more than 70 were regarded as susceptible.

Inoculation test for evaluation of resistance to powdery mildew

Nine apple cultivars, grafted on to JM8(ARM8), new size-controlling apple rootstock (Soejima *et al.* 1998), and 8 apple families raised by crosses among 'Jonathan', 'Megumi', 'Golden Delicious', *M. robusta* and *M. sieboldii*, were tested for resistance to powdery mildew in 1998 and/or 1999. In June to early July the two-month-old grafted plants and four-month-old hybrid seedlings were sprayed for inoculation with a suspension of conidia ($2\sim3\times10^5$/ml in 1998, $1\sim2\times10^5$/ml in 1999) collected from mildew-infected apple seedlings grown in a glasshouse. Reaction of the plants and seedlings to the pathogen was rated visually 10 and 30 days after inoculation in 1998. In 1999 rating was carried out 21 days after inoculation. The most seriously infected leaf in each plant was rated according to the rating scale of Markussen et al. (1995). Plants with a score of 0 (no visual symptoms) were evaluated as highly resistant, 1 (rate of infected leaf area less than 12.5%) or 2 (12.5~25%) were resistant, scored 3 (25~50%),
4 (50~75%) and 5 (more than 75%) were regarded as susceptible.

Results and Discussion

Difference of resistance to Valsa ceratosperma among Malus and Pyrus species and cultivars

Lesion length on excised dormant twigs of *Malus* and *Pyrus* species inoculated with *Valsa ceratosperma* isolate AVC-12 is shown in Table 1. The lesion length of 'Shirobana robusta' belonging to *M. robusta*, 'Waringo' belonging to *M. asiatica*, 'Elstar', 'HAC 9', 'Fuji' and 'Tsugaru' belonging to *M.* x *domestica* exceeded 20 mm, while the canker length was less than 10 mm in 'Matsuo No.1' and 'MO-15' belonging to *M. sieboldii*, in *M. platycarpa*, and *M. toringoides*, in 'Hanakaido' and 'Nokaido' belonging to *M. halliana*, in 'Japanese Crab' belonging to *M. floribunda*, in *M. scheideckeri*, and *M. toringo*, in 'HongHaiTang' belonging to *M. micromalus*, in *M. florentina*, in 'Nikkozumi' belonging to *M. baccata*, in 'La France', 'Brandywine', 'Aurora', 'Kieffer' and 'Bartlett' belonging to *Pyrus communis*, and in 'Kosui', 'Gold Nijisseiki' and 'Hosui' belonging to *P. pyrifolia*.

The result suggest that several wild *Malus* species and accessions such as 'Nikkozumi', *M. florentina* and 'HongHaiTang' are available as gene sources for resistance to Valsa canker as well as *M. sieboldii* which is known to be resistant to the canker (Bessho *et al.* 1994). Most

of pear and Japanese pear cultivars investigated in our study were also resistant to the canker. Introgression of the resistance to apple cultivars from *Pyrus* species and cultivars may be an alternative strategy. However it seems difficult to acquire intergeneric hybrids between apple and pear or Japanese pear because some report showed that all intergeneric hybrids died in their seedling stage (Crane and Marks 1952, Shimura *et al.* 1980).

Table 1. Length of necrosis on excised, dormant twigs of *Malus* and *Pyrus* species inoculated with *Valsa ceratosperma* isolate AVC-12

Species and Cultivars	Lesion length (mm)				
Shirobana robusta	28.3	*M.soulardii*	15.4	Virmorin	11.3
Elstar	23.6	Senshu	15.3	ShanDingZi-1	11.2
HAC 9	22.7	Sansa	15.2	BianYeHaiTang 83034	11.0
Waringo	22.2	Karafutozumi	14.8	*M.prattii*	11.0
Fuji	20.8	HeNanHaiTang	14.7	*M.floribunda* 88071	10.5
Tsugaru	20.8	*M.robusta* Bailey	14.5	Tarehanakaido	10.5
Liberty	18.5	*M.sikkimensis*	14.3	ShanDingZi-2	10.3
Criterion	18.4	*M.X adstringens*	14.2	*M.orientalis*	10.2
Granny Smith	18.3	*M.arnoldiana*	14.0	*M.praecox*	10.2
M.orthocarpa	18.2	HuaHong	14.0	Matsuo No.1	9.8
M.honanensis	18.0	ShanDingZi-4	14.0	*M.platycarpa* 73031	9.8
Himekami	17.8	*M.cerasifera*	14.0	MO-65	9.7
Rome Beauty	17.8	*M.sieversii*	13.8	*M.toringoides*	9.7
ChuiSiHaiTang	17.3	Bracteata	13.7	Hanakaido	9.4
HuBeiHaiTang 83051	17.3	*M.rockii*	13.5	Japanese Crab	9.3
Spartan	17.3	Miyamakaido	13.4	Sargent Crab	9.3
Mandschurica-1	17.2	Yellow Autumn C	13.2	*M.scheideckeri*	9.3
M.yunnanensis	17.0	Le Conte	12.8	*M.toringo*	7.8
M.major	16.8	*M.kirghisorum*	12.8	Nokaido	7.8
M.X hartwigii	16.8	HaiTangHua	12.3	HongHaiTang	7.7
Grabrata	16.5	*M.turesii*	12.3	La France	7.5
Miyagi-1	16.3	Hanyaehanakaido	12.0	Brandywine	7.2
Robusta	16.2	Mildew Immune Sdl.	12.0	*M.florentina*	7.0
ShanDingZi-3	16.2	*M.ioensis*	11.8	Kosui	6.8
M. angustifolia	16.0	*M.tschonoskii*	11.8	Gold Nijisseiki	6.0
M.bracteata	15.8	Hayanarisanashi-1	11.6	Nikkozumi	5.7
Jonathan	15.8	Jiringo	11.5	Aurora	5.3
Rinki	15.8	Robusta No.5	11.5	Kieffer	5.3
M.X gloriosa	15.8	Sanashi 63	11.5	Hosui	4.8
Empire	15.5	Veitchii	11.5	Bartlett	4.0

Table 2. Segregation of resistance to *Valsa ceratosperma* in apple families

Cross	Number of plants inoculated	Number (Frequency) of plants observed		
		Resistant	Moderately Res.	Susceptible
Akane x Sansa	11	0 (0 %)	2 (18 %)	9 (82 %)
Fuji x Iwakami	16	0 (0 %)	0 (0 %)	16 (100 %)
Jonathan x Starking Del.	22	0 (0 %)	1 (5 %)	21 (95 %)
Sansa x Jonathan	33	0 (0 %)	2 (6 %)	31 (94 %)
Megumi x Sansa	9	0 (0 %)	0 (0 %)	9 (100 %)
Senshu x Splendor	34	0 (0 %)	0 (0 %)	34 (100 %)
Sansa x Akane	16	0 (0 %)	0 (0 %)	16 (100 %)
Fuji x Splendor	15	0 (0 %)	0 (0 %)	15 (100 %)
Total	156	0 (0%)	5 (3%)	151 (97%)
Jonathan x MO-15	73	1 (1%)	8 (11%)	64 (88%)
Hayanarisanashi x Megumi	32	4 (13%)	17 (53%)	11(34%)
Total	105	5 (5%)	25 (24%)	75 (71%)

Inheritance of resistance to V. ceratosperma

Frequency distributions of lesion length expressed as the relative length to 'Fuji' on dormant twig segments of *Malus* hybrid plants inoculated with *V. ceratosperma* isolate VC96-A, AVC-12 or AVC-55 are shown in Fig. 1. All the individual families showed continuous distribution around family-mean with no evidence of segregation due to major gene effect. The effect of the three different inoculum sources on the distribution of the progeny derived from *Malus sieboldii* ('MO-15') to the relative lesion length seemed to be small.

Segregation of resistance to *V. ceratosperma* in apple families is shown in Table 2. In 8 progenies derived from crosses between susceptible cvs. such as 'Akane', 'Sansa', 'Fuji', 'Iwakami', 'Jonathan', 'Starking Delicious', 'Megumi', 'Senshu' and 'Splendor', no plants were found to be resistant. Three progenies possessed a few plants evaluated as moderately resistant. On the other hand, 'MO-15' and 'Hayanarisanashi' belonging to *M. sieboldii* gave progenies which included resistant plants. Descendant of 'Hayanarisanashi' had more than 50% in the moderately resistant class. 'Hayanarisanashi' had a higher proportion of resistant or moderately resistant plants in its progenies than 'MO-15'.

Inheritance of resistance to powdery mildew (Podosphaera leucotricha)

Reaction of apple cultivars to powdery mildew is shown in Table 3. No visual symptoms were observed after spore inoculation in 'Sanashi 61', 'Sanashi 63', 'Hayanarisanashi', 'MO-15', 'Miyamakaido' and 'Nikkozumi', indicating that these accessions were not only resistant to Valsa canker but also had a high level of resistance to powdery mildew. 'Shirobana robusta', belonging to *M. robusta* which was considered to have a dominant allele of the major gene *Pl* 1(Knight and Alston 1968), produced some sporulating spots and was rated as resistant. Many large sporulating lesions were observed on the most severely infected leaf in commercial apple cultivars ; most of the leaves infected completely.

Table 3. Reaction of apple cultivars to *Podosphaera leucotricha*.

Cultivar(Species)		Incidence	Resistance grade
Sanashi 61	(*M. sieboldii*)	0	Highly resistant
Sanashi 63	(*M. sieboldii*)	0	Highly resistant
MO-15	(*M. sieboldii*)	0	Highly resistant
Hayanarisanashi	(*M. sieboldii*)	0	Highly resistant
Miyamakaido	(*M. sieboldii*)	0	Highly resistant
Nikkozumi	(*M. baccata*)	0	Highly resistant
Shirobana robusta	(*M. robusta*)	2	Resistant
Fuji	(*M.* x *domestica*)	5	Susceptible
Jonathan	(*M.* x *domestica*)	4	Susceptible

Table 4. Segregation of resistance to *Podosphaera leucotricha* in apple families.

Cross	Number of plants inoculated	Number (rate, %) of plants observed		
		Highly resistant	Resistant	Susceptible
Jonathan x Shirobana robusta	24	0 (0%)	13(54%)	11(46%)
Jonathan x Sanashi 61	15	14 (93%)	0(0%)	1(7%)
Sanashi 63 x Jonathan	22	22 (100%)	0(0%)	0(0%)
Jonathan x MO-15	57	57 (100%)	0(0%)	0(0%)
Jonathan x Miyamakaido	290	223 (77%)	24(8%)	43(15%)
Jonathan x Kobanozumi	96	59 (62%)	9(9%)	28(29%)
Hayanarisanashi x Megumi	9	7 (78%)	0(0%)	2(22%)
Jonathan x Golden Delicious	21	0 (0%)	0(0%)	21(100%)

Segregation of resistance to powdery mildew in apple families is shown in Table 4. The percentage of resistant plants was 54% in a progeny of *M. robusta* ('Jonathan'x'Shirobana robusta') which was considered to have a dominant allele of the major gene *Pl* 1. In the progenies of *M. sieboldii*, all plants were rated as highly resistant in 2 progenies ('Sanashi 63'x'Jonathan' and 'Jonathan'x'MO-15'). One progeny ('Jonathan'x'Sanashi 61') gave only 1 susceptible plant (7%) all the other plants were highly resistant. When 'Miyamakaido', 'Hayanarisanashi' and 'Kobanozumi' belonging to *M. sieboldii* were used as a cross parent, 15%, 22% and 29% of each progeny were susceptible, respectively. One progeny derived from a cross between commercial cultivars, 'Jonathan'x'Golden Delicious', gave no plants rated as highly resistant or resistant.

Data from progenies of *M. sieboldii* suggest that high level of resistance of *M. sieboldii* to powdery mildew is controlled by oligogenes. 'Sanashi 61', 'Sanashi 63' and 'MO-15' are considered to have at least one dominant gene pair controlling high resistance. Since the level of resistance to powdery mildew is quite different phenotypically between *M. sieboldii* and *M. robusta*, it appears that major gene(s), which are supposed to exist in *M. sieboldii*, can be

different from the mildew resistant gene *Pl* 1 in *M. robusta*.
In the progenies of *M. sieboldii* high proportion (62 ~ 100%) of hybrid plants were highly resistant to powdery mildew. Some of the plants are expected to be resistant to Valsa canker when the progeny size is adequately large, because resistance of *M. sieboldii* to the canker is transmitted to their progeny polygenically. In fact, a few plants in the progenies of 'Jonathan'x'MO-15' and 'Hayanarisanashi'x'Megumi' had a good level of resistance to Valsa canker with a high resistance to powdery mildew. Such plants will be useful breeding materials for resistance breeding to Valsa canker and powdery mildew in the work at Apple Research Center, NIFTS.

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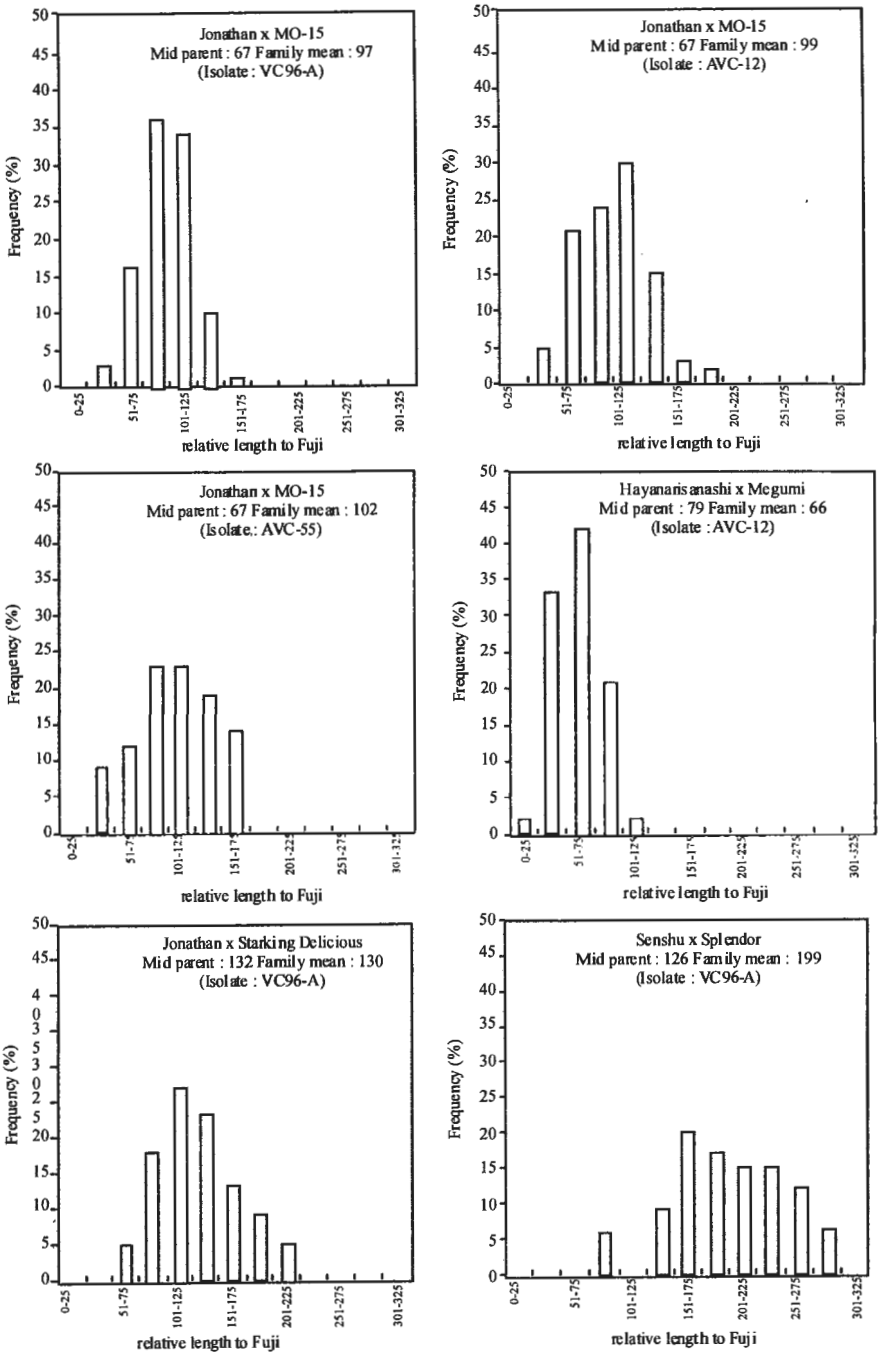

Fig. 1. Frequency distribution of canker length on excised dormant twigs of *Malus* hybrid plants inoculated with *Valsa ceratosperma*

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An European project : D.A.R.E. - Durable Apple Resistance in Europe (FAIR 5 CT97-3898)

Durable resistance of apple to scab and Powdery-mildew : one step more towards an environmental friendly orchard

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Abstract : Most of today's apple cultivars are susceptible to scab (*Venturia inaequalis*) and powdery-mildew (*Podosphaera leucotricha).* The use of large amount of fungicides in orchard raises ecological problems, consumer's health concerns in addition to the economic cost. The D.A.R.E. project aims at developing knowledge, methodologies, plant and pathogen material to achieve durable genetic resistance towards these pathogens in the future. The resistance/susceptibility of European cultivars towards several *V. inaequalis* and *P. leucotricha* inocula was assessed. A large part of this plant material showed partial or complete resistance to numerous *V. inaequalis* inocula. The European collection of *V. inaequalis* strains was settled. A core-orchard including partial and complete scab resistant cultivars was planted in each participating country. This orchard network will enable partners to observe the possible appearance of new *V. inaequalis* races and to assess the plant resistance stability in different environmental conditions. Monoconidial strains of *P. leucotricha* were isolated and are now tested on a range of apple cultivars to define whether physiological races exist or not. The genetic mapping of major genes and QTLs involved in fungi resistance is in progress. A gene pyramiding strategy was initiated by crossing genotypes carrying complete and partial resistance to mildew and scab. Consumer's preferences were tested with scab resistant cultivars.

Key words : *Malus domestica, Venturia inaequalis, Podosphaera leucotricha,* genetic mapping, gene pyramiding, consumer's preferences

Introduction

The D.A.R.E. project focuses on the durable resistance of apple to its main fungi diseases, scab (*V. inaequalis*) and powdery mildew (*P. leucotricha).* Five closely linked aspects are carried out : (1) characterisation of the resistance status of a wide range of apple cultivars, (2) assessment of the pathogenicity variability of the two fungi, (3) genetic dissection of partial resistance (4) development of new breeding strategies and (5) market study and consumer preferences for new resistant cultivars. The progress of the work is described below (extracts of the D.A.R.E. newsletter, issue n° 2, July 1999).

1. Characterisation of the resistance status of a wide range of apple cultivars

Twenty-two apple cultivars and eight new hybrids carrying partial scab resistance were chosen by the partners of the D.A.R.E. project in their own collections. They were tested in 1998 and 1999 in the glasshouse for their susceptibility/resistance to known races (race 1, race 6, race 7) and natural inocula of *Venturia inaequalis* present in different european countries. These tests had three main objectives : to improve the methods of assessing partial resistance to scab, to test the virulence and aggressiveness of various *V. inaequalis* inocula and to study the

behaviour of cultivars with partial resistance to scab. Scab resistance/susceptibility symptoms (Chevalier *et al.*, 1991) and severity (Croxall *et al.*, 1952, modified by Parisi *et al.*, 1993) were assessed. Various traits were used to identify the level of resistance/susceptibility of each cultivar : type of resistance symptoms, rate of sporulation, incidence (number of scabbed leaves), mean leaf severity and sporulation density. Significant variability has been identified between the inocula tested. Many cultivars and hybrids showed typical macroscopic and microscopic symptoms of partial resistance : they will be selected as race-non-specific resistant individuals and will be used in crosses for developing new genotypes. Four new selected hybrids show very interesting broad-spectrum resistance; this confirms the relevance of breeding strategies, which aim at associating major resistance genes and "polygenes" in the same genotype.

Other cultivars and wild apple species have been tested for their susceptibility/resistance to several local inocula of *Podosphaera leucotricha*. The tests will be continued with improved experimental conditions.

2. Assessment of the pathogenicity variability of V. inaequalis and P. leucotricha

One part of the work devoted to scab in the D.AR.E. project concerns the establishment of the core collection of *V. inaequalis* strains. This is the first time that such work has been done in Europe, though some laboratories do have important research collections. The collection currently includes 314 monoconidial strains from 8 countries and 47 cultivars and species of *Malus* (table below). The establishment of the collection is nearly achieved.

The strains showing the most sporulation were chosen for the test *in vivo*. The final objective is to test the pathogenicity of 40 strains, representing the greatest variability (about 5 strains per country). The test is performed in a growth chamber, under controlled conditions with a host range including 8 cultivars. Up to now, 23 strains have been tested; the work will be continued throughout the year.

We expect that the results will help us to answer different questions. The first is to identify the presence in Europe of strains of races 6 and 7, virulent to the *Vf* gene (Parisi *et al.*, 1993 ; Roberts and Crute, 1994). There is only one report of the presence of race 6 in Europe, in Germany, while there are 3 reports of the presence of race 7, in Great Britain, The Netherlands and France (Parisi, 1997). But these races are probably present in others countries or regions; however, we need more information on this point. The second question concerns the behaviour of the cultivars included in the host range, and the parents of the progenies studied in the project with resistance under polygenic control. What will the pathogenicity of the different strains be on these cultivars ? The results of the tests will provide a better understanding not only about fungus variability, but also the host's resistance and its genetic determinants. Finally, we hope to be able to maintain a set of strains which gives an estimation of the variability of the fungus in Europe. We anticipate the results will provide a good choice of strains for further tests of plant material in the framework of the D.A.R.E. project. The european collection of *Venturia inaequalis* strains will be available for all the partners, and could provide good support for others studies, such as the study of diversity within the fungus using molecular markers.

Core-orchards have been established in all the countries participating in the D.A.R.E. programme. The plant resistance stability to scab and the presence of new *V. inaequalis* races will be assessed in 2000 and 2001 on well established trees. In 1999, partners are developing an appropriate methodology to score scab symptoms in the orchard.

Establishment of the collection of *V. inaequalis* strains

Inoculum origin, (partner, country)	Species of *Malus* and cultivars of *Malus domestica*	Number of strains isolated	Number of strains that can be tested
CPRO- DLO Wageningen, NL	Alkmène, Belle de Boskoop, Cox's Orange Pippin, Discovery, Elstar, Golden Delicious, Jonagored, *M. floribunda* #821, *M. robusta*, Otava, Pinova, Prima, Santana	56	13
CRA Gembloux, B	Cox's Orange Pippin (Queen Cox's), Egremont Russet, Elshof, Golden Delicious, Jonagold-Jonica, Lombarts Calville, Président Roulin, Radoux, Reinette Clochard, Reinette Etoilée, Belle de Boskoop	47	11
HRI East-Malling, UK	Russian seedling, *M. robusta, M. arnoldiana, M. floribunda* #821, *M. zumi* (open pollinated), *M. baccata flexilis, M. lemonei, M. baccata gracilis, M. atrosanguinea, M. sp.* Mary Charlton, *M. sp* almey, *M. sp* winter gold	63	13
DCA-BO Bologna, I	Durello di Forli, Decio, Golden Delicious, Red Chief Delicious, Renetta Grigia di Torriana, TN10-8	26	6
NAGREF Naoussa, GR	Golden Delicious, Granny Smith, Red Chief Delicious, Starking Delicious	9	4
INRA Angers, F	Gala, Golden Delicious, Granny Smith, Judeline, Smoothee, TN10-8, Top Red	27	7
BAZ Ahrensburg, Dresden, D	Alkmène, Coop 9, Fiesta, Firiki, Florina, Golden Delicious, Lombarts Calville, *M. floribunda* #821, Prima, Priscilla, Schweizer Orangenapfel	61	11
ETHZ-FAW Zürich, Wädenswil, CH	Fiesta, Golden Delicious, Idared, *M. floribunda* #821,Topaz	25	5
TOTAL	**47**	**314**	70

With regard to mildew, several strains of *P. leucotricha* from different european countries were isolated. This pathogen is an obligate parasite and must normally be maintained on the host plant. An *in vitro* methodology has been developed to conserve it. The monoconidial isolates are then used to inoculate a range of apple cultivars to see whether physiological races exist or not.

3. Genetic dissection of partial resistance taking into account pathogen variability

Numerous molecular and biochemical markers were found in 5 progenies derived from crosses between susceptible and resistant cultivars. The map of the two parents already studied during the previous european project on apple (EAGMAP, Maliepaard *et al.*, 1998) was nearly saturated. The resistance/susceptibility to scab of the 4 other progenies is in

progress. The aim here is to map QTL conferring resistance to *V. inaequalis* taking into account the new races of this pathogen.

Genetic mapping of two major mildew resistance genes (Plw, Pl-D12) has begun using bulk segregant analysis. Several potential AFLP markers for mildew resistance were identified. These include 8 markers for Pl-W resistance and 7 markers for Pl-D12.These bands will be re-isolated, cloned and sequenced. Appropriate sequences will be used to design primers to convert the AFLP markers into the more user friendly SCARS.

Many resistance gene analogues (RGA) have been found in apple cultivars. Apple DNA amplified with degenerate PCR primers gave high degrees of similarity to major resistance genes or receptor-like kinases from other plant species in genomic databases. These resistance gene analogues can be used as RFLP probes or CAPS markers for mapping resistance loci.

4. Development of new breeding strategies

Several crosses between genotypes carrying different major resistance genes to mildew and scab were performed. Some progenies obtained were tested for their susceptibility to mildew and showed a high level of resistance. Crosses between cultivars, carrying partial resistance to scab, were also performed. Marker assisted selection for both mildew and scab resistance has been started.

5. Market study and consumer preferences for new resistant cultivars

Sensory evaluation of different scab resistant cultivars was undertaken with trained and untrained panellists in laboratory conditions. The results were analysed and compared with physical and biochemical measurements on fruits. Consumer tests on a wider scale in supermarkets were also performed. These experiments will be pursued till the end of the project. The opinions of the consumers will be of a great help in promoting new resistant cultivars on the market.

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Preliminary evaluation of new gene transfer strategies for resistance to fire blight in pear

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Abstract : Fire blight is a major disease of the European pear (*Pyrus communis* L.), caused by the necrotic bacterium *Erwinia amylovora*. Different gene transfer strategies have been used to enhance resistance to fire blight. A significant reduction of symptoms was observed for some transgenic clones expressing the attacin E, T4 lysozyme or lactoferrin genes.

Key word : Pear, fire blight, resistance, genetiç transformation, attacin, lysozyme, lactoferrin

Introduction

Fire blight, caused by *Erwinia amylovora* is the most important bacterial disease of pear (*Pyrus communis* L.). Different strategies may be considered to enhance resistance to fire blight including the use of lytic peptide genes [Jaynes *et al.* ; 1987] isolated from *Hyalophora cecropia* (attacin E) or bacteriophage T4 (T4 lysozyme), and use of lactoferrin gene (from bovin origin). Lactoferrin is an iron-chelating agent that could compete with the siderophore of *E. amylovora* and could reduce the biological availability of iron for the invading bacteria [Dellagi *et al.*; 1998]. Integration of these genes was attempted since a successful transformation protocol was established for pear by Mourgues *et al.* [1996]. In this study transgenic clones of the variety Passe Crassane were evaluated for their fire blight resistance via *in vitro* inoculation.

Material and Methods

Transformation experiments were conducted as previously described [Mourgues *et al* ; 1996] with EHA 105 carrying the following binary vectors : pFM3002 (attacin E) described by Reynoird *et al.*(1998), pCa2AMVT4 (T4 lysozyme) and, pWiAttCa2AMVT4 (attacin E -T4 lysozyme) described by Ko *et al.* [1998], and pCaLacto (lactoferrin gene driven by CAMV35S promoter). Integration of these transgenes was established by PCR using specific oligonucleotides after DNA extraction from leaves. Ploidy level was checked by flow cytometry.

Disease resistance was determined according to Brisset *et al*, 1988 ten days after leaf inoculation of *in vitro* plants. In total, eighty shoots per clone were inoculated with a virulent strain of *E. amylovora* (CFBP1430, 2.10^7 cfu/ml) during at least 3 experiments. Infection was rated on a wilting scale from 0 (no symptoms) to 3 (whole shoot necrotic) (Fig 1).

Results and discussion

Transformation rate obtained with these binary vectors varied from 0.4 to 1 %, which is in agreement with previously published rate for this variety (Mourgues *et al.* ; 1996). Among 21 transgenic clones obtained, 2 tetraploïds (6A and 1L) were detected by flow cytometry. Such a high frequency of doubling (10%) has already been observed among Passe Crassane transgenic clones previously obtained.

Figure 1. Behaviour of resistant Old Home (wilting note 0) on the left and susceptible Passe Crassane (wilting note 3) on the right, 10 days after inoculation with *E. amylovora.*

Among the 5 attacin E and 5 T4 lysozyme clones, a wide range of variation for fire blight resistance was observed (Fig 2). In each case, one promising clone was identified (clone 1 E and 7 D). The small number of tested clones expressing the combination attacin E – T4 lysozyme did not permit to measure a synergistic effect between these genes.

The *in vitro* growth of lactoferrin clones indicated a reduction of vigor for some of them. Thus, the number of shoots available for fire blight testing was reduced for 2H, 2I, and 2P. Despite this effect, a reduction of fire blight symptoms was observed for several lactoferrin clones (2N, 2L, 2H) (Fig 3).

Transgene expression is currently being evaluated by western-blot, northern-blot and ELISA analysis. All the transgenic clones have been acclimated in the greenhouse, and resistance test on grafted plants will be performed the next year.

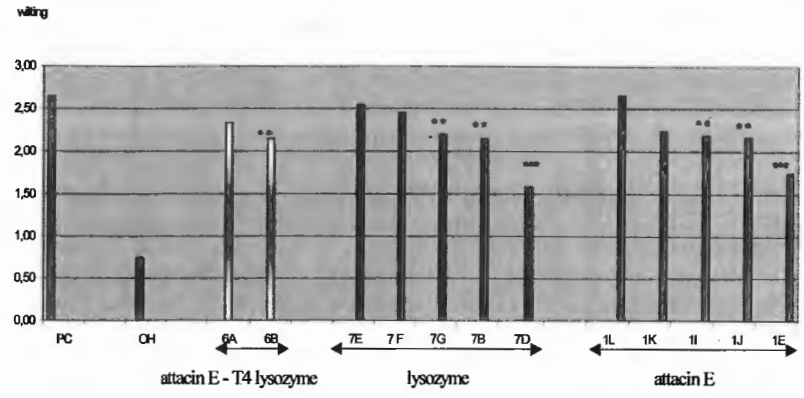

Fig 2. Fire blight susceptiblity of Passe Crassane control (PC), transgenic clones attacin E (1E to 1L), T4 lysozyme (7B to 7G), attacin E-T4 lysozyme (6A and 6B) and the variety Old Home (OH) used as resistant control. Transgenic clone susceptibility is significantly different from control at $p < 0,05$ (*), $p < 0,001$ (**) and $p < 0,0001$ (***) according to Kruskall and Wallis test.

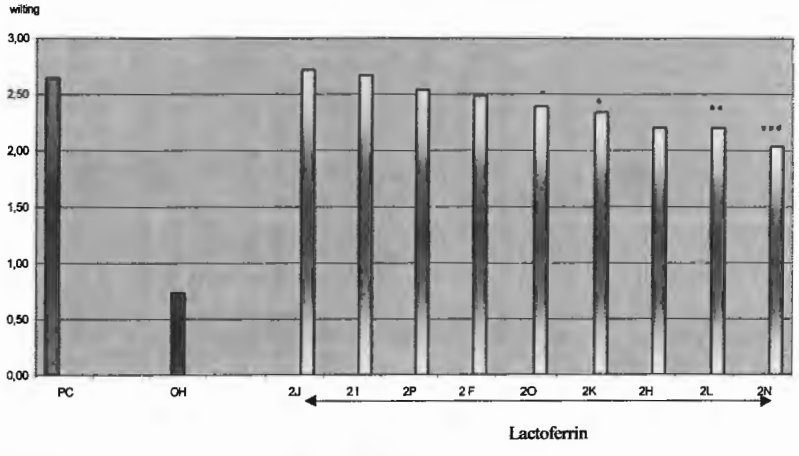

Fig 3. Fire blight susceptibility of Passe Crassane control (PC), lactoferrin clone (2F to 2P) and the variety Old Home used as resistant control. Transgenic clone susceptibility is significantly different from control at $p < 0,05$ (*), $p < (0,001)$ (* *), $p < 0,0001$ (* * *) according to Kruskall and Wallis test. Clone 2J is the only clone which did not amplify a fragment of lactoferrin gene by PCR.

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Selection of apple cultivars with low-susceptibility to fire blight (*Erwinia amylovora*) using a two-step strategy.

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Abstract : After the first report of fire blight in Spain, it became necessary to start the evaluation of the susceptibility to fire blight of local apple varieties, which are extensively used for cider production and that can also become a source of genetic resistance to this and other apple diseases (scab and mildew). The demand for highly resistant cultivars from fruit growers, the large number of cultivars to be tested, and the severe restrictions we face to avoid the spreading of the pathogen from the experimental facilities, prompted us to implement a two-step procedure for the evaluation of 253 local apple cultivars. The first step involves a quick excised leaf assay with the aim to discard those cultivars showing a high susceptibility to fire blight. The second step involves testing grafted cultivars, and is directed to a significant estimation of the cultivar susceptibility to this disease. The correlation between results obtained with both assays was $r = 0.42$ ($P = 0.03$). Our results suggest that this strategy could be useful to speed up the identification of cultivars with low susceptibility to fire blight among a large group of cultivars, by reducing after the first step the sample to be tested, although some interesting cultivars could be erroneously discarded

Key words : resistance, detached leaf assay, shoot inoculation, scab and mildew resistance

Introduction

Since the introduction of *Erwinia amylovora* in Europe, Spain has stayed free of the pathogen for reasons that remain largely unknown. In the summer of 1995, however, a limited outbreak of fire blight was reported in Northern Spain in cider apples (López *et al.*, 1996). In subsequent years, the pathogen has been repeatedly isolated from fruit trees and ornamental plants in different locations in Northern and Central Spain, Navarra being one of the most affected provinces. Several management strategies, such as inspection to identify potentially infected plants and an eradication policy were rapidly implemented to control the spread of the pathogen. The incidence of the disease, if any, is still low in fruit-producing areas, however, considering the rapid progression of the disease in other Mediterranean countries (van der Zwet, 1996), it is necessary to establish appropriate management strategies to minimize the potential losses.

A collection of 253 local cider and table apple cultivars was established by the Instituto Técnico de Gestión Agrícola a few years ago (Lizar, 1996). These cultivars represent a pool of germplasm that could be exploited in the future as a source for resistance against fire blight and other diseases, either in breeding programs or as choice cultivars for cider production. Particularly, field observations carried out during the last 5 years indicate that a portion of these cultivars are potentially resistant to scab and mildew (B. Lizar, unpublished), two of the most important apple diseases. We have thus started the evaluation of this collection for

resistance to diseases, especially to fire blight. Experiments involving inoculation *with Erwinia amylovora* in our institution, however, can only be performed under strict surveillance in a specially dedicated growth chamber, to avoid spread of the pathogen. Additionally, there is an increasing demand for apple cultivars with low susceptibility to fire blight. All these has prompted us to implement a strategy that could allow us to rapidly identify this kind of cultivars by concentrating our efforts in those showing a reduced susceptibility to the pathogen in a quick excised leaf assay. This could allow us to evaluate a manageable number of cultivars each year, while still providing fruit growers with information about cultivars that could potentially show a reduced susceptibility to fire blight in the field.

Material and methods

Biological material

The 253 cider and table apple cultivars used originated from Northwestern Spain and were previously characterized (Lizar, 1996). Selected cultivars were grafted on M9 and kept in 2 l pots in the field; approximately 3 weeks before inoculation they were moved to a greenhouse, and maintained under controlled conditions until inoculation.

Erwinia amylovora UPN500, isolated from a naturally diseased pear tree in Guipúzcoa, was identified by PCR (Bereswill *et al.*, 1992), enrichment ELISA-DASI using monoclonal antibodies (Gorris *et al.*, 1996), and conventional methods, including pathogenicity, and was maintained at –80°C in 20% glycerol.

Inoculation and disease assessment

Young leaves (2 to 4 cm long) were collected from field-grown trees and surface-sterilized. For inoculation, a 10 µl drop of a UPN500 suspension (108 cfu/ml) was placed on an incision made in the midvein with a scalpel. Twenty-one leaves (7 leaves from each of 3 trees) were inoculated per cultivar and placed on moist filter paper inside a transparent plastic container fitted with a hermetic lid. The containers were held in a controlled growth chamber at 20°C with a 16 h photoperiod. Vigorously growing shoots at least 30 cm long from one year old plants grafted in M9 were inoculated by cutting the youngest leaf with a pair of scissors dipped in a 108 cfu/ml suspension of UPN500 (Norelli *et al.*, 1984). Plants were then placed in a controlled growth chamber and incubated as described for inoculated leaves. One or, when available, two shoots were inoculated per plant, and eight plants were used per cultivar. Symptoms were scored after 5 d (excised leaves) or 12 d (shoots) after inoculation using a 0 to 4 arbitrary scale and transformed to percentage using a "necrosis severity index" (NSI), essentially as described (Duron *et al.*, 1987).

After a one-way Anova analysis, the Student-Newman-Keuls test was used to determine differences in means ($P = 0.05$) previously transformed by arcsin $\sqrt{x}$.

Results and discussion

Testing for fire blight resistance is usually carried out using seedlings, asexually propagated material (grafting and budding), or field-grown trees, which requires large amounts of field or greenhouse space. Other researchers have previously tested different methods that might facilitate this task by examining *in vitro*-grown plants or excised leaves (Duron *et al.*, 1987; Norelli *et al.*, 1988; Donovan, 1991; Donovan *et al.*, 1994). A significant correlation was found between the fire blight symptoms produced on excised leaves and on *in vitro* plants, although no correlation was found with symptoms produced on plants grown on the

greenhouse (Donovan, 1991). As pointed out (Donovan, 1991), this might have resulted from an inappropriate inoculation technique for whole plants or because the plants from the greenhouse were not actively growing. The examination of excised leaves offers important advantages, such as a drastic reduction in space needs; the large amount of material that can be assayed in a short period of time, and the abundance of available material. Also, this type of assay has also been successfully used to evaluate pathogenicity and virulence of *Pseudomonas syringae* pv. syringae (Yessad *et al.*, 1992), a pear pathogen. Therefore, we decided to examine the usefulness of the excised leaf assay method to identify those cultivars showing high susceptibility to *E. amylovora*, with the aim to concentrate our immediate efforts in a reduced group of cultivars.

Excised leaf assay

In inoculated leaves from highly susceptible cultivars used as controls, such as Fuji and Earligold, symptoms started to develop 2-3 d after inoculation and necrosis rapidly progressed through the midrib, lateral veins and the lamina tissue. Eventually, the leaves became completely necrotic at day 5, and in certain cases production of bacterial ooze was observed. The NSI rating for these cultivars was generally higher than 60%. As previously reported (Donovan, 1991), we observed a marked decrease of the severity of symptoms with increasing leaf length. For this reason, leaves longer than 4 cm were not assayed. In no case, however, did we observed any unexplained browning of control leaves or the filter paper during the assay.

Inoculated leaves from the 209 different apple cultivars tested showed a differential progression of symptoms after inoculation, most of them being of an intermediate level (92 cvs. had a NSI of 40 to 60%). Cultivars were then ranked according to their NSI in five categories (Table 1). A high level of variability was observed among inoculated leaves of a given cultivar, as it has been previously reported for this type of assay (Donovan, 1991). In most cases, however, separate evaluations of a given cultivar resulted in the placement of the cultivar in the same NSI category or in one of the adjacent categories, suggesting that this method could provide us with an approximate idea of the cultivar susceptibility to fire blight.

Table 1. Distribution of apple cultivars according to their susceptibility to fire blight in an excised leaf assay.

NSI category [a]	N° of cultivars
0-10%	0
10-30%	28
30-50%	89
50-90%	92
90-100%	0
Total	209

[a] NSI, necrosis severity index

Shoot assay

Twenty seven cultivars showing different NSI values obtained with the excised leaves assay, were assayed by inoculating shoots from plants grafted in M9 to evaluate the correlation between both techniques. Other 16 local cultivars, that were chosen because of their agronomic characteristics or because they showed scab and mildew resistance in the field (B. Lizar, unpublished data), and cultivar Granny Smith were included in this assay. The cultivars greatly differed on their susceptibility to the pathogen, and could be classified in 3 susceptibility groups (Table 2), being Granny Smith included in the "Highly susceptible" category with a NSI= 73.4 % ± 3.7. It is important to notice that field observations classify as scab and mildew resistant 8 of the 17 cultivars classified as "Moderately resistant" to fire blight in this assay.

Table 2. Distribution of 43 local apple cultivars according to their susceptibility to fire blight in inoculated shoots grafted in M9

Resistance class	NSI category	N° of cultivars
Moderately resistant	0-40%	17
Susceptible	40-60%	12
Highly susceptible	60-100%	14

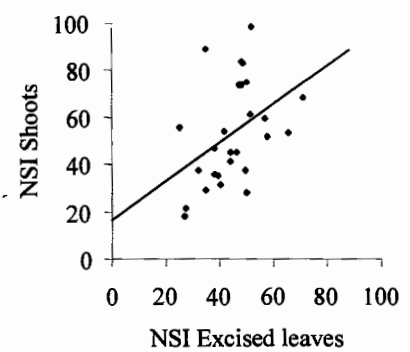

Fig. 1. Relationship between the necrosis severity index (NSI) from shoots grafted in M9 and from excised leaves of the same cultivars grown in the field, and inoculated with *Erwinia amylovora* UPN500.

The correlation between the results for inoculated shoots and the results obtained with excised leaves was r = 0.42 (P = 0.03). The leaf assay, however, tends to underestimate the susceptibility of the cultivars (Fig. 1). Our results suggest that the strategy we followed could be useful to reduce the number of cultivars to test for susceptibility to fire blight in programs that require the examination of a large number of individuals. Nevertheless, it would still be necessary to carry out more experiments before this strategy can be fully validated.

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Scab resistant apple trees and Integrated Pest Management

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Abstract : Varieties displaying resistance against pathogens or noxious insects are of great interest for Integrated Pest Management. INRA has been selecting scab resistant apple varieties for many years (Lespinasse, 1989). A study was carried out about these varieties at the INRA Domaine de Gotheron (Drôme, France) from 1990 to 1993. It demonstrated the resistance stability. The use of these selections allowed to reduce significantly the number of fungicide sprays and to restrict *Panonychus ulmi* Koch population level. Repeated fungicide applications seem to be disruptive for the apple mite fauna and to favour the increase of *P. ulmi* numbers. The use of scab resistant apple trees allowed the natural regulation of *P. ulmi* by predatory mites, especially by *Typhlodromus pyri* Sheuten.

In 2000, we plan to plant new scab resistant selections in two sites in France : Domaine du Bois l'Abbé-Rétuzière (Maine et Loire) and Domaine de Gotheron (Drôme). The resistance stability and the induced control of *P. ulmi* are now assessed. The characterization of the qualitative potential of the new hybrids and the sensitivity to aphids and codling moth, *Cydia pomonella* L. are the present field of research.

Keywords : Scab resistant apple, Integrated Pest Management, noxious arthropods monitoring.

Introduction

Varieties displaying resistance against pathogens or noxious insects are of great interest for Integrated Pest Management. INRA has been selecting scab resistant apple varieties for many years, with an oligo-genic resistance conferred by a group of closely linked genes at locus Vf from *Malus floribunda* 821 (Lespinasse, 1989).

This paper presents : a 4 year study carried out from 1990 to 1993 on scab resistant and non resistant apple trees including a survey of *Panonychus ulmi* populations and its antagonists ; a project of experimentation with new scab resistant hybrids.

Materials and methods

4 year study : 1990-1993

Six scab resistant selections were studied. These were Baujade, X3191, X3189, X3263, X4972*, X4982* (*selections with low sensitivity to the aphid *Dysaphis plantaginea)*. Two susceptible varieties were also studied, that were Melrouge and Early Red One. The experimental orchard included 75 trees per variety and trees were spaced 2 m apart in the row with 4.5 m between rows.

If necessary, acephate and pirimicarb were used to control aphids and granulosis virus was sprayed against codling moth except during the first year of the study.

Visual control was used to monitor the percentage of leaf infestation by *P. ulmi* from samples of 60 leaves (2 to 4 leaves per tree on 15 to 30 trees). Tapping method was also used

to sample predatory mites (Fauvel *et al.*, 1981). On each variety 100 branches were sampled once a month in June, July, August and September.

New planting : 2000-2005

At the experimental station of Gotheron (Valence, Southeastern France), three new scab resistant hybrids (selected by INRA Angers) will be planted with 150 trees per variety and trees were spaced 2 m apart in the row with 4.5 m between rows.

This planting will be managed according to the IOBC guidelines for integrated production of pome fruits (IOBC/WPRS, 1993, 1994).

Results and discussion

4 year study : 1990-1993

Along the 4 years of the study, the number of pesticide sprayings in the scab resistant apple orchards remained 50 % lower than in the susceptible ones (Table 1). This decrease of the total number of sprayings is explained by the suppression of the chemical protection against scab and mites. However the control of powdery mildew and of post-harvest diseases remained necessary for scab resistant trees and required an average of 3.25 fungicide sprayings per year. The number of insecticides was the same for all the varieties (resistant and non resistant ones).

Table 1. Average number of treatments (1990-1993)

	Fungicides	Insecticides	Miticides	Number of pesticides
Resistant selections	3,25	11,25	0	14,5
Susceptible varieties	19	11	1	31

The percentage of leaves infested by *P. ulmi* is always lower for scab resistant hybrids than for susceptible ones, except in August 1991 (Figure 1). For scab susceptible varieties, the percentage of infested leaves is often very high (higher than 60 %) and an average of one miticide (cyhexatin) per year is necessary (ACTA, 1977). In 1992, the very low numbers of red mites for both situations can be partly explained by unusual climatic conditions with frequent summer rainfalls.

Samples of the beneficial fauna allowed to follow the numbers of the specialized predatory mite, *Typhlodromus pyri* and the numbers of other predatory arthropods : Stethorus ladybird, lacewings, predatory bugs (Miridae and Orius) and the predatory mite *Zetzellia* (Figure 2). The diversity and the numbers of these predatory species or groups were always higher on scab resistant hybrids than on susceptible ones.

In 1992, a high level of *T. pyri* numbers was likely to explain the absence of red mites on apple leaves for both situations.

This survey allowed to assess the natural regulation of *P. ulmi* populations by beneficial arthropods, mainly by *T. pyri* on scab resistant apple trees. Recurrent sprayings on non resistant apple trees were likely to imbalance strongly the apple beneficial fauna and to favour *P. ulmi* development. However, the fungicides that were sprayed on non resistant varieties (mainly Inhibitors of Sterol Biosynthesis) were not known to favour *P. ulmi* nor to be

disruptive or highly toxic for *T. pyri*, except triforine, a moderately toxic fungicide for *T. pyri* (Gendrier and Reboulet, 1997).

The control of *P. ulmi* by predatory mites was noted again in 1995 and a similar survey in Dordogne (Southwestern France) reported the same conclusions (Baudry, 1994).

New planting 2000-2005

A 5000 m^2 scab resistant apple orchard will be planted in December 2000 (Figure 3) with 3 new hybrids that will be managed according to integrated production guidelines (IOBC/WPRS, 1993, 1994). This survey will aim to test the field behaviour and the fruit quality of these hybrids and to assess their susceptibility to pests, mainly codling moth *Cydia pomonella* and aphids.

Moreover, this experiment will associate two different sites : (1) the Unité Expérimentale du Bois l'Abbé-Rétuzière (Angers) and (2) the Unité Expérimentale de Gotheron (Valence). The area near Angers is characterized by deep loamy soils well adapted to pome fruit crops. The climate is oceanic with frequent rainfalls which increase the number of scab primary contamination periods. On the opposite, soils in Gotheron experimental station are stone and shallow soils, from 50 cm to 60 cm deep, from old Rhone river sediments (Diluvium alpin). The climate is semi-continental with some mediterranean influences, resulting in a lower number of spring scab primary contamination periods. The behaviour of the scab resistant apple hybrids in these 2 sites will allow to test them for different soils and climates.

One specificity of the orchard in Gotheron is the integration of the agrosystem diversity in its management. Mixed hedgerow planting allow the increase of plant diversity. Moreover, a supervised choice of tree species or plant species to be planted in the inter-row of the orchard contributes to a better management of the agrocenosis (Simon, 1999).

The nineties allowed to assess the stability of Vf scab resistance gene under Valence area climate and to point out the natural regulation of the red mite *P. ulmi* by beneficial arthropods, mainly by *T. pyri*, for scab resistant hybrids.

In the next years, new hybrids will be tested for their field behaviour, their fruit quality and their susceptibility to codling moth and aphids, allowing a complete integration of scab resistant varieties in the apple cropping system.

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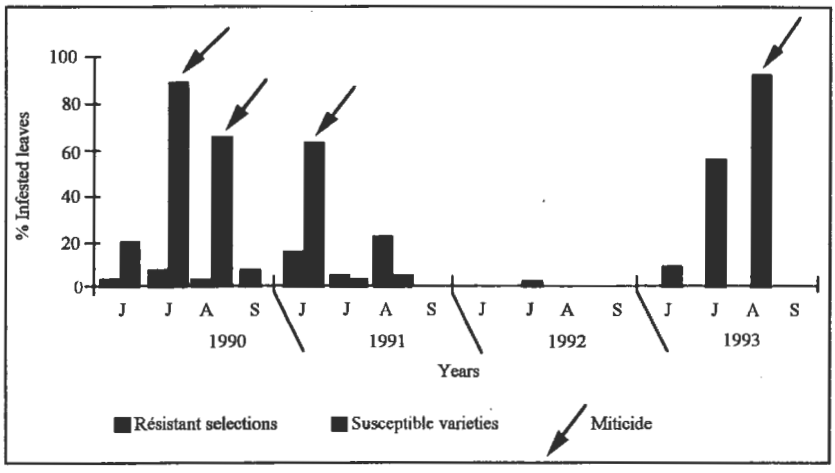

Figure 1. Infestation level of *P. ulmi*

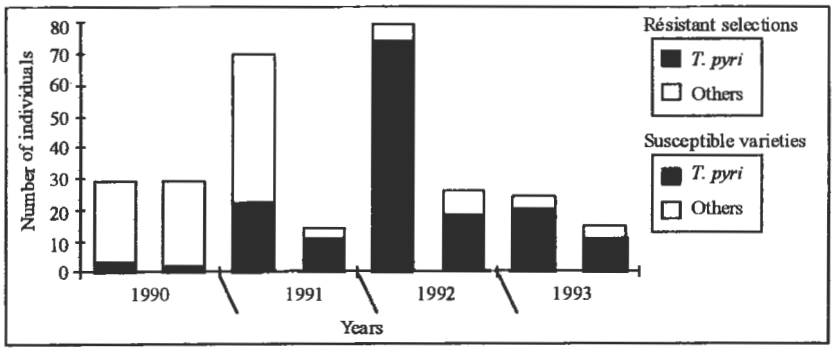

Figure 2. Evolution of predatory mites

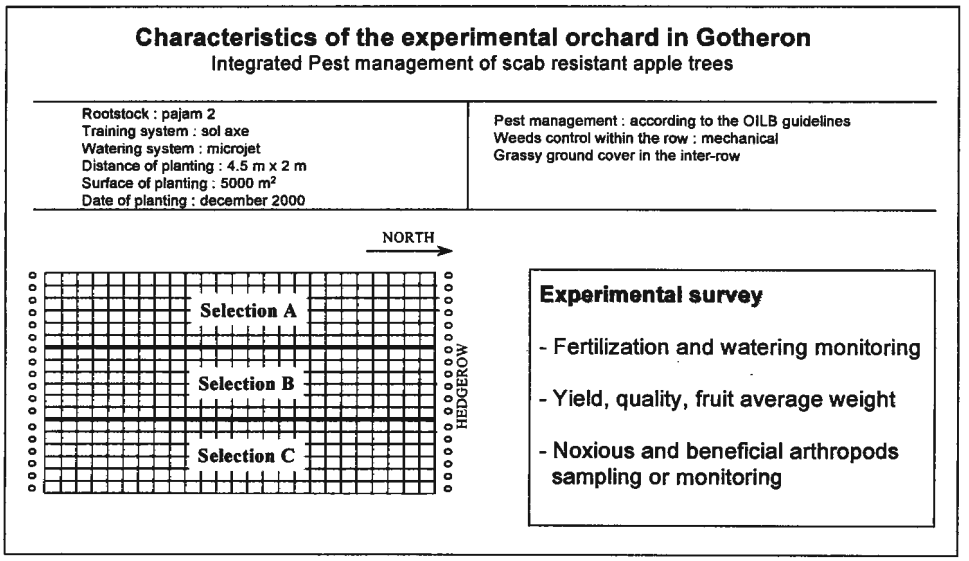

Figure 3. Characteristics of the orchard in Gotheron

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Durability of scab (*Venturia inaequalis*) resistance in apple and combination of different resistance sources.

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Abstract : Different resistance sources were combined in a crossing-scheme and progenies were artificially inoculated with *Venturia inaequalis* in the greenhouse. The parents of the progenies in this test included V_f and polygenic resistance. Twenty one days after inoculation, also a high infection of powdery mildew (*Podosphaera leucotricha*) was observed. The influence of powdery mildew on scab infection was not clear, therefore a second artificial inoculation was done. A deviation from the theoretical 1/1 segregation was observed, which indicates as stated in earlier studies, that genetic factors (minor and modifier genes) in addition to V_f are involved in resistance. Percentage of resistant genotypes within the progeny was, combined with the same parent, higher for Angold as for Goldrush. The contribution of the polygenic parent to the level of resistance was not clear. The same artificial inoculation on other progenies, including V_f and V_r as resistant resources, was carried out in 1992. The 50% most susceptible seedlings were discarded, the others were grafted on rootstock M27 and planted in the orchard. There were no fungicides sprayed on these trees. Field-observations were done in 1999 between the first and the fifteenth of May on the cluster-leaves. Scab symptoms were mostly found on dark green cluster-leaves, sometimes also on petals and sepals. This indicates an early infection at the end of March. In June, scab infection was evaluated on one year old shoots. Results were compared with scab observations done on the same trees in June of 1997. In a third experiment, 20 one-year old branches of 39 cultivars in an unsprayed plot were evaluated on scab in July 1999. Most polygenic cultivars showed scab while Vf-carrying cultivars showed no scab.

Key words : *Malus x domestica,* breeding, polygenic resistance, genetic control

Introduction

The most important apple disease in humid areas is scab caused by *Venturia inaequalis* (Cooke) Wint. The use of less susceptible cultivars offers a way to achieve a significant reduction of fungicide applications in apple. Breeding for resistance is therefore an important objective in most apple breeding programs (Crosby *et al.*, 1992). In the past, a lot of scab resistant varieties have been released. Most of them carry the V_f-resistance derived from *Malus floribunda* 821 (Kellerhals *et al.*, 1993). Recently a *Venturia inaequalis* race that can overcome V_f-resistance has been identified (Parisi *et al.*, 1993). This emphasises the importance of introducing new cultivars with more than one gene for resistance to establish a durable disease resistance (Lateur, 1996).

Apart from the qualitative resistance, quantitative (or polygenic) resistance is also available in Malus and is possibly more durable (Olivier and Lespinasse, 1982).

The apple breeding program at the KUL is not only based on combining cultivars with desirable pomological characteristics with resistant cultivars (qualitative or quantitative), but it is also based on the combination of different resistance sources.

Results of artificial inoculation of seedlings of different crossings (including combinations of parents with V_f and polygenic resistance) are presented in experiment 1.

Experiment 2 describes the results of scab observations in the field on seedlings of pre-screened progenies of different crosses. At that time the most important aim of the breeding program was to combine cultivars with good pomological characteristics with scab resistant cultivars. The sources of resistance for these crosses were V_f from *Malus floribunda* 821 and V_r from *Malus pumila* R#12740-7A.

In a third experiment, different unsprayed cultivars were evaluated to establish their scab resistance under natural field inoculation. Most of them are released by different apple breeding programs throughout the world as being scab resistant. The resistance sources are V_f and polygenic resistance. The aim of observing these cultivars is to evaluate them for further breeding.

Material and methods

Experiment 1

Eight different crosses with parents carrying different resistance sources were made in 1998. The following resistant apple cultivars were used as parents: Discovery, Angold and HL164 are polygenic resistant and 21/4/104, Retina, Coop 30, Coop 34, Vanda and Goldrush include V_f. The parents were cultivars with good pomological characteristics or scab resistant cultivars, the latter to reach the aim of combining different resistance sources.

The resulting seedlings were evaluated after artificial inoculation in the greenhouse with a mixed inoculum of *Venturia inaequalis* collected from different susceptible cultivars. The conidial suspension was composed of $1{,}2*10^5$ conidia/ml and was sprayed on the leaves of young seedlings without drips forming. The incubation took 48 hours at 18°C and 100% relative humidity. Afterwards the seedlings were brought by 20°C and 70% RH. The total number of seedlings for each progeny is presented in table 1. Twenty one days after inoculation, the seedlings were classified into six different classes of scab infection (adapted from Chevalier *et al.*, 1991). The description is given in table 2 and is based on the most infected leaf observed on the plant. After the first artificial inoculation a second was done because a high infection of powdery mildew occurred during the first infection which influenced probably the evaluation on the scab symptoms. For the second infection plants were cut back with one shoot left and inoculation was done on ± 4-5 leaf stage. Evaluation was done 15 days after inoculation.

Table 1. Progenies and number of seedlings included in artificial inoculation test.

Cross	Source of resistance	Number of seedlings
7/10 x Angold	- x polygenic	120
Delcorf x Vanda	- x V_f	192
Coop34 x Discovery	V_f x polygenic	236
21/4/104 x 9/5/206	V_fx -	48
Retina x Coop 30	V_f x V_f	44
7/10 x Goldrush	- x V_f	264
HL 164 x Goldrush	polygenic x V_f	200
6/281 x Discovery	- x polygenic	212

Table 2. Description of different classes of scab symptoms(Chevalier *et al*, 1991)

Class	description
0	No visible symptoms
1	"pin-point"pits
2	chlorotic lesions (irregular edges), slight necrosis, no visible sporulation
3a	necrotic lesions, some chlorotic lesions, slight sporulation (occasionally)
3b	chlorotic and necrotic lesions, sporulation
4	high sporulation

Experiment 2

The same artificial inoculations as described in experiment 1 were carried out in 1992 on several progenies mentioned in figure 2. Seedlings assigned to class 3b and 4 were considered susceptible and therefore discarded. The others (one tree per genotype) were grafted on rootstock M27 and planted in the orchard. These trees were not treated with fungicides. Observations were done in June 1997 in three classes: no symptoms, slightly infected or severe infection. The second observation was done in May 1999 on part of the trees. A third observation took place in June 1999 based on a more detailed scale : 0=no infection, 1=pin-points,2=chlorotic lesions, no visible sporulation, 3= some necrotic lesions, slight sporulation, 4=many necrotic lesions and sporulation, 5= many necrotic lesions and sporulation, also on nerves, 6= some lesions on fruits, 7= many lesions on fruits.

For the three observations a tree was considered susceptible or infected as soon as some lesions with sporulation could be observed (with slightly and severe symptoms or class 3 – class 7).

Experiment 3

In an unsprayed plot, 39 cultivars (figure 3) were evaluated on scab infection on 13 July 1999. For each cultivar 20 one year old shoots distributed over five trees were observed to sort all leaves in one of the six reaction classes given in experiment 1.

The percentage of infected leaves (class 3a, 3b and 4) was calculated for each cultivar.

Results

Experiment 1

In this experiment we artificially infected eight different progenies twice. Segregation rates for all seedlings together after each of the artificial inoculations are given in table 3. The percentage of seedlings considered as resistant (class 0 to class 2) was 58% in test 1 and 81% in test 2 ; 91% of the genotypes, considered as scab resistant in test 1, were also resistant in test 2, but 69% considered as susceptible in test 1 were resistant in test 2.

In figure 1, the percentage resistant seedlings of each progeny for the two different scab infection tests is illustrated. The seedlings assigned to class 0,1 or 2 were considered resistant. Crosses between a susceptible parent and a polygenic parent gave more resistant seedlings as expected. Depending on the parent, V_f x susceptible resulted in more (with Vanda and 21/4/104) or less (with Goldrush) resistance as expected. V_f x polygenic resistance when Goldrush is combined with HL164 gave less and when Coop 34 is combined with Discovery gave more resistance than expected. A cross between the two V_f-cultivars Retina and Coop 30 resulted in less than 75% resistance.

Table 3. Observations of two scab infections tests on the same seedlings (total number of 802 in both tests). Segregation of seedlings among classes in both tests and % of seedlings that were classified in test 2 in the same group as in test 1.

	Second obs.						
First obs.	total test 1	0	1	2	3a	3b	4
Total test 2	100%	69%	0%	12%	3%	12%	3%
0	19%	78%	0%	10%	2%	7%	3%
1	3%	58%	0%	19%	8%	15%	0%
2	36%	80%	0%	13%	2%	5%	1%
3a	15%	66%	0%	9%	5%	14%	6%
3b	18%	54%	0%	13%	5%	23%	5%
4	9%	51%	0%	9%	4%	28%	7%

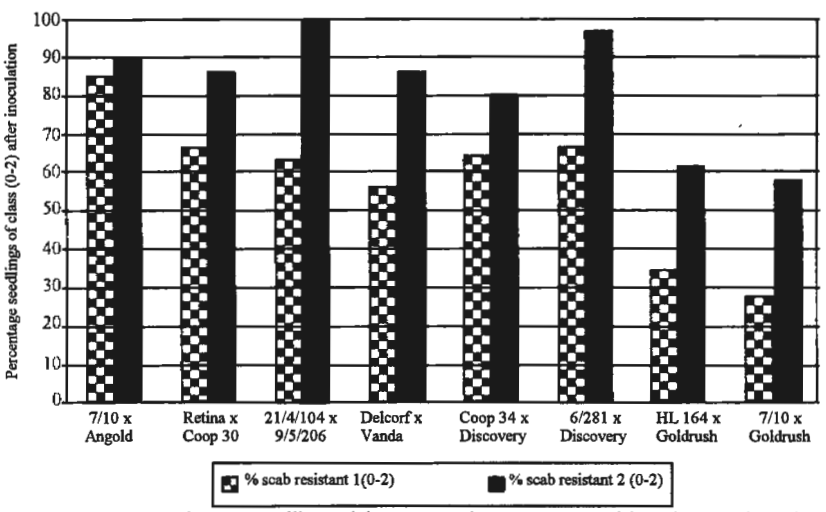

Figure 1. Percentage resistant seedlings (class 0 to class 2 are considered as scab resistant) for each progeny for the two scab infection tests (scab resistant 1 = first infection, scab resistant 2 = second infection).

Experiment 2

Although all trees were evaluated as resistant in 1992 after artificial inoculation in the greenhouse, we can conclude that not all of them were resistant in the field (figure 2).

There is good correlation between the observations done in June 1997 and those done in June 1999. Both years were characterised by high scab infection pressure.

Selection after artificial infection in the greenhouse and selection after natural infection deviates depending on the cross. Differences are small in crosses with Telamon and mostly large with Delbard Jubilée as susceptible parent. The deviation for Delbard Jubilée depends on the resistant parent: small when Priam and Nic43 is used and large for crosses with Liberty. Progenies without symptoms in May can show infection in July and vice versa but the latter only in a small amount.

Experiment 3

In figure 3, cultivars on an unsprayed plot with scab symptoms are indicated. Twenty seven other cultivars containing the V_f-gene cultivars showed no scab (not shown). Varieties containing polygenic resistance showed mostly sporulation: HL 938, HL164, Lord Lambourne Red, Nabella and HL2128 showed severe infection. HL 517 and HL442 were moderately infected, 7.113, HL480 and HL 446 were lowly infected and HL1547 and Angold (not indicated) showed no infection.

Discussion

Experiment 1

The differences between the two scab infection tests can be caused by high temperatures in the greenhouse during the second inoculation resulting in a lower infection rate. Between the first and second infection, seedlings were cut back. This can possibly lead to a certain resistance of the new leaves (induced resistance).

Using the classification of Chevalier *et al* (1991), most research stations consider plants in class 0 to class 3a, sometimes even class 3b as resistant. Considering durable resistance, in our opinion, a more severe classification is needed. At the Fruitteeltcentrum, resistant plants are plants without symptoms and belong to class 0, 1 or class 2. The less susceptible plants belong to class 3a.

The 1 susceptible: 1 resistant segregation in the progeny of the V_f-cultivar Goldrush with the susceptible parent 7/10 (this genotype is an offspring of a cross with certain resistance but not the V_f-gene) is not found. This means that the recognition of the V_f-gene does not always guarantee for a sufficiently strong defence mechanism. If we would consider class 3a as resistant, the 50:50 segregation could be reached. As stated in other studies (Lamb and Hamilton, 1969), not only the major V_f-gene, which is the most important gene, is involved in resistance, but also the whole complex. Other genes, inherited from both parents, are also involved in resistance, which is also proved in the other progeny Delcorf x Vanda (60% resistant genotypes).

Comparing the % resistant seedlings of the progeny 7/10 x Goldrush with the progeny 7/10 x Angold, the resistance of Angold (with polygenic resistance) is handed on most of the offspring's. This can indicate the resistance is present in all Angold gametes (one resistance locus with sister-alleles of equal value (=contrasting alleles) or many different loci on different chromosomes which results in gametes with sufficient alleles for resistance). If Angold is crossed with a V_f-parent we expect a more stable resistance.

The results of the cross HL164*Goldrush showed a lower percentage resistant seedlings than expected, only 35% after the first inoculation. Blazek and Paprstein (1994) obtained the same results for crosses with some polygenic scab resistant parents, as did Lateur (1994, 1996), without incorporating the V_f scab resistance gene.

In the progeny of Coop 34 (V_f) x Discovery, % resistant plants is not higher as in the progeny 6/281 x Discovery. In this case V_f seems subordinated to polygenic resistance.

As conclusion, more stable and durable resistance could be obtained combining a good polygenic cultivar such as Angold and a V_f-cultivar. In this V_f-resistance, more genes are involved in which V_f is responsible for the recognition and initiation of the defence-process.

Experiment2

As stated before (E. Pauwels and J. Keulemans, in press), after selection in the greenhouse according to the 1 susceptible: 1 resistant segregation as we did in 1992, apparently still many genotypes can be susceptible in the orchard. This indicates once more that there is more than

one gene involved. Hrazdina *et al.* (1997) discovered phytoalexines which also plays a role in the defence-mechanism.

Comparing the different crosses with Delbard Jubilee as mother parent, the rate of susceptibility in the orchard depends in the cross combination. Considering the low rate of susceptibility in the crosses with Telamon (which contains the columnar gene), growth habit of the susceptible parent could be involved.

Late infection on genotypes not infected in May can occur which can indicate that there is a different susceptibility between the clusterleaves, petals and sepals comparing with leaves developed later in the season.

Experiment 3

In our region, the V_f-resistance has not overcome by *Venturia inaequalis* which can indicate that race 6 (Parisi *et al.*, 1993) is not present in the inoculum in the orchards. Also other plots in Belgium are still free of this race (Lateur, personal communication).

Apparently, there are many differences between polygenic cultivars. This polygenic resistance is also very important in a breeding program. Breeders need a high level of resistance which must be inherited in the progenies. The more genes involved and the more gametes with sufficient resistance-alleles, the more stable the resistance. Better is also if resistance genes do not operate as a cascade but in a parallel way.

Conclusions

The knowledge about the different resistance-mechanisms including the recognition and response of the V_f-mechanism is till now insufficient. The mechanism of polygenic resistance operates differently and seems to be a more constitutive system and not based on recognition. Also the genetic control of the different mechanisms is still unclear.
These knowledges are necessary for a more specific breeding.

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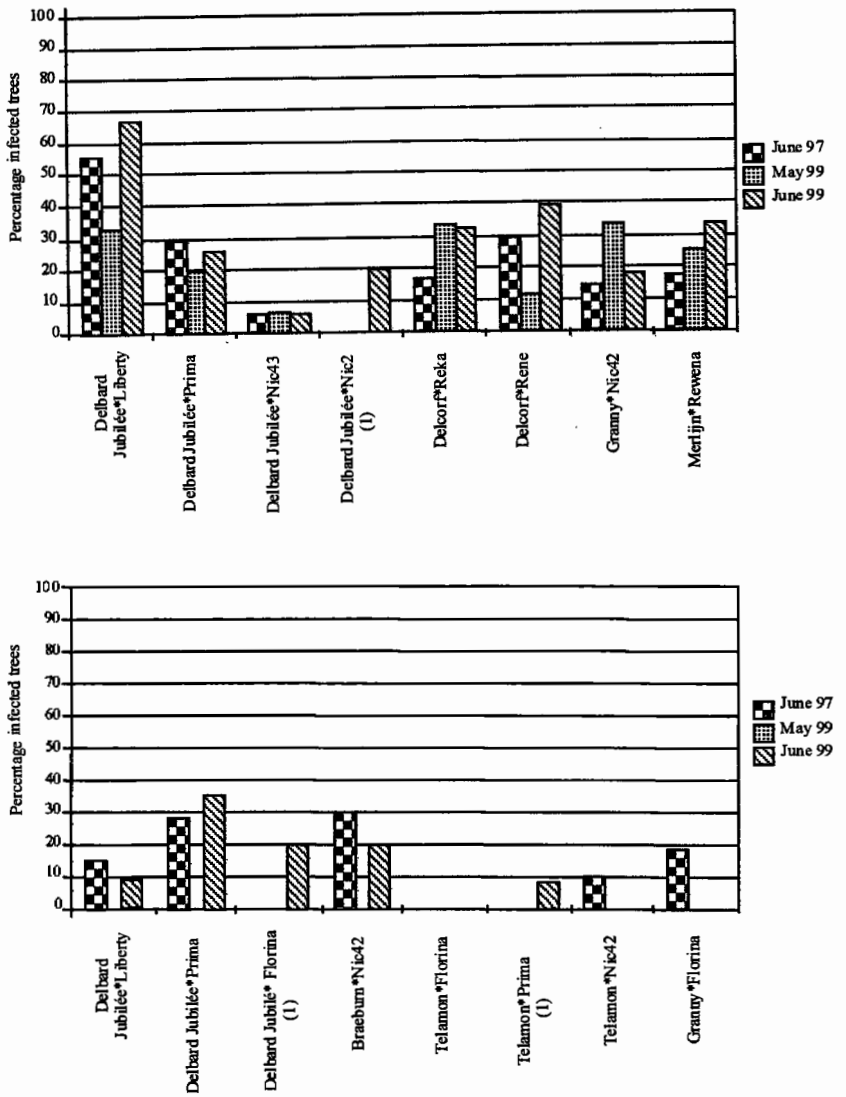

Figure 2. Results of scab observations in the field. In May 99 flowers were evaluated and in June 97 and June 99 observation was done on leaves. In May 1999 not all trees of each progeny were evaluated.

*(1) No observations were done in 1997 on Delbard Jubilée*Florina, Telamon*Prima and Delbard Jubilée*Nic42.*

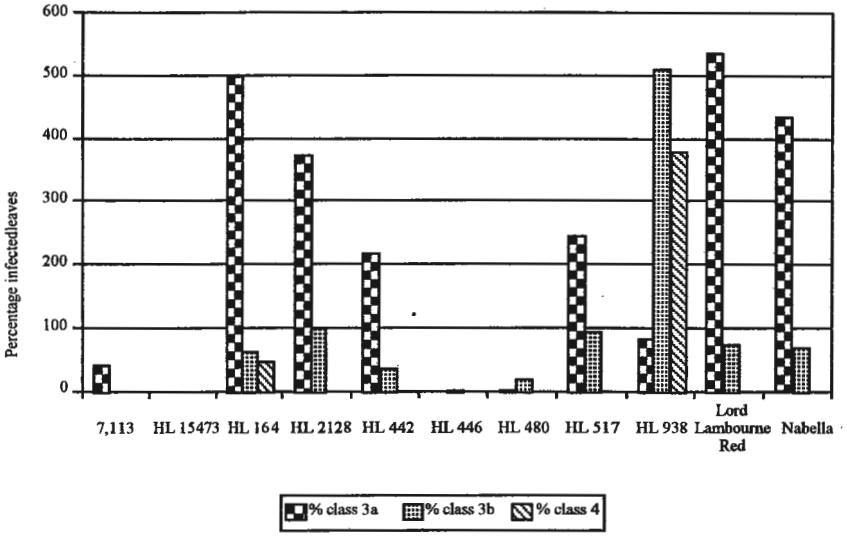

Figure 3. Observations of infection (infected leaves in class 3a, 3b and 4) on polygenic cultivars in an unsprayed plot in July 1999

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Identification of Molecular Markers Linked to Mildew Resistance Genes *Pl-w* and *Pl-d* in Apple

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Abstract : Powdery mildew, caused by *Podosphaera leucotricha*, is one of the most important diseases of apples in Britain. In order to reduce the reliance on single, major gene resistance to the disease in the future, molecular markers are being developed which will enable plant breeders to identify and pyramid genes in new varieties. At H.R.I.-East Malling we are developing markers for *Pl-w* and *Pl-d*, which are resistance genes derived from the *Malus* selections 'White Angel' and D12 respectively. Segregating populations were generated from crosses of the susceptible cultivar 'Fiesta' with E295-4, a resistant selection derived from 'White Angel', and with A871-14, a selection derived from D12. Glasshouse and field assessments of seedling-susceptibility have been made over four seasons and AFLP markers have been identified using a bulked segregant approach.

Key Words : Powdery mildew, *Malus*, molecular markers, resistance.

Introduction

Powdery mildew is one of the most important disease problems faced by apple growers in the U.K. The causative agent (*Podosphaera leucotricha*) affects the foliage and stems of growing shoots as well as the blossom, fruit and yield. To control infection, applications of fungicides are required from the tight cluster stage until terminal shoot growth ceases and may be necessary every seven days during periods of rapid growth. Alternative methods of disease control need to be developed for economic and environmental reasons.

The incorporation of genes conferring resistance to mildew is an important part of many apple breeding programmes and most of the apple varieties released from European programmes during the past 25 years carry some resistance to mildew. Genetic studies have indicated that much of this resistance is under polygenic control and is rarely sufficient to allow significant relaxation of spray regimes (Alston, 1983). More effective control would require the incorporation of major resistance genes such as *Pl-1* and *Pl-2*, identified from *Malus robusta* and *M. zumi* respectively (Knight & Alston, 1968), which may be found in many modern breeding lines. However an overreliance on these genes could lead to the widespread breakdown of resistance, and a number of physiological races of mildew able to overcome specific resistance genes have been reported already (Krieghoff, 1995). If such genes are to remain useful to breeders, better strategies must be adopted.

The development of more durable resistance is dependent on combining various major genes in breeding lines, preferably against a background of polygenic resistance. A breeding line whose resistance depends on accumulated partial effects of numerous resistance genes should exert little selection pressure on the pathogen (Pedersen & Leath, 1988). Such gene pyramiding is possible only when the presence of individual genes can be identified. *Pl-1* is known to demonstrate a specific necrotic reaction to infection (Alston, 1983) but other genes cannot be distinguished as easily. Successful pyramiding therefore relies on the development of molecular markers and marker assisted selection. Molecular markers are also useful for the

early identification of resistant seedlings, which improves the efficiency of breeding programmes.

Although markers linked to *Pl-1* and *Pl-2* have been identified (Markussen *et al.*, 1995; Seglias & Gessler, 1997; Dunemann *et al.*, 1999; Gardiner *et al.*, 1999 and Gianfranceschi *et al.*, 1999) there are no DNA based markers for the identification of the two other resistance genes *Pl-w* and *Pl-d*. These genes were identified respectively from the ornamental crabapple 'White Angel' (Batlle & Alston, 1996) and from D12, derived from the D series of open pollinated crab apples from Northern Italy (Visser & Verhaegh, 1976). Both genes are thought to give greater protection than *Pl-1* and *Pl-2* against mildew (Alston, 1983). Molecular markers that enable breeders to identify *Pl-w* and *Pl-d* are therefore required for gene-pyramiding to progress.

Recently developed techniques such as bulk segregant analysis (Michelmore *et al.*, 1991) and AFLPs, amplified fragment length polymorphisms (Vos *et al.*, 1995), have greatly aided the process of marker detection.

This work is a continuation of that initially reported in 1996 at the IOBC Workshop, Croydon (Evans, 1997) and describes the progress towards the identification of AFLP markers linked to *Pl-w* and *Pl-d* and the development of 'breeder friendly' markers.

Methods

Plant material

The apple progenies E623 and E624 were produced in 1995 specifically for marker analysis. E623 consists of 250 seedlings derived from a cross between the susceptible cultivar 'Fiesta' and the resistant selection E295-4 ['Gloster 69' x 'White Angel']. A cross between 'Fiesta' and resistant selection A871-14 ['Worcester' x D12] resulted in the 208 seedlings of E624.

Disease assessment

The progenies were assessed for mildew symptoms in the glasshouse for two seasons before being planted out in the field in cordon rows. They were then assessed for primary and secondary mildew over two more seasons. Inoculum levels were high enough in the glasshouse and in the field to infect the seedlings naturally. The disease descriptors used were Pl-GH-1, Pl-F-1 and Pl-F-2, developed by the European Apple Genome Mapping Project (King, 1996).

Bulk composition and AFLP analysis

Leaf samples were collected from seedlings of E623 and E624, frozen in liquid nitrogen and stored at –80°C. 12 resistant (score 0) and 12 susceptible (score 4 or 5) were selected from each population for DNA extraction and bulk segregant analysis. DNA was extracted from 2g leaf material using a modified version of the protocol described by Dellaporta *et. al.*, (1983).

Aliquots of the DNA extracts were diluted to the same concentration (300ng/µl) and pooled to create the bulks, each bulk consisting of DNA from 12 resistant or 12 susceptible individuals.

AFLP analysis was used to identify markers by bulk segregant analysis. Both sets of bulks have been screened with 128 primer combinations, 64 from the 'Large genome' and 64 from the 'Small genome' AFLP kits (Gibco).

Results

14 bands associated with mildew resistance and 3 with susceptibility have been identified as distinguishing the bulked samples of population E623. In population E624, 16 bands for

resistance and 14 bands for susceptibility have been found. The AFLP reactions revealing these markers are currently being confirmed, with 12 resistant and 12 susceptible individuals being run alongside the bulks. To date, two of these bands have proved to be only weakly linked, occurring in only 7 and 10 of the resistant individuals. However, a third, an AFLP fragment of 100 bp, was found to occur in all 12 resistant but no susceptible individuals in population E623. The fragment has been gel isolated, re-amplified, cloned and sequenced.

Discussion

Bulked segregant analysis, coupled with the AFLP technique, was found to be an effective way of finding potential markers in our progenies segregating for mildew resistance. Using this method, we have detected several fragments probably linked with *Pl-w* and *Pl-d.* Cosegregation analysis of the remaining samples will be carried out to confirm linkage. When linkage is established, these markers can be used to form the basis of a screen in breeding to allow pyramiding of resistance genes to commence.

Progenies incorporating *Pl-1, Pl-2, Pl-w* and *Pl-d* have been produced and will be tested for our markers and with primers developed for the detection of *Pl-1* and *Pl-2.* Seedlings found to be carrying more than one major resistance gene may then be selected for use in further crosses.

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After Lofthus, we all said "this is the top", after Kent, we said "this is just one step higher than the top" and now we were almost in heaven.

Clem Verheyden
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