IOBC/WPRS

Working Groups
"Integrated Control of Cereal Crops"
and
"Integrated Control in Oilseed Crops"

OILB/SROP

Les Groupes de Travail
"Lutte Intégrée en Céréales"
et
"Lutte Intégrée en Cultures d'Oléagineux"

International Conference on Harmful and Beneficial ~~Microorganisms in Grassland, Pastures and~~ Turf

Paderborn (Germany)
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Edited by
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Preface

Grassland, pastures and turf play a major role in agriculture and landscape in Europe and throughout the world. However, there is no international scientific organisation dealing with the problems of benificial and harmful micro-organisms in grassland, or the ways in which they may be controlled in an environmentally acceptable manner.

Professor Volker Paul and his team from the University of Paderborn in Germany therefore organised the first "International Conference on Harmful and Beneficial Microorganisms in Grassland, Pastures and Turf", which was held from the 4 - 6th October 1993 at Paderborn. The organizers worked together with Prof. K. Krohn (Fachbereich Chemie, University Paderborn) and Dr. M. Poehling (Fachbereich Agrarwissenschaften, University of Göttingen) who is the convenor of the group covering international control of diseases in cereal crops within the International Organisation for Biological Control of Noxious Animals and Plants West Palaearctic Regional Section (IOBC/WPRS). More than seventy scientists from Europe, the US, and New Zealand attended the conference, which is the third to be organised in Paderborn dealing with agricultural production, plant pathogens, pests, integrated control and the environment. The first two conferences were held at Paderborn in 1988 and 1991.

The scientific Committee succeeded in presenting a program of current interest and high scientific content, which the editors of this volume have tried to match with a special publication comprising the papers and posters presented at the Conference. Authors were given instructions for the preparation of papers in advance, and each manuscript was then checked carefully for the grammar and phrasing in English (JT). Manuscripts were also submitted on disk so that rapid corrections and reformatting could be carried out (KK). The editors have not only tried to edit the book very rapidly, but also to produce the highest standards of print, layout, and drawings by use of advanced text editing techniques.

Finally, we would like to thank Mrs. M. Näther, Mrs. Zukowski and Mr. H. Adam for their help, and Dr. Minks from the IPO-DLO who supported this new mode of publishing, and naturally all of the authors for their cooperation and patience.

Karsten Krohn, Volker H. Paul, Jane Thomas

Address of the Parliamentary State Secretary of the Federal Ministry of Food, Agriculture and Forestry, Wolfgang Gröbl,

at the
Conference on Harmful and Beneficial Microorganisms in Pastures, Turf and Grassland
on 4 October 1993
(delivered by Dr. Walter Däschner)

Ladies and gentlemen,

May I use the opening of your three-day international conference on microorganisms in pastures, turf and grassland to convey the regards of the Parliamentary State Secretary of the Federal Ministry of Food, Agriculture and Forestry, Wolfgang Gröbl. He is very sorry not to be able to speak to you in person as he has other commitments today.

Meadows, pastures, grassland as elements of the landscape, public greens and private lawns, and the flora and fauna to be found there, are of increasing importance in many ways. It is not only farmers and agricultural policy makers who, in the course of the re-orientation of Europe's agricultural policy, are interested in the prospects of whether, how and to what economic purpose grassland can be used in a less intensive way or arable land be converted into grassland.
Experts, too, who are committed to the cause of environmental protection or landscape management have to take a closer look at grassland and its role.

Some 5 243,000 hectares of permanent grassland accounts for about 31 per cent of the total agricultural land in Germany of approx. 16 950,000 hectares (1992 figures). Thus permanent grasslands are both economically and, above all, ecologically an important factor for our country.

In view of its importance it is remarkable that grassland is given - compared to arable crops - relatively little attention in research, in practical farming and also in the public discussion both at national and international level.

This is why I am all the more pleased that this international meeting of experts in Paderborn does focus its work on the great variety of different types of grassland.

You, ladies and gentlemen, intend to look, among other things, at the interactions between diseases and pests and their environment - it is for the first time in Europe that this subject will be dealt with comprehensivelyand in depth.

The great interest and the need to have closer cooperation among scientist and farmers to exchange experience on this important subject becomes evident from the large number of participants in this meeting, comprising experts from the European Community, Eastern Europe and other regions of the world.

Another encouraging factor is that the safeguarding of quality and yields of forage grasses as well as a good turf cover through healthy grass species and varieties obtained by resistance breeding in the field is given particular attention at your meeting. And - also with regard to fodder quality and related animal health questions - research into the field of grass endophytes should be of particular interest.

This conference can be expected to give major incentives to research workers, extension services and practical grassland farming.

Parliamentary State Secretary Gröbl would like to wish your conference every success. And I hasten to add my best wishes, too. We hope that our guests from abroad will spend a pleasant time in Germany.

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Aspects of efficient resource use in grassland production

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ABSTRACT

In recent years grass production in Europe has been characterized by a trend towards extensification which is supported by public budgets. In order to develope economically viable extensive systems, the production costs have to be lowered by increasing the efficiency of the inputs. A high efficiency of resource use is also advantageous from an ecological point of view. Extensification measures do not automatically ensure a high resource use efficiency. Examples of simple input-output relationships and also aspects of more complex systems are shown in the paper which give evidence of the requirement and the potential to optimize the inputs in extensive grass production systems.

Keywords: grassland herbs, grass species, nutrient turnover, resource use, white clover

INTRODUCTION

Extensification measures in grass production in Europe are widely supported by public budgets aiming at a decrease of surplus production but also at a maintenance of biodiversity, i.e. to protect threatened habitats and species. One could assume that besides this little attention is paid to the development of economically viable extensive systems. A main principle of a competitive production is to decrease the costs by increasing the efficiency of the input factors. Looking for an efficient resource use will bring about an economical as well as an ecological advantage. The more efficient the input factor, i.e. nutrient, the lower the risk of environmental pollution with unused nutrients, i.e. by leaching, and the more money will be saved. What will happen if grass production is de-intensified? As a rule, extensification measures seem to be accompanied with an increase of the efficiency of resource use. But this conclusion does not mean that the highest efficiency will always be achieved with the reduction of the inputs. As de Wit (1991) has shown even with extensive systems an input factor may be inefficiently used if other factors are in suboptimum supply and thus potentially pollute the environment. In order to meet environmental as well as economical goals it seems necessary to focus practical and scientific efforts on the optimization of the resource use in extensive grass production systems. This paper reviews some possibilities to increase the effectiveness of grass production. Simple input-output relations as well as some aspects of more complex systems will be considered.

YIELD RESPONSE TO N SUPPLY

According to their botanical composition grass swards show different abilities to convert supplied nitrogen into DM yield even at low levels of fertilisation. This has been proved not only with different grass species (Cowling & Lockyer 1965, Theiß & Opitz von Boberfeld 1992) but also with different varieties within a species (Bugge 1988). Sound results of the efficiency of supplied nitrogen will only be obtained if nitrogen returns by the grass are included. Figure 1 shows the nitrogen yields of shoot biomass of different grass swards with increasing nitrogen fertilisation. Cocksfoot (*Dactylis glomerata*) was superior to perennial ryegrass (*Lolium perenne*), i.e. with ryegrass available nitrogen was not completely used even at zero fertilisation. In the second example of Figure 1 the grass-white clover mixture hardly responded to fertiliser nitrogen and outyielded the grass sward by far, especially at a low level of fertilisation.

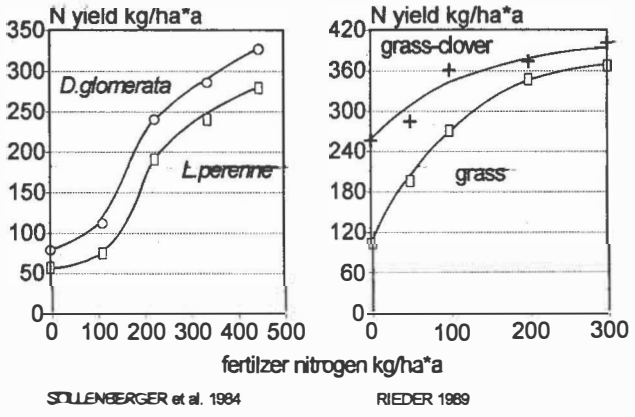


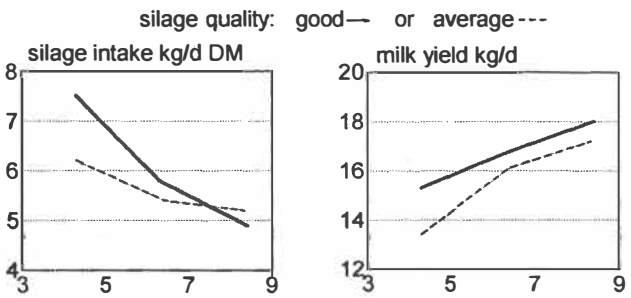
Figure 1: Nitrogen yield of different grass species/swards with increasing nitrogen fertilisation

This is of course a result of symbiotic nitrogen fixation of white clover which is most effective when no fertiliser nitrogen is applied. These simple examples give evidence of a significant margin to optimize the inputs even in low input systems, for instance by selecting suitable grass species and varieties. In addition, more examples of simple relationships could be given which also demonstrate the opportunities to optimize extensive grass production systems. This concerns for example the performance and nitrogen fixation of white clover varieties (Laidlaw 1988, Evans et al. 1992), the combining ability of grass species and varieties with white clover under grazing and cutting (Collins & Rhodes 1989, Evans et al. 1989), the disease resistance of grassland plants, the effectiveness of mycorhiza and endophyte infection on performance characteri-

stics or the water use efficiency where water is in limited supply. But there is still considerable research work necessary to make progress in the efficiency of resource use of low input systems.

GRASS UTILIZATION BY ANIMALS

The first aim of any agricultural grass production is to produce fodder for ruminants. Thus, looking at the resource use of grass production, the utilization of grass by cattle or sheep must be included apart from yield characteristics. An example of the significance of forage quality on animal performance is given in Figure 2. Silage intake of dairy cows and milk yield differed markedly between good and average silage quality.



composition of the forage

	concentrate	good silage	average silage
DM %	87	29.6	28
CP %[1)]	19.4	16	11.1
MADF %[2)]		28.9	40
IVDOM %[3)]	80.9	68	60
pH		4.2	4.4
ME MJ/kg[4)]		10.8	9.4

1) crude protein 2) acid detergent fibre
3) in vitro digestibility of organic matter 4) metabolizable energy

PHIPPS et al. 1987

Figure 2: Silage dry matter intake and milk production of British Friesian heifers as influenced by silage quality and concentrate feeding

The advantage of a good silage quality compared to an average silage was higher at low concentrate feeding. Less performance of average silage could not be compensated for by increasing the offer of concentrates. The feeding of concentrates is regarded as contributing mainly to surplus nutrient balances of intensive dairy farming; therefore it should be limited (Aarts et al. 1992). Thus, the importance of the herbage quality will increase. But will it be possible to meet the requirement of a high quality roughage as well as a low cost grass production?

DEVELOPING A LOW INPUT SYSTEM

Considering the yield potential of grass clover swards without fertiliser nitrogen, the animal performance of white clover based grass production systems has been investigated in several countries in recent years. Ernst & Heiting (1991) in Germany for instance, did an experiment with dairy cows comparing an intensive with a low input clover based system. Mineral nitrogen fertilisation was either 308 kg per ha or nil. Total N input including slurry ammonia was 364 or 29 kg per ha. Stocking rates were adapted to the yield potential with 3.3 cows per ha for the intensive and 2.2 cows per ha for the low input system. The DM yield was 133 dt per ha in the intensive and 77 dt per ha in the low input system. Milk yield was either 14,000 or 8600 kg fat corrected milk per ha and a. That means a yield reduction of about 40 %. It is interesting that the average yield per cow was nearly the same for both systems. Obviously, the forage value of the low input system was not poorer compared to the grass nitrogen system. This is also demonstrated by an investigation done in Giessen comparing the nutritive value of white clover with perennial ryegrass (Table 1).

Table 1: Nutritive value of *L. perenne* and *T. repens* of a spring and a summer harvest

	CP %	ADF %	NEL MJ/kg	Mg %	Na %	Ca %
			spring cut			
L. perenne	11.1	23.3	6.78	0.12	0.18	0.46
T. repens	28.3	14.5	6.31	0.28	0.44	1.50
			summer cut			
L. perenne	13.6	30.7	5.46	0.21	0.09	0.70
T. repens	23.2	24.1	5.75	0.29	0.20	1.20

White clover was characterized by a high CP, Mg, Ca and Na and a low ADF content, that means it was a quite valuable forage. Concerning the energy content, ryegrass showed higher values in spring cut forage but was lower in summer cut forage. Feeding trials with lactating cows (Thomson et al. 1985) also showed a high nutritive value and a

good milk performance of white clover feeding ration. Some other large scale experiments have been conducted in Western and Central Europe, which showed similar results to the experiment conducted by Ernst & Heiting (Davies et al. 1993, Vipond et al. 1993, Wilman & Williams 1993). As a result it seems to be possible to develope clover based low input-low cost grass production systems with a decreased risk of environmental pollution. But there are still some questions which will have to be answered if clover based systems are going to be sustainable. White clover will maintain its productivity only if enough nutrients like P, K, Mg are available and the pH value is not too low. If a system is designed without any nutrient input then the cycling of nutrients has to be improved. As the output of the nutrients with milk and sold livestock is relatively small, there must be a good potential for nutrient recovery. Grassland herbs are known to contain minerals like P, K, Mg, Ca to a greater extent than grasses. Experiments in Giessen have shown a two- to threefold higher Mg and Ca content of common herbs than perennial ryegrass. Besides this, dandelion (*Taraxacum officinale*) was found to have K contents of more than 5 % of DM. Thus, it was investigated whether herbs can improve the nutrient status of a grass sward, i.e. whether they may operate as a nutrient pump (Hopkins 1993). In a field experiment in Giessen, perennial ryegrass was either sown alone or in binary mixtures with yarrow (*Achillea millefolium*), plantain (*Plantago lanceolata*) or dandelion. Nitrogen fertilisation was 90 kg per ha and year; three cuttings per year were taken. Figure 3 shows results from a spring cut. Binary mixtures showed slightly higher yield than ryegrass monocultures. The herbs took a share of about 20-30 % in the DM yield of the mixtures.

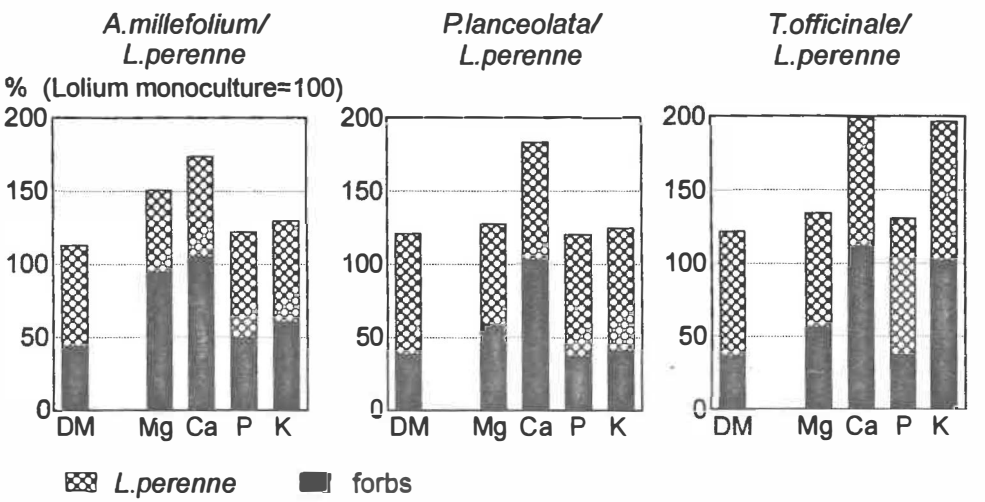


Figure 3: Dry matter and plant nutrient yield of different binary mixtures of forbs with *L. perenne* in relation to a *L. perenne* monoculture

The contribution of the herbs to the nutrient yield was much higher. For example, inclusion of yarrow to about 30 % into a *Lolium* sward increased the yield of Mg by 50 %, of Ca by 75 %, of P by 20 % and of K by 30 %. Very promising results were found for dandelion. A yield share of about 20 % increased the Ca and K yield by 100 %. In pasture systems these nutrients will be turned over by grazing animals and mainly be supplied to the regrowth.

CONCLUSIONS

The hypothesis has been proved suitable that even with extensive grass production systems there is a considerable margin to optimize the inputs and therewith the efficiency of resource use. Realizing this, European grassland farmers should recognize the more and more restricting economical framework as a challenge to increase the efficiency of grass production. This would decrease the costs and would also be advantageous from an ecological point of view. There is still a big need for agricultural research work to increase the efficiency of inputs in extensive grass production systems.

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Breeding and use of forage plants in Europe

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The time available for this paper is not sufficient to cover all diverse species used in Europe for forage production. Therefore, I shall concentrate on gasses as the main part of herbage seed used all over Europe. The species grown in Europe can be seen from the chart "Grass seed consumption" (EEC-Estimate)

Grass seed consumption (EEC-Estimate)		
Species	to/year	%
Festuca Pratensis	4.100	3
Poa pratensis	5.600	4
Poa palustris/Poa trivialis	300	
Lolium perenne (high persistent)	29.600	
Lolium perenne (interm.and persistent)	24.300	41
Lolium perenne (low persistent)	800	
Loliulm multiflorum	35.800	27
Phleum pratense	3.200	2
Festuca rubra	17.300	13
Dactylis glomerata	3.700	3
Agrostis canina	500	
Agrostis gigantea	100	
Agrostis tenuis (others)	700	
Festuca ovina	1.300	1
Lolium hybridum	2.800	2
Arrhenaterum elatius	100	
Festuca arundinacea	2.900	2
Poa nemoralis	100	
Total	133.200	

The main species are Perennial Ryegrass (41%), Italian and Westerwolth Ryegrass (27%) and Red Fescue (13%). All other species are of minor importance in terms of volume. But, we have to be aware that the majority of grasses are grown in mixtures and that, based on the calculated critical seed rate of a species within a mixture, small amounts

of seeds of a species are quite often to great importance for a consistent and sustainable award originating from that mixture.

Dealing with so many species is a heavy task for plant breeders. In addition, we have to bear in mind the multiple ways and application for using grass species. Based on technical and market experience, I tried to make a survey, which shows the different uses of grass seeds and the correspondent volume of seeds.

The chart "Grass seed - type of use" shows, that roughly one half is used for forage production (29,3 + 20,8 + 7,5% and the other half only for green cover on both, the agricultural side and the amenity side.

Grass seed: type of use

	to/year	%
Forage on		
- permanent grassland	39.000	29.3
- arable land	27.800	20.8
Intercropping for		
- forage	10.000	7.5
- green manuring	5.800	4.4
green cover/		
erosion control	8.500	6.4
Turf/amenity grasses	42.100	31.6

And quite often you see the same species used for

forage production in permanent grassland
forage production on arable land
forage production by intercropping
green manuring
erosion control
turf,

as it is true for instance for Perennial Ryegrass.

The chart "Potential use of the main grass species" shows, to which extent grass species are used for different purposes.

Potential use of the main grass species

	Forage		Intercroping			
	permanent grassland	arable grassland	forage	green manuring	Green cover/erosion control	Turf/amenity/ landscaping
Fesluca pratensis	X	X				
Poa pralensis	X				X	X
Lolium perenne	X	X		X	X	X
Lolium multifl.		X	X	X		
Phleum pralense	X	X				
Festuca rubra	X			X	X	X
Dactylis glomerata		X		X		
Festuca arundinacea		X				

For each purpose a special ability or special characteristics of the plants is needed. So, consequently, the breeder has to define accurately the breeding goals according to the intended use. This makes it very difficult to breeder of grass species, because the breeding goals are so different and most frequently, conflicting, that he has to run separate programs for the same species, as shown in the chart "Multiple breeding goals for Lolium perenne".

Multiple breeding goals for Lolium perenne, (examples)

haracteristic	Forage		Intercropping		Green cover/ero-sion control	Turf/amenity/ landscaping
	permanent grassland	arable grassland	forage	green manuring		
Growth	fast	very fast	very fast	slow	slow	very slow
Growth Heigth	high	very high	very high	short	short	very short
Sward	very dense	dense	dense	very dense	very dense	very dense
Persistency	very high	not needed	n.n.	n.n.	high	very high
Resistence to diseases	+++	++	+	+	++	+++
Dry matter yield	high-very high	very high	very high	low	low	very low

The differentiation into the many into the many and conflicting breeding goals is threatening the profitibility of the breeding business, because the breeder cannot create varieties for a market volume of 50.000 or 3.700 tons, but for 10.000 or 1.000 tons considering the potential different uses. This leads us to the question,

- how much a breeder can invest in breeding new improved varieties?
- how much can he spend for diversifying his selection activity by increasing the number of characteristics in question?

This is a very difficult question to answer. The majority of grasses are peranial plants and are constantly attacked and affected by a cosiderable number of diseases. Considering the present economic situation and the desired improvement of grass varieties, we as plant breeders need not only a better understanding of plant diseases and how plants react to diverse attack, but mainly efficient and fast practical methods for selecting high numbers of genetically different plants, or genotypes. In this way, we may afford expenditure on special disease resistant grass varieties.

Thank you all for all assistance and support so far and all good cooperation.

As President of ASSINSEL, the worldwide breeders' Association, may I wish you successful days in exchanging knowledge and views and so contributing to the great challenge we are faced, the genetic progress to the benefit of all farmers feeding their ruminants, controlling the erosion of their soils or all who really love the wonderful sustainable green lawn not affected by diseases.

Breeding for disease resistance in forage grasses with special emphases on 'costs of resistance'

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ABSTRACT

The achievements of resistance breeding in the last 20 years are shown. As a tool, the sample regression coefficient between the year of release and the score of resistance is used for a range of cultivars of perennial ryegrass (*Lolium perenne*). This paper deals with red thread, leaf spot, rust and fusarioses. The possibilities for increasing resistance to red thread and rust are shown from our own experiments. For rust and leaf spot the influence of increasing resistance on yield is demonstrated. For rust it was found that an increase of resistance of one point decreased the yield around 0.7 % to 1.5 %. The yield decrease for leaf spot was greater, between 1.2 % and 5.4 % for every one point increase in resistance. Only in one experiment was a yield increase (0.3 %) observed with increasing resistance. The impact of these results on fodder crop breeding is discussed.

KEYWORDS: forage grasses, leaf spot, resistance breeding, red thread, rust, yield

INTRODUCTION

The 16th meeting of the Fodder Crops Section of Eucarpia in 1990 had the motto 'Fodder crops breeding: Achievements, novel strategies and biotechnology'. It was concluded that, based on published evidence, the increase in DM yield for fodder crops in the last 25 - 40 years was about 0.5 % per year (NIJS and ELGERSMA, 1991). Since this meeting achievements in fodder crops and amenity grass breeding have been discussed eg WIJK (1993) presented data on the improvement in quality characters of amenity grass in recent years.

All these discussions came to the conclusion that in forage grasses it was possible to achieve certain yield increases and in the amenity grasses certain quality increases, but that disease characters have not been fundamentally changed.

These conclusions have been reached by examining individual characters in cultivar lists, but there has been little attempt to examine the relationship between characters. This paper aims to establish the influence of improving resistance on yield.

MATERIAL AND METHODS

Change of resistance level was estimated on basis of cultivars of perennial ryegrass (*Lolium perenne*) in the National Lists of France, Germany and the United Kingdom (ANONYM, 1993, BUNDESSORTENAMT, 1991 and STRI, 1993). For France and

Germany the sample regression coefficient was calculated between the year of release and the disease score of all registered cultivars of perennial ryegrass. The result was transformed to the change of resistance in the last 20 years. As there is no year of release from the STRI in UK only cultivars which are also described in Germany and/or France were used. Here the first year of listing was used.

In all the calculations and scores used there was no distinction between species of disease eg. only 'red thread' is used not *Laetiseria fuciformis*, *Limonomyces rosipellis* or *Limonomyces culmigenus*. The same was true for rusts and for leaf spots. Under the classification 'fusarioses' we find all the harmful influences on the grasses during the winter, including infections by fungi (BUNDESSORTENAMT, 1991).
All scores are expressed on a 1 to 9 scale, where 9 means the most resistant.

For estimation of the potential for selection for red thread resistance our own experiments were used at Verneuil and Le Chatelier in France. These amenity experiments are sown in two replicates with plots of 2 m² each. Red thread occours every year. Between the two replicates and between the two locations the sample correlations coefficients (r) were calculated.

For rust resistance, our experiments with *Lolium multiflorum* were used. The populations in this experiments were infected artificially by the method described by BIRKENSTAEDT, 1990. Plants which showed no rust sporulation were selected. The offspring of these plants were further screened in France under conditions of natural infection.

To investigate the impact of rust and leaf spot resistance on yield there have been parallel experiments in Hof Steimke and in France.
The rust experiments were sown in autum 1992 and spring 1993 respectively. In Hof Steimke yield experiments used a plot size of 10 m² in a standard design. In France row experiments (row length 2 m) were used. These rows served only for rust screening. In all experiments in Hof Steimke no severe rust attack was recorded, except in the 4th cut of the intermediate maturity group where some rust occoured. This rust was scored and the calculation for this cut was done independently.

The leaf spot experiments were sown in spring 1989 and spring 1990 respectively. In Hof Steimke yield experiments used a plot size of 10 m² in a standard design. In France row experiments (row length 2 m) were used which served only for leaf spot screening. In all experiments in Hof Steimke no severe leaf spot attack was recorded. For the calculations the total DM-yield of the 3 years were considered in relation to the leaf spot scores in France.

RESULTS

For estimation of the success of selection procedures for red thread, rust, leaf spot and fusarioses during last 20 years the sample coefficient of regression between the scores and the years of release was calculated based on the cultivar lists from France, Germany

and the United Kingdom. The results are shown in **Table 1**. It is obvious that in France there is little selection progress for all diseases over the 59 cultivars examined. For red thread the increase of 0.2 of a score point was not significant, whereas the progress of rust resistance and leaf spot resistance was significant with increases of 0.8 and 1.4 respectively.

Table 1: **20 years change (1973 - 1993) on a 9 to 1 scale for disease resistance of perennial ryegrass**

	France	Germany	United Kingdom
Turf			
Number of cultivars	59	71	40
Red thread	0.2	- 0.6*	- 0.02
Rust	0.8*	-	-
Leaf spot	1.4*	-	-
Fusariose	-	-0.3	-
Forage			
Number of cultivars		65	
Rust	2	0.2	

- disease resistance have been expressed on a 1 to 9 scale, where 9 is most resistant

In Germany 71 cultivars were evaluated. For red thread there had been a significant decrease in resistance of 0.6 in the last 20 years. For the fusarioses there had been a non significant decrease of 0.3. For the 65 forage cultivars evaluated there had been a slight progress of 0.2 in the last 20 years, but this change was not significant.
Finally in the United Kingdom the older cultivars show the same susceptibility of red thread as the newer cultivars.

In extensive amenity experiments in France it was not possible to find any significant correlations between the replicates or between the locations (**Table 2**).

Tabelle 2: **Sample correlation coefficient (r) between two replicates and two locations of red thread resistance of perennial ryegrass**

location	number of lines	correlation coefficient
Verneuil	125	0.06
	69	0.06
Le Chatelier	75	0.01
	141	0.18
between locations	165	0.20

For rust the results indicate that it is possible to increase the level of resistance of a population in only a few generations. In an unselected basic population of *Lolium multiflorum* an average rust score of 4 was found. Another population was scored with an average of 5 after one cycle of artificial infection with rust. In two other population it was possible to increase the rust resistance even more. In populations with two and three cycles of artificial infection the scores were 5.5 and 6.0 respectively. This shows that it was possible to increase the level of resistance with every cycle of selection.

In the next two experiments the aim was to show the effect of increased resistance on the yield. Parallel experiments in Germany and France were used. In Germany it was possible to estimate the yield more or less without disease attack. On the other hand there had been a servere rust attack in the one experiment in France and a heavy leaf spot attack in the other.

In **Table 3** the results of three parallel experiments are combined.

Table 3: Influence of a change from a rust susceptible cultivar (2) to a rust resistance cultivar (8) on yield of perennial ryegrass 1993

heading group	N	yield change in %	cuts
early	103	- 4.1*	1 - 4
intermediate	71	- 4.1*	1 - 3
late	64	- 9.0*	1 - 3

- yield was measured in Germany, rust was scored in France

In the early and intermediate heading group the increase of the resistance level of 6 points (from susceptible 2 to resistance 8) was associated with a yield decrease of 4.1 %. In the late heading group the yield decrease was larger at 9.0 %. In the experiment with the intermediate heading group there was a 4th cut in addition to the three shown in the table. In this cut there was also a heavy rust attack on some breeding lines in Hof Steimke. If this 4th cut is calculated separately slightly smaller yield depressions (3.5 %) are found on the basis of the French rust attack. This is shown in **Table 4**. It is also possible to present this calculation on the basis of the German rust attack itself. For this very different results are found, with no yield depression occuring. When only the 4th cut, the cut with the rust attack in Germany is considered, a yield increase (+ 6.9 %) for the resistant breeding lines is found. There was a significant relationship between the rust score in France and the rust score in Germany of $r = 0.56^*$.

Table 4: **Influence of a change from a rust susceptible cultivar (2) to a rust resistant cultivar (8) on yield of intermediate perennial ryegrass 1993 depending on the location of rust scoring**

location of rust scoring	cuts	yield change in %	
France	1 - 4	- 3.9*	
		1 - 3	- 4.1*
		4	- 3.5
Germany	1 - 4	+ 1.2	
		1 - 3	+ 0.2
		4	+ 6.9*

Similar calculations were done with another series of experiments from 1989 to 1992. In this case the total yield of the three years were compared to the leaf spot score in France. The first two experiments had only low numbers of breeding lines, so results must be treated with caution. In the early heading group a yield loss of 32.4 % was found, with an increase of 6 points for resistance. The early to intermediate heading group was the only one with a slight yield increase (1.6 %) combined with a resistance increase. Experiments with the lines of the intermediate and late heading group contained a wide range of material, in this case the results can be considered as reliable. In these experiments yield depressions of 7 % and 11.9 % respectively were found combined with an increase of 6 points for leaf spot resistance. The results are shown in **Table 5**.

Tabelle 5: **Yield loss of a leaf spot resistance breeding line (8) against a leaf spot susceptible breeding line (2) in perennial ryegrass 1989 - 1992**

heading group	N	yield loss in %
early	25	- 32.4*
early - intermediate	39	+ 1.6
intermediate	117	- 7.0*
late	64	- 11.9*

yield was measured in Germany, leaf spot was scored in France

DISCUSSION

The resistance level of cultivars was relatively constant during the last 20 years. The situation of the amenity grasses in France seems to be slightly better than in Germany. This may be explained by the different listing procedures in the two countries. In France a special VCU estimate is necessary for cultivar listing whereas in Germany it is sufficient to fullfil the DUS-requirements.

That there was no breeding progress in red thread resistance can be explained by results of own experiments which show that it is nearly impossible to select for red thread resistance due to the lack of uniformity of natural infection and there was no agreement between replicates. This result is confirmed by observations of other breeders (ROULUND, 1993 personal communication). The scores of resistance for red thread published by the different plant variety offices have to be considered with caution. There is a considerable need for research on this disease, which is very common in France (GONDRAN, 1993) and elsewhere.

As shown by the example of rust it is relatively simple to make progress in breeding for resistance. However, there has been little actual progress during the last 20 years. This may be explained by the fact that every increase in rust resistance is combined with a yield decrease. The same is true for leaf spot resistance.

As long as the variety testing authorities emphasise dry matter yield in preference to resistance there will not be any fundamental chance in the level of resistance. But there are some positive signs as it was shown recently by THOMAS (1993) and FREUDENSTEIN (1993). So in the United Kingdom rust resistance has been given some value especially in those cultivar types which are mainly used for forage conservation. At the German plant variety office the resistance to *Xanthomonas* evaluated by artificial infection has been used for the description of a variety for some time. It would be desirable to get a certain yield bonus for every increase in resistance score.

In practical breeding it is difficult to integrate these demands within a breeding programme. The aim of every breeder is to develop a cultivar with a wide adaptation combined with a high yield and good disease resistances and there is a negative relationship between these aims. However, there are exceptions to this and with the help of a wide network of experiments which combines sites for yield estimation and sites for resistance it should be possible to select lines which have a high yield combined with good resistances and a wide adaptation - though breeding progress is likely to be very slow.

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Biology/Ecology of Harmful and Beneficial Microorganisms on Grasses

Significance of virus infections on pasture and turf grasses in Central Europe

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ABSTRACT

The incidence of viruses on grasses in Central Europe is reported, and the characteristics of viruses as pathogens of grass are discussed.

Only three gramineaceous viruses are considered economically important: barley yellow dwarf, ryegrass mosaic and cocksfoot streak viruses. The occurence of epidermic of these viruses depends on the abundance of their vectors. Reactions of plants to virus infections have been described in terms of symptom expression, growth patterns, and effect on yield. Possible ways of avoiding virus infection or yield losses caused by viruses are discussed.

Keywords: viruses, gramineae, spread, symptoms, tolerance

VIRUSES AS PATHOGENS OF GRAMINEAE

Viruses, like fungi and bacteria are widespread pathogens of plants. Nearly 1 000 different viruses are known to be pathogens of plants but relatively few of them are economically important. In Europe more than 26 gramineae infecting viruses have already been identified (Table 1) and probably some more may exist. Recently, a new virus has been isolated from *Lolium* species which obviously is common at least in Germany. Grass viruses belong to several different groups and differ in several respects such as host ranges, transmissibility, frequency, influence on infected plants and consequently in the damage they cause. Most of the graminaceous viruses infect wild growing as well as cultivated grasses including cereals and maize. Therefore grasses in pastures and other grasslands are important reservoirs from which viruses can be transmitted to cereals. One of these viruses, brome mosaic virus, infects dicotyledonous plants (e.g. beech tree, *Fagus silvatica*, or elder, *Sambucus racemosa*) as well. Out of 5 to 10 frequent but mainly cereal infecting viruses only three, barley yellow dwarf virus (BYDV), ryegrass mosaic virus (RMV) and cocksfoot streak virus (CfSV), are economically important pathogens of forage grasses.

Table 1

Viruses Isolated from Gramineae

(excluding mainly cereal infecting viruses)

Potyviruses
ryegrass mosaic virus
cocksfoot streak virus
Agropyron mosaic virus
Holcus streak virus
Spartina mottle virus
Anthoxanthum mosaic virus
wheat streak virus

Potexviruses
Foxtail mosaic virus
"*Lolium* virus"

Hordeumviruses
Anthoxanthum latent blanching virus

Geminiviruses
wheat dwarf virus

Crypticviruses
Lolium cryptic virus(es)

Rhabdoviruses
Festuca leaf streak virus
cocksfoot stripe mosaic virus

Bromoviruses
brome mosaic virus

Sobemoviruses
Cynosurus mottle virus
cocksfoot mottle virus
cocksfoot mild mosaic virus
Molinia streak virus

Phytoreoviruses
Arrhenatherum blue dwarf virus
Lolium enation virus

Luteoviruses
barley yellow dwarf virus(es)

Assessing viruses as pathogens requires knowledges of their biology and their epidemiological behaviour. Viruses differ from other pathogens in their requirement of vectors to be spread. Vectors may be insects, mites, nematodes or fungi which aquire viruses from source plants and transmit them into new hosts. Therefore epidemic occurrence of viruses often correlates with vector abundance which is affected by environmental conditions. An epidemic spread of viruses requires the occurrence of sufficient numbers of virus sources and a high proportion of virus carrying vectors. Insects sometimes carry viruses from sources over long distances to new host.

SPREAD OF VIRUSES

Viruses may be transmitted by specific or non-specific vectors, either persistently or non persistently. For instance both *Agropyron* mosaic and ryegrass mosaic viruses are transmitted non persistently but specifically only by the eriophyd mite *Abacarus hystrix*. Immediately after virus aquisition vectors are able to transmit.
Persistently-transmitted viruses can only be transferred after a passage of 24 to 48 hours through the vector. After that latent period, a few minutes are sometimes sufficient to transmit the virus into new hosts. BYDVes are persistent and vector specifically transmitted and based on the principal vector species more than five strains of BYDV have been characterized; ***Rhopalosiphum padi*** is the most effective vector of BYDV-RPV, whereas ***Macrosiphum (Sitobium) avenae*** transmits BYDV-MAV most efficiently.

In general non persistent viruses can also be transmitted mechanically which is a common measure to propagate viruses for investigations. In nature spread by mechanical inoculation has seldom been observed. For instance *Cynosurus* mottle virus (CyMV), transmitted non persistently by the beetle *Lema melanopa* is occasionally found on *Cynosurus cristatus* and *Agropyron repens*. In plots of *Cynosurus cristatus* it has been observed that small patches of plants infected by CyMV have been spread in the course of the year through cuttings by lawn-movers.

Viruses, with the exception of barley stripe mosaic virus which does not infect grass naturally, are not seed transmitted. Thus germinating plants are virus free. Plants become infected after vectors transmit viruses from host sources into newly sown fields.The number of primary infected plants depends on the number and distance of host sources, and on the numbers and movement of virus carrying vectors, their transmission efficiency, and also from attractiveness of the plant species to the vectors. On average seldom more than 5 to 10% of vectors are virus carriers and in general a comparable number of plants receive primary infections. From these primary infected plants secondary spread within fields occurs, mainly by the progenies of the original vectors. In contrast to annual crops, perennial plants in pastures, turfs and other grass areas are threatened by virus infection year after year. While during the first years of growth relative few plants become infected, the rate of infected plants raises with the age. The speed of spread depends again on the activity of vectors and also on the mode of transmission. During a study of 4 000 single plants of *Lolium perenne* the proportion of infected plants increased yearly and five years after planting nearly all became naturally infected by different viruses.

These observations recorded on single plants differ from those of on self-regenerated grasslands. There infected plants become out-competed by healthy ones and finally an unstable ratio of infected and uninfected plants results which changes yearly and also during the course of each year. This fact may be one of the reasons that from several countries ratios of between 1 to 70% virus infected plants in pastures or grasslands have repeadedly been reported. Investigations in Germany led to similar results.

SYMPTOMS OF VIRUS INFECTED GRASSES

Plants react to virus infections with characteristic symptoms which are different from those induced by other pathogenic and non pathogenic agents. Intensity of symptom expression depends on the virus species, the host species and environmental conditions such as temperature, light intensity but sometimes also soil conditions (e.g. humidity). Light green streaks or stripes are the most distinct symptoms which appear first on the youngest rolled leaves. After infections by luteoviruses, e.g. BYDV, whole leaves can sometimes become completely yellow or red. Vein swellings (enations) on the under surface of leaves can be a third type of symptomes caused by reoviruses such as *Lolium* enation or *Arrhenatherum* blue dwarf viruses. Stunting and tillering of plants are also common reactions of virus infected plants. Based on these symptoms virus infected plants can

normally be recognized, but because several viruses induce rather similar symptoms, individual viruses cannot be diagnosed visually. For instance cocksfoot, *Dactylis glomerata*, produces similar symptoms when infected by cocksfoot mild mosaic, cocksfoot mottle, cocksfoot streak or maize dwarf mosaic viruses, which belong to different virus groups. On the other hand symptoms caused by RMV range from mild mosaics to severe necrosis depending on the reaction of individual *Lolium* cultivars and in case of very mild symptoms infected plants may only be diagnosed as virus infected by direct comparisons with uninfected plants of the same cultivar. Symptom expression is much more severe when plants have mixed infections of two or more viruses. Therefore an unequivocable identification of a virus requires special diagnostic methods like electron microscopy or serological techniques or biological tests (e.g.mode of transmission or examination of host ranges) or modern techniques such as PCR. Each of these tools is also suitable for the detection of viruses in latent infections.

Since many grasses have small leaves the often inconspicuous virus symptoms make the discovery of infected plants within grassland sometimes rather difficult. In general those plants infected by aggressive viruses (e.g. BYDV) remain smaller than healthy ones. Their leaves remain shorter and the number of inflorescenses can drastically be reduced. Decreased cold resistance and increased susceptibility to secondary infections by other pathogens and to non pathogenic influences are further consequences of virus infections. Stressed plants become overgrown by more rigorous healthy plants.This may explain why virus disease in self-regenerated grassland have seldom been noticed and that our knowledge on the influence of viruses on yield and quality of forage grasses is very limited.

Symptom expression in general correlates with reduction of photosynthetic active surface and consequently leads to reductions of green tissue as well as seed production. Virus infections furthermore reduce organic matter, water soluble carbohydrate content and organic matter digestibility. In the fifth year after planting the green material of the 4 000 single plants of *Lolium perenne* of the trial mentioned above was reduced to less than 10% compared to uninfected plants. On self-regenerated fields comparable yield reductions have never been observed and are not expected because of the generally smaller number of infected plants.

RESISTANCE TO VIRUSES

Nevertheless the yearly decreasing yield of pastures and grasslands is at least partially caused by viruses. The unknown number of infected plants and secondary infections by other pathogens as well as environmental influences make the calculation of yield losses induced only by viruses difficult. To avoid yield reductions plants have to be resistant to a broad complex of yield reducing factors.

Even if most grass species are susceptible to the most important virus, BYDV, growth of resistant cultivars can be a helpful measure to reduce virus infections or yield

losses caused by viruses. Some experiences have already been collected on virus resistance of cereals but most of the selected resistant lines or cultivars are actually susceptible to viruses but tolerate them. Cultivars which do not propagate viruses, referred to as "immune", have been found only occasionally (e.g. some barley cultivars which are immune to barley yellow mosaic virus).

Only a few examples of resistance to virus in forage grasses are available but preliminary investigations have revealed several types of resistance. From the different patterns of growth inclused by virus infections, it can be deduced that tolerance to viruses is a common property of numerous plants. In order to avoid yield reductions caused by viruses the selection of tolerant lines or cultivars appears to be the most feasible approach at present. Such plants should be less damaged by viruses than highly sensitive ones. In general, in most breeding stations great numbers of infected plants are present and tolerant plants have therefore been unconsciously selected. The high level of mostly undiscerned infected plants results from persistent cultivation of single plants and their vegetative propagation for several years. Only those plants relevant to the aims of the breeder are used, regardless of whether they are virus infected or not. Because several viruses are common in breeding stations selected plants may be tolerant against some of them. The disadvantage of tolerance is its polygenic determination, and it is therefore a very unstable property of plants. Tolerance can be overcome by changed climatic conditions. Therefore especially for cross-pollinated plants breeding of virus resistance in general appeared to be difficult.

Some grass species unimportant as forage grasses as e.g. *Leymus angustifolius* or some lines of *Thinopyrum intermedium*, obviously not become infected by BYDV. In case these species really are immune to BYDV efforts are necessary to proof their use as sources of resistance in future breeding programs.

From recent investigations it can be concluded that some plants may be resistant to virus transmission. Even if susceptible to BYDV, plants did not become infected or only occasionally after repeated inoculations. Possibly due to special morphological structures of leave tissue insects may be unable to place the stylet and therefore the virus particles into phloem cells. From observations that insects seem to favour some plants it may be concluded that leaf colour or secondary plant substances are further factors of resistance to viruses. However, the use of these properties for resistance breeding is unlikely to be appropriate.

Chemical treatments successfully reduce virus infections in cereal crops even if primary infections can not be prevented because vectors survive on treated plants as long as needed for virus transmission. Pesticides used in annual forage crops should lead to comparable success. The control of virus infections of perennial crops is much more difficult and requires several treatments a year without any guarantee that infections will be avoided. Plants can be colonized by vectors again after the diminishing effect of pesticides 2 weeks after treatment. Because of the very limited effects, the use of pesticides for controlling virus infections in grasslands is not recommended.

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First results on the development of a serological routineassay specific for gramineous Xanthomonads

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Introduction

Bacterial wilt of forage grasses can cause considerable damages to different species of fodder grasses (SCHMIDT & NUESCH 1980; PAUL & FREUDENSTEIN 1989). The causal agent is *Xanthomonas campestris* pv. *graminis* (Xcg) which was first described by EGLI et al. 1975.

During the last ten years the Laboratory for Biotechnology and the new Laboratory for Molecular Phytopathology of the University of Paderborn have worked intensively 1n this field. Basic methods for artificial infection and a diseases assessment scale (PAUL & BIRCKENSTAEDT 1989) have been established. As an official testing site for the Federal Office for Plant Varieties it was our task to screen varieties during the registration process for the Official Variety List of Germany.

Because there was a need for a rapid and simple identification method for the bacterium we concentrated on the development of a routine serological assay.

Material and Methods

Production of Antibodies

Polyclonal antibodies were raised in rabbits after immunization with a recently isolated strain of Xcg. Immunization and bleeding was carried out by Dr.Frank Niepold at the Federal Biological Research Centre in Braunschweig.

Cultivation of Plants and Preparation of samples

For all tests with plant material *Lolium multiflorum* cv. Remy plants were grown in a climate chamber at 20°C for 6 weeks. At this age the plants were

inoculated with a Xcg suspension diluted to an OD_{630} of 2.0. The tillers and leaves of the plants were cut with scissors of which the blades had been dipped into the bacterial suspension. At different times after inoculation samples were taken and stored at -20°C.
Plant extracts were prepared by grinding 2 g of frozen plant material with 15 ml extraction buffer (PBS pH 7.4 containing 0.6% w/v polyvinylpyrrolidone, 0.25% b-mercaptoethanol and 10mM EDTA). Until further use the samples were stored at -20°C.

Electrophoresis and Western Blotting

For testing the antiserum Xcg cells were harvested from shake cultures incubated overnight (1% Yeast extract, 2% D-Glucose-Monohydrate, 120 rpm at room temperature) and a crude protein extract was prepared. For this 30 ml of the culture were centrifuged for 30 min.at 15.000 rpm / 4°C (JA20, Beckmann Instruments). The supernatant was discarded and the pellet was resuspended in 1 ml Lysing Buffer (50 mM Glucose, 10mM EDTA, 25mM Tris pH 8.0). To 500µl of this suspension 200µl of freshly prepared lysozyme solution (3.5 mg /ml) were added and then incubated for 30 min.at 37°C. After this 3.7 ml Extraction Buffer (200 mM Tris, 500 mM Sucrose, 5 mM EDTA, 5% v/v b-Mercaptoethanol, 3% w/v SDS, 0.01% w/v Bromphenol-Blue) was added and the samples were boiled for 10 min and stored at -20°C until electrophoresis.

Electrophoresis was carried out in a 6% Stacking Gel and a 12% Separation Gel at a constant current of 6 mA for approximately 19h in a Hoefer SE 600 System cooled to 5°C. After electrophoresis gels were stained with Coomassie R 250 (Staining solution : 0.25% w/v Coomassie Blue R250, 40% v/v MeOH, 10% v/v Acetic Acid; destaining solution: 30 % v/v MeOH, 5% v/v Acetic Acid) or transferred to a Hoefer TE70 Blotting Chamber and blotted onto a nitrocellulosemembrane (NCM)(Schleicher&Schuell BA 85) at a constant voltage of 7 V for 1 hour. The NCM was then blocked with 3% w/v bovine serum albumin in TN Buffer (150 mM NaCl, 10 mM Tris pH 8.0) for 1 h. After incubating the NCM with the antiserum (500fold diluted with TN-Buffer) for 1 hour the membrane was washed three times for 10 minutes with TN-Buffer containing 1% v/v Triton-X-100 and 0.1 % v/v Tween 20. After a final washing step with TN-Buffer the enzyme conjugated secondary antibody (Anti-Rabbit-IgG conjugated with alkaline phosphatase provided by Boehringer Mannheim,1000fold diluted with TN-Buffer) was incubated with the NCM for 1 h. Triple washing with TN-Buffer for 20 min was followed by a final washing step with Aq.dest. During the last washing the Substrate Solution I (12 mg Naphtol-AS-MX-Phoshate; Serva in 30 ml Aq.dest.) was prepared and filtered. After this Substrate Solution II (180 mg Fast Red, 60 µl

1M $MgCl_2$ and 6 ml 1M Tris pH 8.0 filled up to 30 ml with Aq.dest.) was filtered into Substrate Solution I. The NCM was then incubated in the filtrate until satisfactory staining of the membrane occurred resulting in red bands on the NCM. Staining was stopped by washing the NCM with Aq.dest.

Dot-Immuno-Binding-Assay

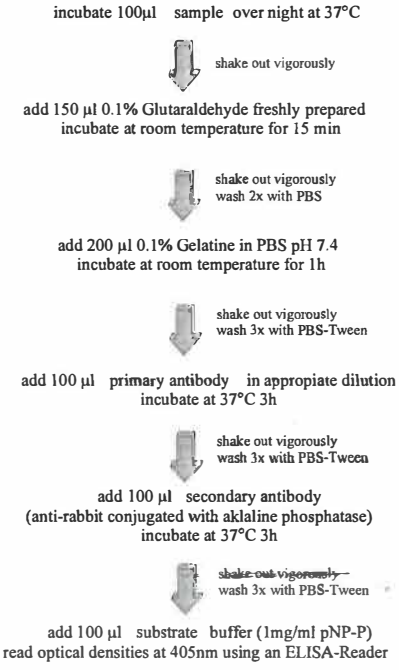
For Dot-Blot 3µl droplets were placed on marked positions on an NCM. After airdrying of the samples the membrane was incubated in a drying oven at 60°C for 30 min. and then the protocol for western blots was applied to NCM starting with 1h blocking. In addition to Fast Red as an alternative subtrate Nitro-Blue-Tetrazolium(NBT) was used. For this 33µl NBT stock solution (75 mg /ml NBT in 70% v/v Dimethylformamide) and 25 µl 5-Bromo-4-chloro-3-inolyl-phosphate (BCIP) stock solution (50 mg/ml in Dimethylformamide) were added to 20 ml Staining Buffer (0.1 M NaCl, 50mM $MgCl_2$, 0.1M Tris pH 9.5). After development of dark blue spots the reaction was stopped with Stopping Buffer (0.5 mM EDTA, 20mM Tris pH 7.5).

Immuno-Sticks

For the Test Nunc Immuno®-Sticks were used. 1 ml of either samples or controls was incubated overnight at 37°C. After blocking for 1 h with blocking reagent (3% w/v Bovine Serum Albumin Fraction V in TN-Buffer) the samples were washed twice with tap-water followed by 1 h incubation with 1 ml antiserum diluted 500fold with TN-Buffer. After washing as before sticks were incubated for 1h at 37°C with 1m of the enzyme conjugated antibody (see above). After washing as mentioned above the Substrate Solutions I and II (see above) were produced and 1 ml was filled in each stick. Development of red colour on the sticks was observed and reaction was stopped by washing with tap water. Sticks were photographed and stored in Aq.dest. for further documentation.

ELISA-Tests

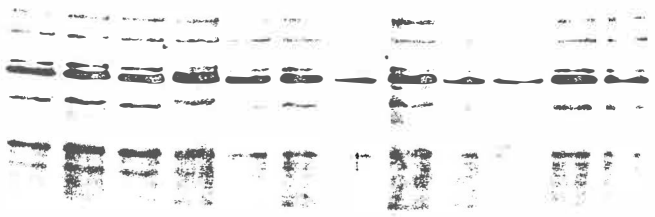
For all tests Dynatech Immulon I 96 well flat-bottom ELISA-Plates were used. The ELISA protocol of STEAD 1992 was slightly modified, but all buffers were used as described there (see Fig.1). Just before use the substrate solution was prepared and added to the plates. Extinctions at 405 nm were measured using a Dynatech ELISA-Reader MR 7000 connected to a Personal Computer for further evaluation of data. As a blank sample buffer and as a control a Xcg suspension (OD_{630} 0.1) was used.



Results and discussion

SDS electrophoresis showed good resolution of bands after running for 19h at a constant current of 6 mA. After western blotting and incubation with the rabbit-anti-xanthomonas-serum and subsequent staining of the NCM typical banding patterns for several *Xanthomonas* pathovars as documented in Figure 2 could be observed.

Xanthomonas campestris pv. *campestris* could be clearly distinguished from the other pathovars. Bands which could be observed in the lanes with *Pseudomonas* and *Erwinia* strains are due to the fact, that there is little unspecific binding of the secondary antibody (HEIN, unpublished data). It became quite clear, that all pathovars of *Xanthomonas* pathogenic to *Graminea* especially the pathovars *graminis*, *arrhenateri*, *poae*, *phlei* and even *translucens* could easily be detected with the antiserum used for this tests.



Slot1 PP, Slot 2 PSS, Slots 3-14 XCG, Slot 15 EA, Slot 8 NCPPB 2700
(see.Fig.4 for Abbrev.)
Figure 2 : Western Blot of Different Xanthomonas Pathovars

column no.													
1	2	3	4	5	6	7	8	9	10	11	12	13	14
	15		16	17									
remy	xcg	2	3	4	7	8	10	12	17	19	21	24	25
	28	remy	xcg										
days past infection													
sample													

Table 1: Specification of samples in Figure 3 (remy = uninfected control, xcg = suspension OD 0.1)

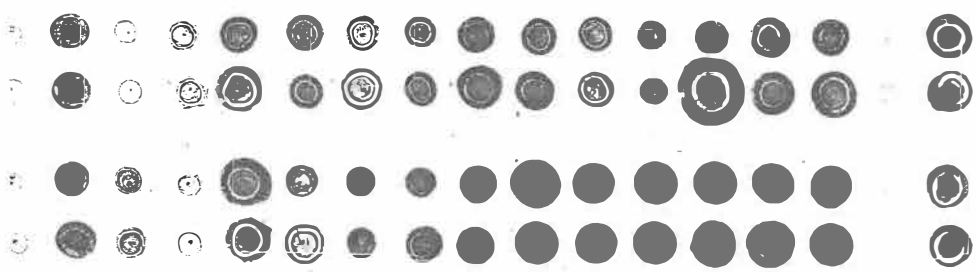


Figure 3 : Dot-Immunobinding Assay of samples extracted from artificially infected *Lolium multiflorum* plants after staining with NBT

At first a Dot Immunobinding Assay was used for the detection of Xanthomonads in plant material. For this samples were taken at different times after inoculation with Xcg. Two days past infection bacteria could be detected and after seven days an increase of antigenic material in the samples could be observed (see Fig.3 and Table 1). The dots in the first and the 16th column (label remy) are extracts from uninfected control plants, which showed no activity. For control in the column two and in the last column a bacterial suspension diluted to an OD_{630} of 0.1 was dotted.

In a second stage a Double-Antibody-Sandwich-ELISA-Procedure was developed. To ensure the observed reaction in western blots was specific several *Xanthomonas*, *Pseudomonas* and *Erwinia* strains were checked for their reaction in ELISA. For this suspension cultures of the strains were cultivated on a rotary shaker overnight, diluted to an OD_{630} of 0.1 and then incubated on the microtiter-plates. We found a strong reaction for most of the examined *Xanthomonas* strains and a little cross reaction with some *Pseudomonas* strains (see Fig. 4)

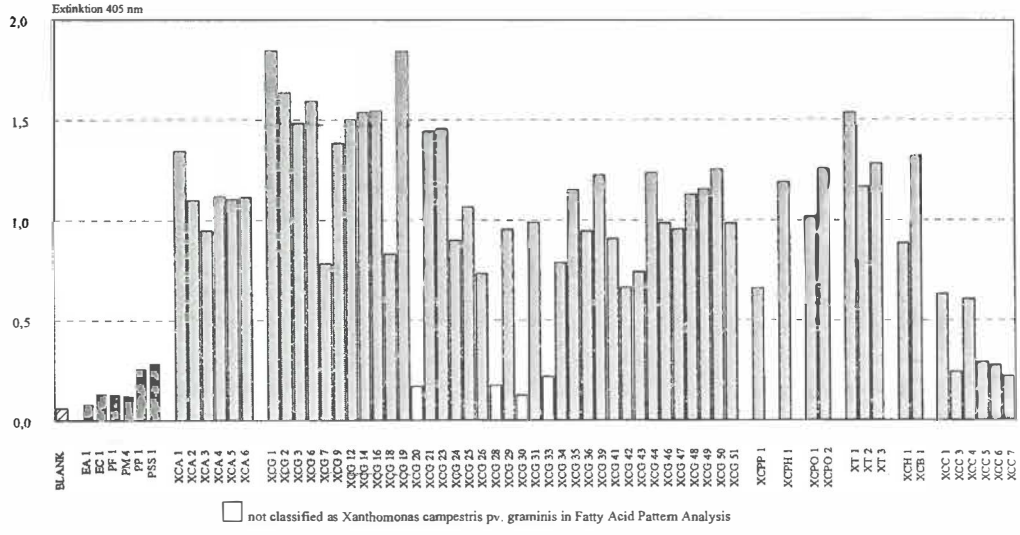


Figure 4 : Reactions of several strains in ELISA

EA = *Erwinia amylovora* EC = *Erwinia chrysanthemii*

PF = *Pseudomonas fluorescens* PP = *Pseudomonas putida*

PSS = *Pseudmonas syringae* pv. *syringae* XCB = X.c.pv. *begoniae*

XCA = *Xanthomonas campestris* pv. *arrhenateri* XCPO = X.c.pv. *poae*

XCG = X.c. pv. *graminis* XCC = X.c.pv. *campestris*

XCPP = X.c.pv. *phlei-pratense* XCPH= X.c.pv. *phlei*

XT = *Xanthomonas translucens* XCH = X.c.pv. *hyacinthi*

Numbers represent several strains of the same pathovar

In Fig.4 there are four strains of the XCG group, which gave no positive reaction in ELISA. After analysis of the fatty acid methyl ester pattern it became obvious, that these strains do not belong to the *Xanthomonas* group (HEIN, unpublished data). The strain labelled with XCG 48 is the pathovar reference strain NCPPB 2700 showing the same reaction as all other *graminis* pathovars.
After this the dose response relationship between the number of bacteria used in the test and the extinction at 405 nm was examined. Serial dilutions of a Xcg suspension were plated on petri dishes and the number of colony forming units was determined. From this the concentration of bacteria at a certain optical density was calculated. Then a serial dilution of a Xcg suspension was incubated in six replicates on a microtiter plate and after incubation with the antibodies extinction at 405 nm was determined. The results are shown in Fig.5.

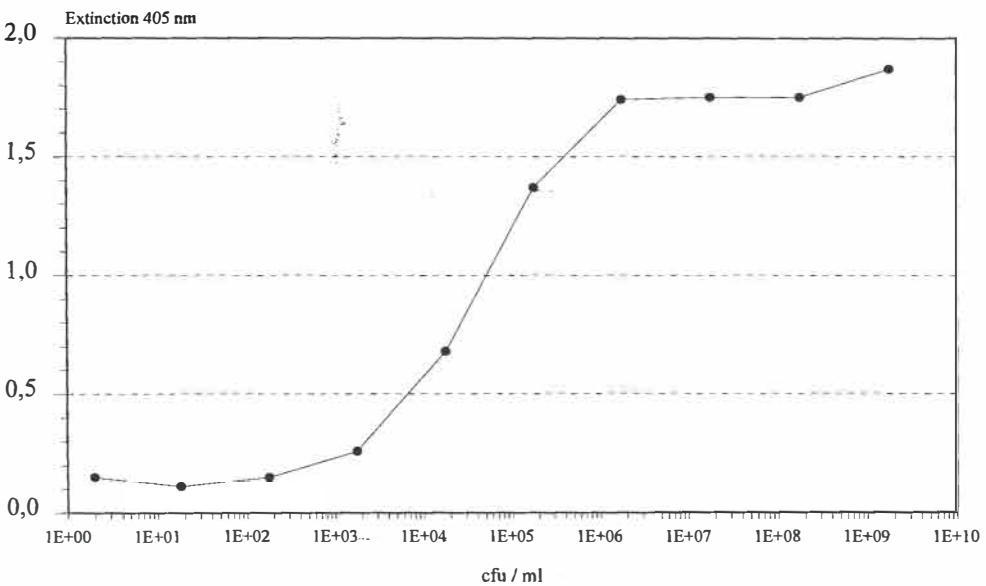


Figure 5 : Standard curve for X.c.graminis suspension cultures with different concentrations of colony forming units (cfu)

It can be seen that there is a linear relationship between 2e+3 and 2e+6 cfu / ml. Below the concentration of 2e+3 cfu/ml detection was not successful because up to this point there was no significant increase in the extinction. Above 2e+6 cfu/ml no further increase of E_{405} could be observed, probably due to the fact that the substrate was depleted or there might have been a sterical hindering of the antigens because of the large amount of bacteria applied to a single well. This has to be confirmed in further investigations.

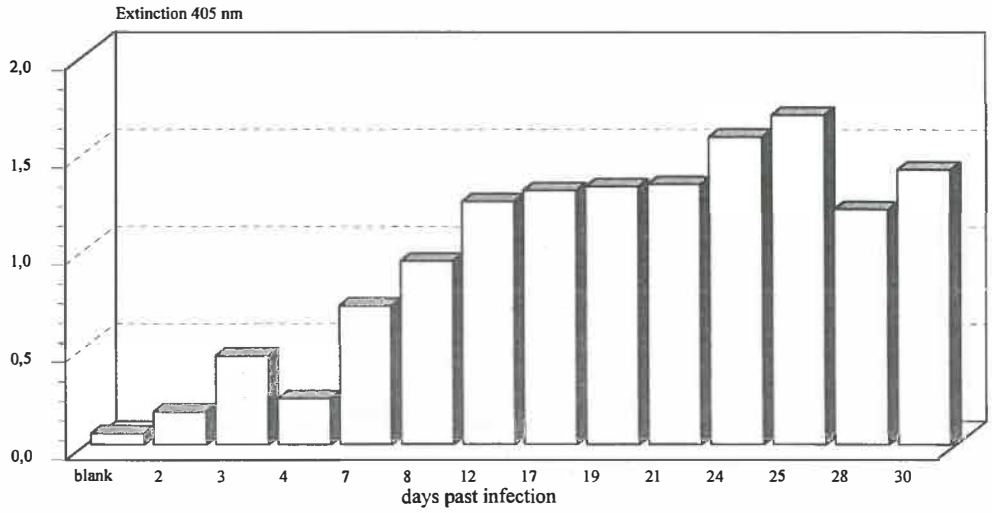


Figure 6 : Detection of *X.c.*pv. *graminis* in *Lolium multiflorum* after artificial infection

Fig.6 shows the result of a first ELISA-test with artificially infected plant material. The same samples as in the dot-immuno-binding experiment were used. Again at 7 days after infection a significant increase of antigenic material in the plants could be observed.

First symptoms like drying of leaves and chlorotic stripes could be observed on the plants two and half week past infection. Six weeks past infection nearly all of the remaining plants were scored with a 9 according to PAUL & BIRCKENSTAEDT 1989. This shows the ability of the newly developed test system, to detect *Xanthomonas* campestris pv. *graminis* in symptomless plants.

ELISA techniques are widespread and common in many research laboratories. Because of automatization in commercial ELISA systems large series with many samples can be processed at a time. Connected personal computers allow the processing of data and documentation. All these facilities are very costly and not necessary for small experiments or for detecting initial infection in a field trial.

For this reason a rapid identification method, which provides qualitative results without a large input of manpower and material, was developed, applying an available system for the detection of plant pathogens. This test system is based on Immuno®-Sticks which were filled with the reagents necessary for the test and washed with tap water. First experiments were carried out with plant material from

a field trial with Italian rye-grass, which had been inoculated with Xcg suspension in spring 1993. Samples were taken and extracted as described above.

Samples taken from an infected plot of a field trial with Italian ryegrass also showed positive reactions.Samples from the climate chamber trial, which had been analyzed with the dot-immuno-binding-assay and ELISA, have been examined with the Immuno®-Stick method and even there it was possible to detect Xcg in the plant material at 7 days past infection. Further experiments are being carried out and data will be published soon.

Summarizing it can be said, that the polyclonal antibodies raised in rabbits showed good activity on western blots, dot-blots, ELISA and Immuno®-Sticks. With the last three test systems it was possible to detect *Xanthomonas campestris* pv. *graminis* in plant material derived from either climate chamber or field trials. More importantly it was possible to detect the bacteria in symptomless plants. A new and easy-to-use test to decide whether a sample is infected with Xanthomonas was invented with the Immuno®-Stick method.

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Further Histological Investigations on the Pathogenesis of *Xanthomonas campestris* pv. *graminis* to *Lolium multiflorum* cv. Remy

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ABSTRACT

Eight week-old plants of *Lolium multiflorum* cv. Remy were inoculated with *Xanthomonas campestris* pv. *graminis* by clipping their leaves with scissors the blades of which had been dipped in a bacterial suspension. One day after inoculation bacteria could be detected in the tracheas close to the inoculation point of the leaf and at the base of the inoculated leaf. Seven days after inoculation by cutting all infected leaves contained bacteria and fibrillar material in the xylem vessels, the protoxylem lacunae at the top of the blades, and the basal portions of the sheaths. Invasion of bacteria inside the plant continued until the fifth week after infection. The oldest leaf with mature tissues shows more infected vascular bundles than the younger leaves, the sheaths of which are enclosed by the older ones. Tracheas, tracheids and protoxylem lacunae of the main vascular bundle are the most invaded tissues. The identity of *Xanthomonas c. graminis* could be verified by immunofluorescence. Healthy leaves of the same plant and leaves of the adjoining shoots can be infected via xylem vessels from an inoculated leaf.

Keywords: Bacterial wilt disease, immunofluorescence, Italian rye grass, *Lolium multiflorum*, pathogenesis, *Xanthomonas campestris* pv. *graminis*.

INTRODUCTION

Bacterial wilt of Italian ryegrass, caused by *Xanthomonas campestris* pv. *graminis* first described by Egli et al. (1975) has become an important bacterial disease in Europe (Channon et al. 1984). Schmidt & Nuesch (1984) reported that bacteria develop in the xylem vessels. This finding was confirmed in a histological study by Masuch et al. (1989).

MATERIAL AND METHODS

In a first experimental series 8 week-old plants of Italian ryegrass (*Lolium multiflorum* cv. Remy) were inoculated with *Xanthomonas campestris* pv. *graminis* by clipping their leaves with scissors the blades of which had been dipped into a bacterial suspension. In a second experiment only the blade of the first developed leaf was inoculated with *Xanthomonas c. graminis*. Eight areas of the *Lolium* plant had been chosen for detailed microscopical investigation: 4 areas of the inoculated leaf from the inoculation point of the blade to the base of the sheath, 2 areas of the subsequent uninoculated leaf, and 2 areas of the adjoining secondary shoot. Samples of the leaf blades and leaf sheaths were prepared by fixation in 2.5% glutardialdehyde in 0.2 M phosphate buffer (Sabatini et al. 1963) for 1 h followed by 1 h postfixation in 1% phosphate buffered OsO_4 (Palade 1952). The samples were dehydrated in an ethanol series and embedded in styrene methacrylate (Kushida 1961). Semi-thin sections were cut on a LKB Ultrotome III, stained with toluidine blue (Trump et al. 1961) and photographed with Leitz Orthoplan. Block samples for the study in the scanning electron microscope were trimmed and cut to get smooth surfaces. The embedding medium was extracted with different solvents, the sample was dried at the critical point afterwards followed by a sputter coating of gold. Electron microscopic studies were performed with Hitachi H 3010 scanning electron microscope. To confirm *Xanthomonas c. graminis* as a pathogen on *Lolium multiflorum*, indirect immunofluorescence labelling studies were performed using primary, polyclonal (Hein et al. 1993) and secondary (FITC anti rabbit) antibodies. Polyclonal antibodies were raised in rabbits. Fluorescence pictures were photographed using a Leica Aristoplan microscope (filter system I 3). Fluorochrome staining with acridinorange was used in a dilution of 1:20.000 in destilled water for 15 minutes. In order to reisolate bacteria present in leaf tissues petri dishes with YDC (yeast-dextrose-carbonate) agar were inoculated with extracts from vascular tissues.

RESULTS

According to the first experimental series, it is obvious that the two large xylem vessels of the median leaf blade bundle, the tracheas, are the principal sites of bacterial invasion. The chloroplast containing mesophyll cells, surrounding the vascular bundle are not primarily infected (Figure 1).

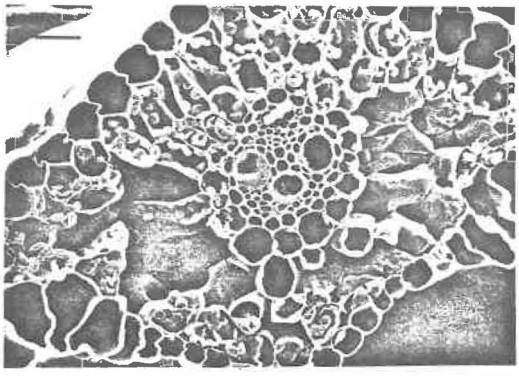

Figure 1: SEM picture of the median part of a leaf cross section of *Lolium multiflorum* infected by *Xanthomonas c. graminis*. The xylem vessels and the protxylem lacuna are occluded by *Xanthomonas* bacteria and fibrillar material. Bar = 50 μm.

Either only one of the two tracheas is colonized by bacteria, or both are invaded. In the next stage, the protoxylem lacuna can be found filled with bacteria and/or fibrous material (Figure 2).

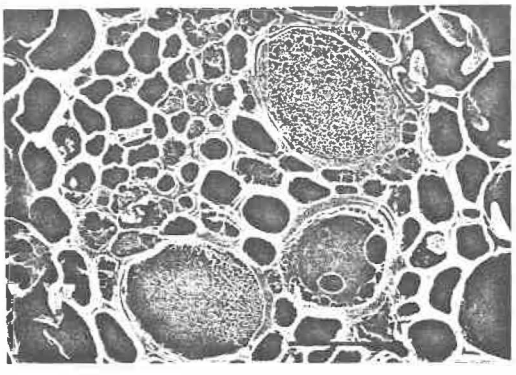

Figure 2: SEM picture of a vascular bundle of a leaf blade of *Lolium multiflorum* . The two xylem vessels and the protxylem lacuna are occluded with fibrous material derived from *Xanthomonas* bacteria. Bar = 5 μm.

Seven days after inoculation all the leaves contained bacteria and fibrillar material in the xylem vessels and the protoxylem lacunae of the leaf blades and the sheaths. Invasion of

bacteria inside the vascular bundles continued until the fifth week after inoculation. The oldest leaf with mature tissues showed more infected vascular bundles than the younger leaves the sheaths of which are enclosed by the older ones. Tracheas and protoxylem lacunae of the median vascular bundle were the most invaded tissues. Parenchyma cells adjoining the protoxylem lacunae showed disrupted cell walls. Thus, the opened parenchyma cells were invaded by bacteria, too. Inside the tracheas invaded by bacteria the longitudinal vessels were not continuously filled with bacterial cells. There were short regions occluded with bacteria and fibrillar matrix and other regions inside the vessels that were free of bacteria and fibrillar matrix. This finding indicates a discontinuous distribution of bacteria within the plant.

In a second experiment the spreading of *Xanthomonas c. graminis* in the inoculated leaf itself and the invasion into healthy leaves of the same shoot and adjoining secondary shoots of Italian ryegrass were investigated. Therefore only the blade of the first developed leaf was inoculated with *Xanthomonas c. graminis* by clipping the leaf with scissors the blades of which had been dipped into a bacterial suspension. Thus we can study successive stages of infection from the inoculation site to the leaf blade of the adjoining secondary shoot. This is possible, because the secondary vascular systems of the root and the shoot form a continuous structure. During the first four days after inoculation bacterial infection spread into this leaf blade. Two days later, the 6th day after inoculation, the sheath of the infected leaf was also colonized by bacteria, including the junction of blade and sheath (Table 1). Already 8 days after inoculation all parts of the first and the second leaf of the main shoot and all the leaves of the adjoining secondary shoot including leaf sheath and leaf blades contained bacteria in the tracheas, tracheids, and xylem parenchyma cells. These vascular tissues of the second leaf of the main shoot and those of the adjoining shoots had not been inoculated. They had probably been infected by bacteria passing the nodal plate of the transition region via xylem vessels. Ten days after inoculation the remaining tip of the blade of the inoculated leaf had died off. On the 12th day the whole leaf blade had died off. To confirm *Xanthomonas c. graminis* as pathogenic to *Lolium multiflorum*, the bacteria of the investigated tissue areas were reisolated and cultivated on YDC-agar. The yellow colour of the bacterial culture and the dome-shaped plaque indicated the presence of *Xanthomonas c. graminis*. A further diagnosis was made with polyclonal antibodies visualized by immunofluorescence. Infected vascular bundles are fluorescing. The two tracheas and the protoxylem lacuna show intense signs of infection (Figure 3). Both *Xanthomonas* bacteria and fibrillar material can thus be detected, indicating that this material occluding xylem vessels and protoxylem lacuna had been produced by bacteria. After having confirmed the presence of *Xanthomonas c.* pv. *graminis* by immunofluorescence a specific staining of bacteria was performed with acridinorange using the fluorescence microscope technique (Figure 4).

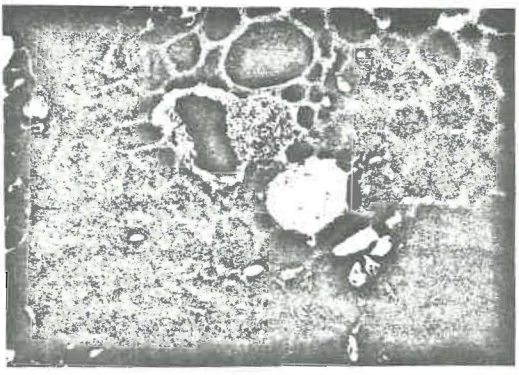

Figure 3: Immunofluorescencing picture of a vascular bundle of a leaf blade of *Lolium multiflorum*. One trachea and the protoxylem lacuna show intense signs of infection. Bar = 10 μm.

Bacteria selectively fluoresce light brown. Using acridinorange staining even single bacteria can be identified.

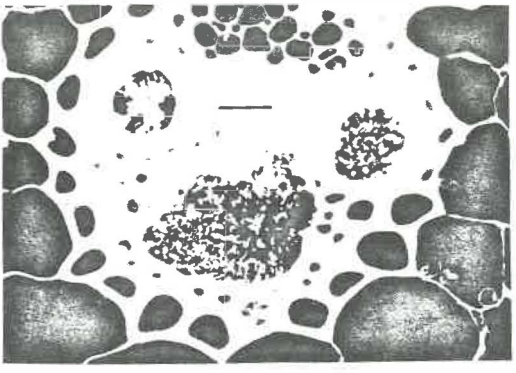

Figure 4: Fluorescence microscope picture of a vascular bundle of a leaf blade of *Lolium multiflorum*, stained with acridinorange. Single bacteria can be identified. Bar = 10 μm.

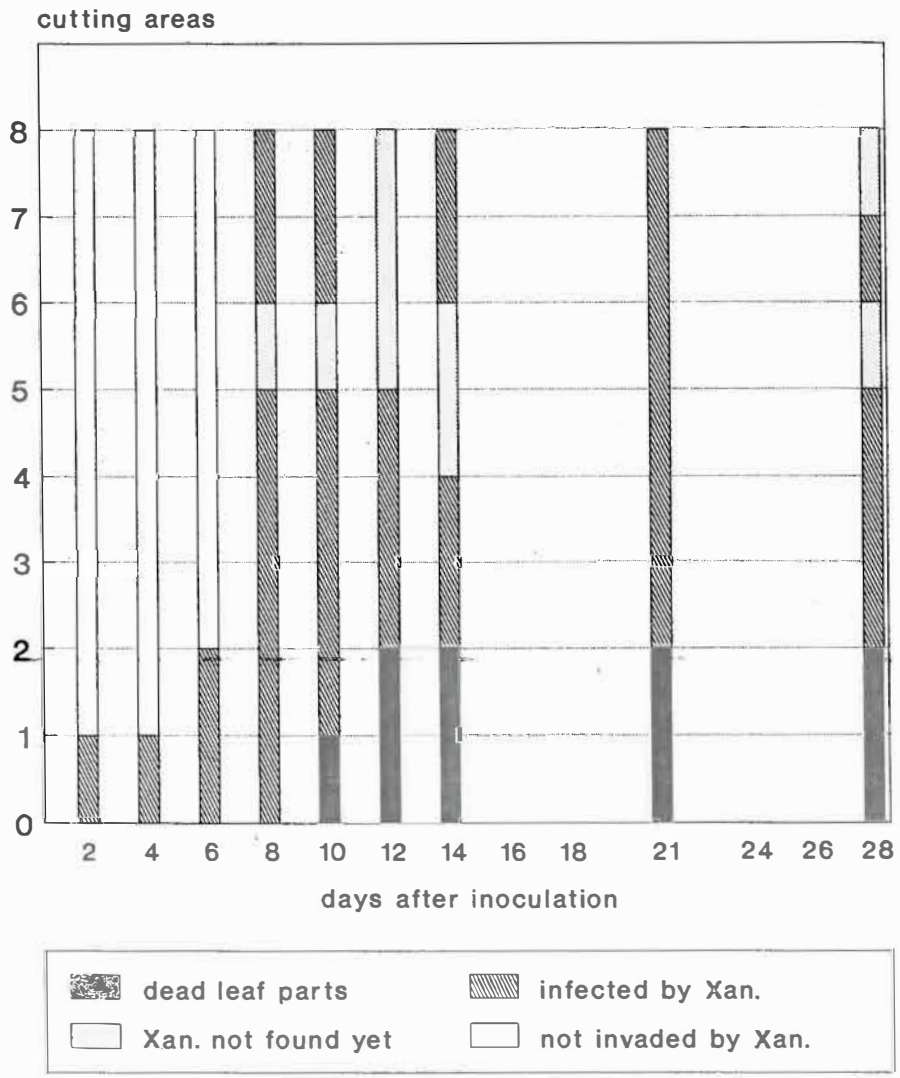

Table 1: Spread of *Xanthomonas campestris* pv. *graminis* in the leaf tissues of *Lolium multiflorum*. Eight areas of the *Lolium* plant had been chosen for microscopical investigations: 4 areas of the inoculated leaf (1 = inoculated area of the blade; 2 = junction of blade and sheath; 3 = top of the sheath; 4 = basal part of the sheath), 2 areas of the following not inoculated leaf (5 = sheath; 6 = blade) and 2 areas of the adjoining secondary shoot (7 = sheath of the first leaf; 8 = blade of the first leaf).

DISCUSSION

Infection of Italian ryegrass with *Xanthomonas campestris* pv. *graminis* could easily be induced by inoculating the leaves as reported by Leyns et al. (1988). The inoculation site in the leaf proved to be of vital importance for the development of the disease, because ten days after inoculation the tip of the blade of the inoculated leaf had died off, and 12 days after inoculation the whole leaf blade had died off. In the young plants which had been used in our experiments the macroscopical symptoms like chlorotic and necrotic stripes along the leaf axis were not developed as strongly as reported for older ones (Egli et al. 1982, Leyns et al. 1987). It can be confirmed that the bacterial wilt disease caused by *Xanthomonas c.* pv. *graminis* is mainly a vascular wilt disease or a tracheobacteriosis as described by Egli et al. (1975). Because of the method of inoculation by clipping the leaf blades with scissors, the xylem vessels or tracheas and the protoxylem lacunae have been observed as initial sites of bacterial invasion (Masuch et al. 1989). Infection of healthy leaves does not occur via contact between diseased and healthy leaves as Leyn et al. (1988) have worked out. Our results elucidate that healthy leaves of the same plant and leaves of the adjoining shoots can be invaded via xylem vessels from an infected leaf. As described by *Boyd and Avery* (1936) for Triticum the polyarch vascular cylinder of the root is connected to that of the leaves by the presense of a plate-like vascular tissue. This plate is termed the nodal plate and represents the transition region where root and shoot meet. The root vascular tissue merges into the nodal plate. Out of the upper side of this plate emerge the shoot vascular bundles. The length of the transition region is short. The transition region coincides with that of the cotyledonary traces. Bell (1976) confirmed this fact for *Lolium multiflorum*. The identity of *Xanthomonas c. graminis* causing the symptoms of this tracheobacteriosis can be verified by immunofluorescence using polyclonal antibodies.

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Taxonomic analysis of *Xanthomonas campestris* p.v. *graminis, X.c. arrhenatheri, X.c. poae* and *X.c. phlei* by means of phytopathological, phenotypic and fatty acid analysis methods

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KEY WORDS: Xanthomonas, Grasses, Infection Method, Strains, Virulence Tests, Biochemical Tests, Fatty Acid Pattern Analysis.

ABSTRACT

Bacterial wilt of grasses is caused by *Xanthomonas campestris* ssp. *Xanthomonas* Pathovars mainly pathogenic to grasses were examined. To evaluate their characteristics host range studies and virulence tests with artificially infected plants were carried out. We used two methods for artificial infection of plants. Growth and behaviour on different nutritive media and several biochemical tests were performed. Fatty acid methyl ester fingerprints were identified and compared using a gas chromatograph and the MIDI Software Package. All data were collected in a SPSS-Datafile and strains were classified using cluster analysis.

INTRODUCTION

The Laboratory for Phytopathology, Department of Agriculture in Soest, has worked with bacterial wilt of grass for many years. The pathogen is called *Xanthomonas campestris* and has the pathovars *graminis, arrhenatheri, poae* and *phlei*. It causes striped chlorosis, curling and wilting symptoms on leaves and tillers and death of the plant.

In cooperation with plant breeders a very efficient infection- and selection-method and a disease assessment scale have been developed. (Paul & Birckenstaedt 1989). For ten years the Laboratory for Phytopathology has been an official testing station of the *Bundessortenamt* for the variety trials assessing the susceptibility of grasses to bacterial wilt. A large collection of strains with numerous isolates from all important grass species has accumulated. Working with these isolates we

observed different reactions after artificial infection of plants. To examine these effects further detailed studies (Fig. 1) of virulence have been carried out. For numerous isolates host range studies were performed including all important grass species, each represented by one variety (Fig. 2).

Fig. 1: Survey of the trials and assessments

Test	Assessment
2 virulence tests	mean of disease score
42 biochemical tests	yes/no
fatty acid pattern analysis	MIDI - Microbial Identification System MIS
cluster analysis	SPSS 5.0 for Windows (average linkage between groups, pearson correlation coeffcient, z-score-transformation) (Deichsel & Trampisch 1985)

Fig. 2: List of varieties used for climate chamber trials (1987/88 and 1992/93)

Species	Abbr.	Common Name	Variety
Agrostis tenuis	AT	Colonial Bentgrass / Rotes Straußgras	Tendenz
Agrostis stolonifer	AS	Creeping Bentgrass / Weißes Straußgras	Listra
Alopecurus pratensis	AP	Meadow Foxtail / Wiesenfuchsschwanz	Alko
Arrhenaterum elatius	AE	Tall Oatgrass / Glatthafer	Odenwälder
Dactlyis glomerata	DG	Orchardgrass / Knaulgras	Iris
Festuca ovina	FO	Sheep fescue / Schafschwingel	Livinia
Festuca pratensis	FP	Meadow fescue / Wiesenschingel	Cosmos
Festuca rubra	FR	Red fescue / Rotschwingel	T. Roemer
Lolium multiflorum	LM	Italian ryegrass /Welsches Weidelgras	Remy
L.m. var. westerwoldicum	LMW	Westerwolths ryegrass / Einj.Weidelgras	Lifloria
Lolium perenne	LP	Perennial ryegrass/Deutsches Weidelgras	Morenne Gremie*
Phleum pratense	PhP	Timothy grass / Wiesenlieschgras	Rasant
Poa pratensis	PP	Kentucky Blue Grass / Wiesenrispe	Erte
Trisetum flavescens	TF	Golden Oat Grass / Goldhafer	Trisett 51

* one trial with Gremie all others with Morenne

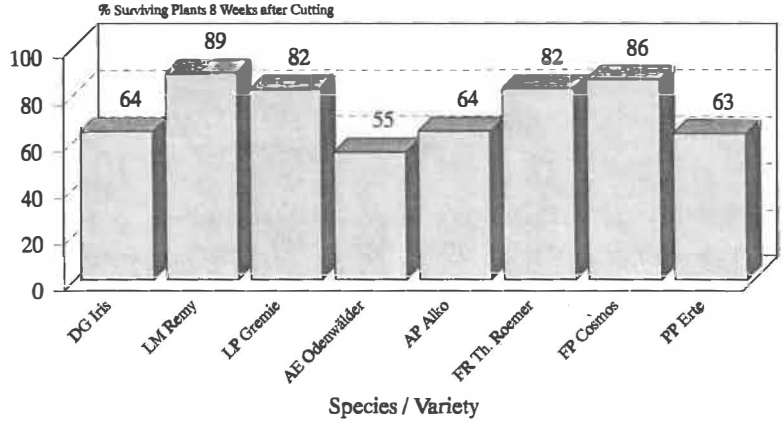
VIRULENCE TESTS

Fig. 3: Survey of the virulence tests

Trial Number	Date	Duration	Grass Species	Strains	Infection Method
1	1987/88	2 years	9	44	cutting and pricking
2	1992/93	2 years	14	27	cutting

The infection method of "cutting" (Fig. 3) was carried out with scissors, of which the cutting edge had been contaminated with a suspension of bacteria. In addition to cutting as infection method "cutting and pricking" was also used. The pricking edge was some blunt nails wrapped in a cloth soaked in bacterial suspension. Apart from the different number of species the main difference between the two trials consisted of the infection method. During the first test it became clear that the method "cutting and pricking" showed a difference between grass varieties, in their ability to regenerate. However, the method appeared to be unsuitable for testing a broad spectrum of different grass species because some showed severe damage which was not related to *Xanthomonas* infection, as demonstrated by the results with the water control (Fig. 4).

Fig. 4: Methods for artificial infection. Influence of cutting and pricking with 0,01 M $MgSO_4 * 7\ H_2O$



The loss of plants due to mechanical injury was very high, 10 to 15 % at least and up to 45% in some cases.

The incidence of injuries after application of both methods was also assessed in cloned plants (*Lolium multiflorum*, Limulta): After four weeks the disease

assessment was 4.7 with "cutting" in contrast to 7.2 with "cutting and pricking". The correlation was not significant. Therefore we can conclude that mechanical injuries following an inoculation with pricking increase the disease severity score. For this reason all further experiments with artificial infection were carried out only with cutting.

Figure 5 shows selected data from our host range studies, (mean values of scores from 31 plants). The plants have been inoculated with cutting (1992/93) and cutting and pricking (19987/88) and assessed according to Paul & Birckenstaedt (1989).

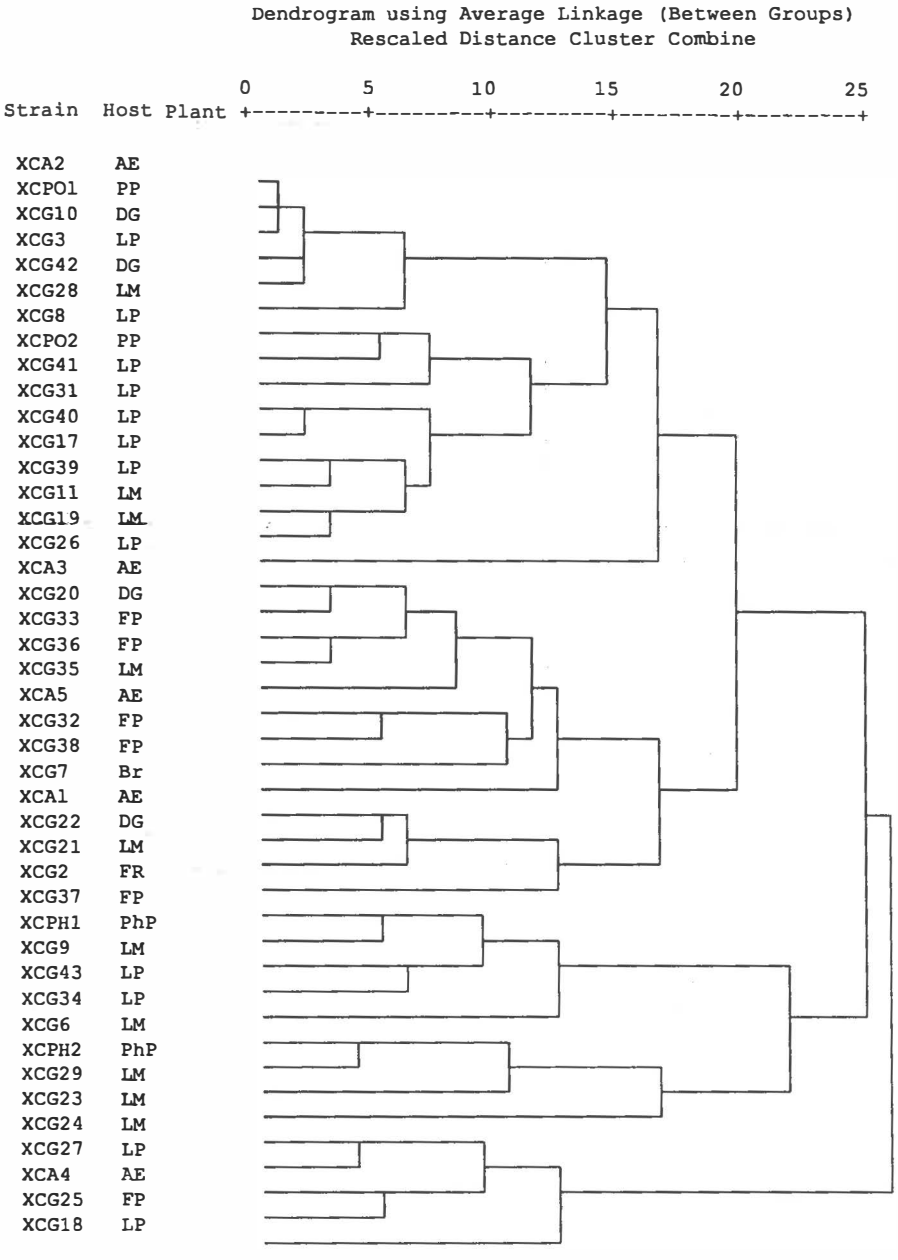
Fig. 5: Virulence of four *Xanthomonas campestris* p.v. *graminis* isolates with same origin

Year	1987/88	1987/88	1992/93	1992/93
Isolate	XCG36	XCG36	XCG 50	XCG 51
LM	8,10	5,50	7,10	8,30
LP	7,90	4,80	3,20	5,80
LMW	-	-	6,90	8,10
FP	5,40	5,70	2,40	7,30
FR	5,60	5,00	3,10	6,20
FO	-	-	2,90	6,40
PP	-	-	3,20	3,20
DG	5,00	6,70	2,80	5,10
AE	8,30	5,90	5,80	3,60
TF	6,00	5,40	3,60	5,70
AT	-	-	2,30	4,10
AS	-	-	3,10	3,00
PhP	7,70	8,20	3,70	3,70
AP	4,40	7,00	3,50	2,40
Mean	6,48	6,00	3,84	5,20
Std.Dev.	1,42	1,02	1,53	1,86
Var.Coeff.	0,22	0,18	0,36	0,40

The results show a better differentiation on the basis of the coefficient of variation between the species in the second virulence test. This is due to the omission of pricking during the infection so that the effect of the bacteria is not masked by strong mechanical damage.

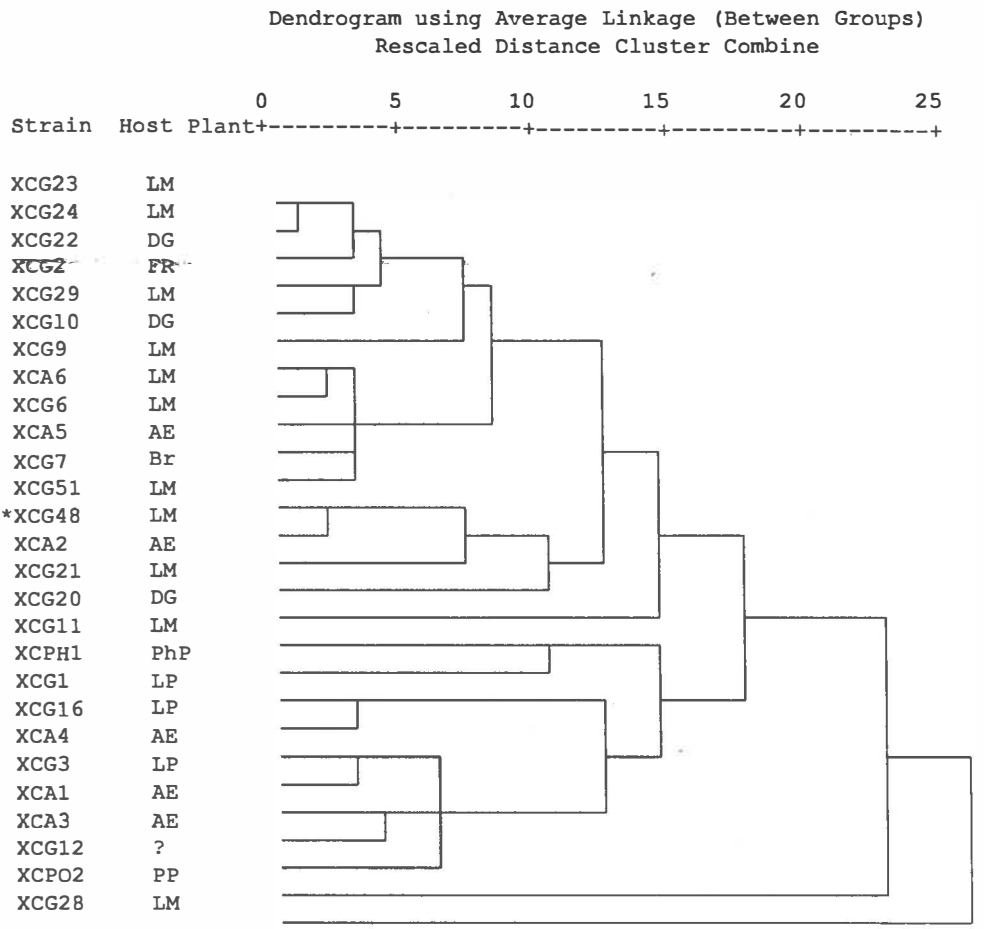
The cluster analysis of the virulence test 1987/88 resulted in the following dendrogramm (Fig. 6).

Fig. 6: Numerical analysis of disease scores after artificial infection with cutting and pricking



No grouping on the basis of the host plant from which the isolates have been isolated could be observed. Clustering of the data from the trials of the years 1992/93 resulted in slight grouping of some isolates from *Arrhenatherum elatius* (Fig. 7).

Fig. 7: Numerical analysis of disease scores after artificial infection with cutting only



* Pathovar Reference Strain NCPPB 2700

BIOCHEMICAL TESTS

38 isolates were analysed on the following media (Fig. 8).

Fig. 8: Biochemical tests and growth of *Xanthomonas campestris* on grasses

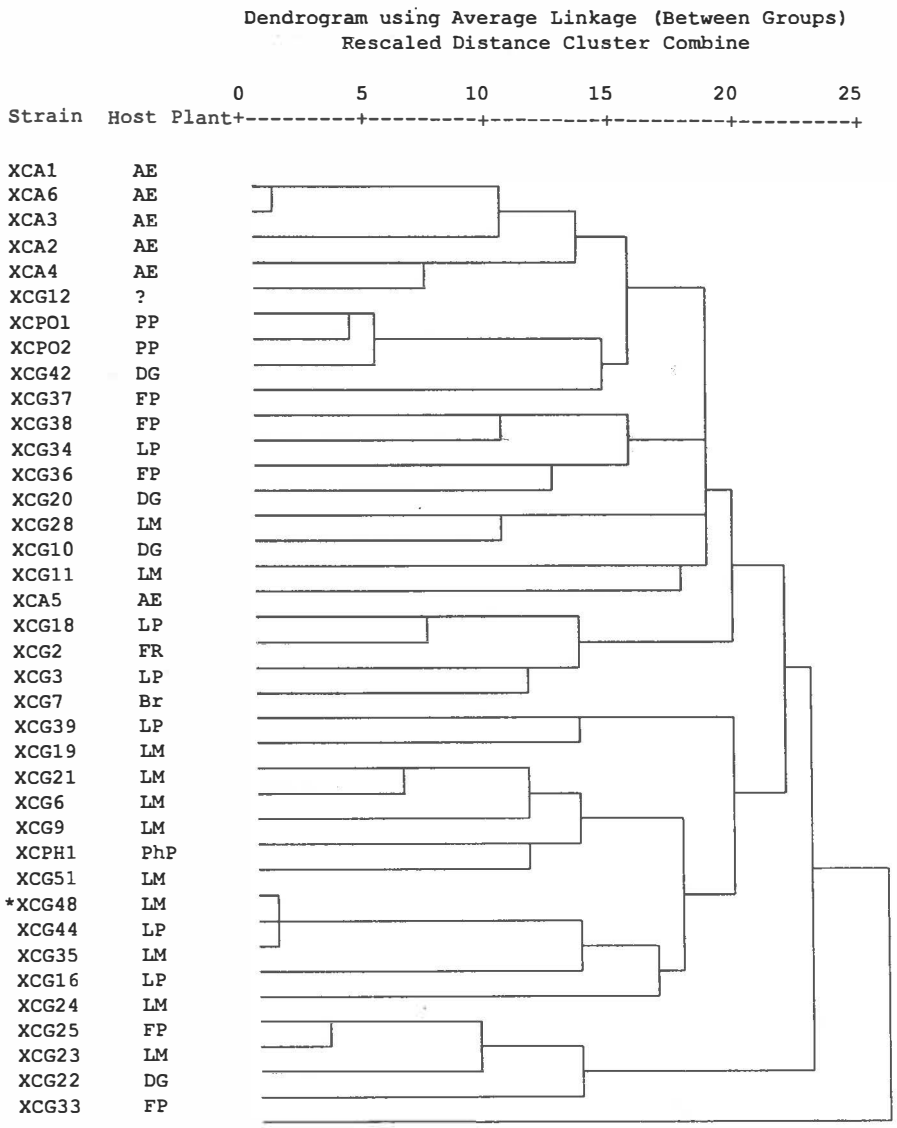
Growth on*		
Media containing		
0.1% (w/v) Cycloheximide		
0.1% (w/v) Tetracycline		
0.1% (w/v) Chloramphenicol		
0.1% (w/v) Sodium dodecyl sulfate		
0.1% (w/v) 2,3,5 triphenyltetrazolium chloride		
1,2,3,4,5% (w/v) Sodium Chloride		
L-Methionine		
L-Alanin		
SNA 5% (w/v) D(+)-Glucose-Monophydrat		
YD with 10,20,30% (w/v) D(+)-Glucose-Monophydrat		
Growth at 10°C, 15°C, pH 4.5, pH 9.5 on YD		
Growth in presence of $Co(NO_3)_2$, $CdCl_2$, $CrCl_3$, ZnO, $Ni(NO_3)_2$		
Checks for		
H_2S-Production from L-Cystein-Hydrochloride		
Esterase Activity on	1%	Tween 20
	1%	Tween 80
Lecithinaseactivity on Egg Yolk Agar		
Ureaseactivity		
Aminopeptidaseactivity		
Acid Production (liquid and solid media) from		0.1% (w/v) D(+)-Glucose
		0.1% (w/v) D(+)-Lactose
		0.1% (w/v) D(+)-Galactose
		0.1% (w/v) D(+)-Maltose
		0.1% (w/v) D(-)-Fructose
Hydrolysis of	Esculin	
	Starch	
	Gelatine	

*Tests performed at 28°C on solid or liquid media depending on the test used

Numerical anaylsis (Fig. 9) of phenotypic features such as growth and behaviour on different nutrient media again demonstrates a separat grouping of the isolates from

Arrhenatherum elatius and an additional grouping of the isolates from *Poa pratensis*.

Fig. 9: Numerical analysis of 42 phenotypic features (growth under special conditions and media)



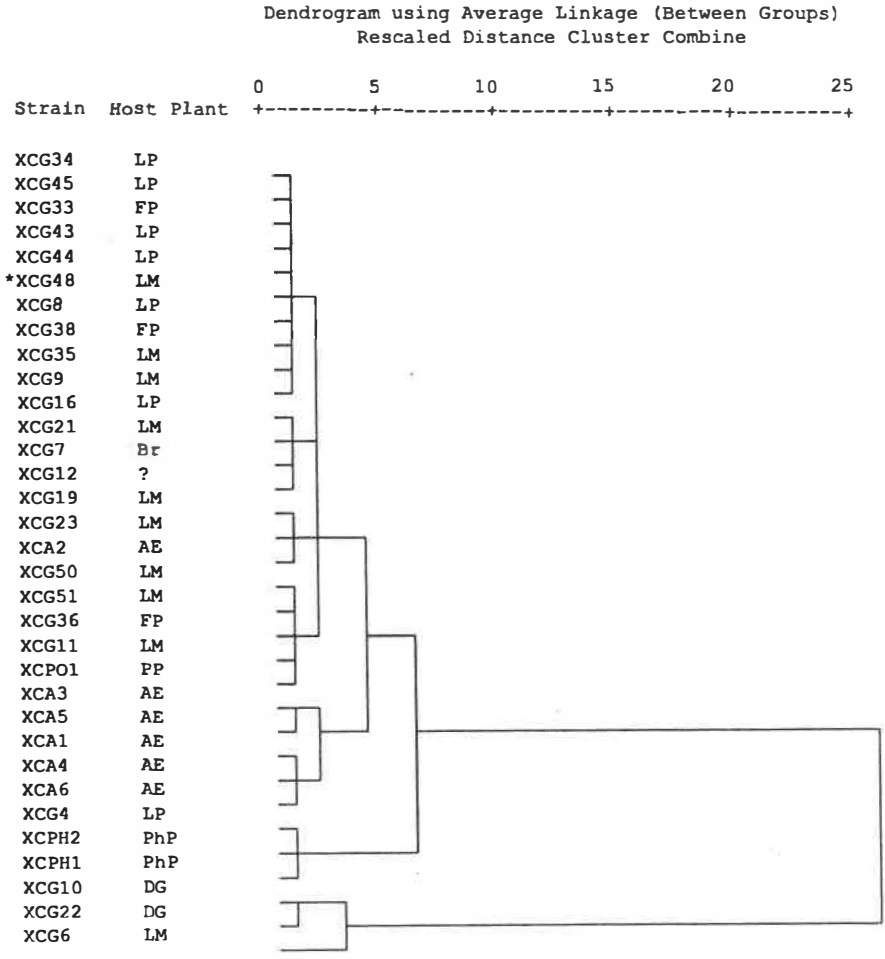
* Pathovar Reference Strain NCPPB 2700

ANALYSIS OF FATTY ACID METHYL ESTER

33 isolates were analysed for their fatty acid pattern. This analysis was made by Dr. Jaap Janse from the Department of Bacteriology Plant Protection Service in Wageningen. We wish to thank Dr. Janse for this kind support.

The isolates were extracted according to Miller & Berger (1985). The analysis was made with the Microbial Identification System. The values of the fatty acid analysis were included into the cluster analysis (Fig. 10).

Fig. 10: Numerical anaylsis of fatty methyl ester patterns



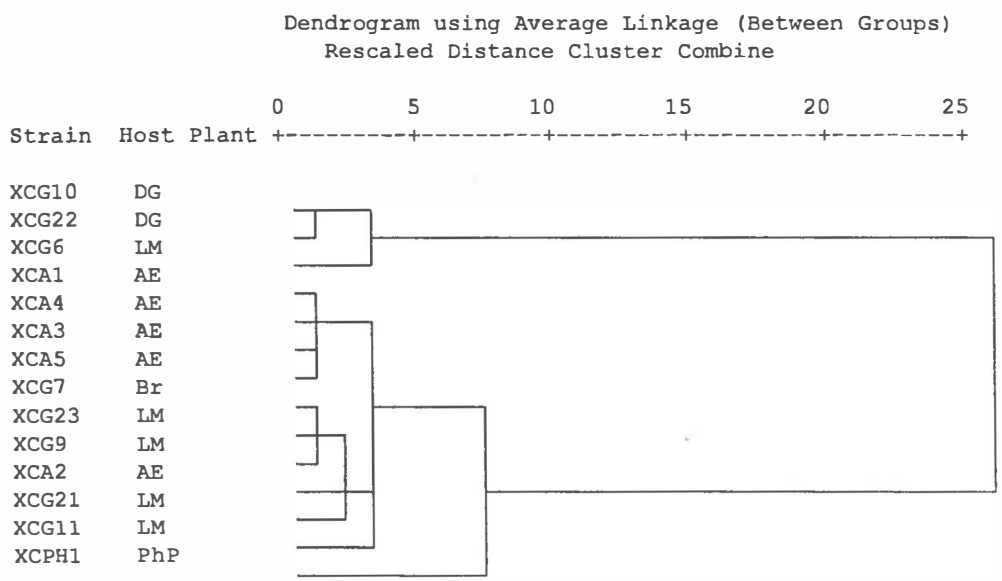
* Pathovar Reference Strain NCPPB 2700

In the resulting dendrogram a distinct grouping of the isolates could be observed. Strains isolated from *Lolium, Festuca, Bromus* and *Poa* are grouped in one cluster, from which two other clusters containing the *Arrhenatherum* and the *Phleum* isolates could be clearly distinguished.

GENERAL EVALUATION

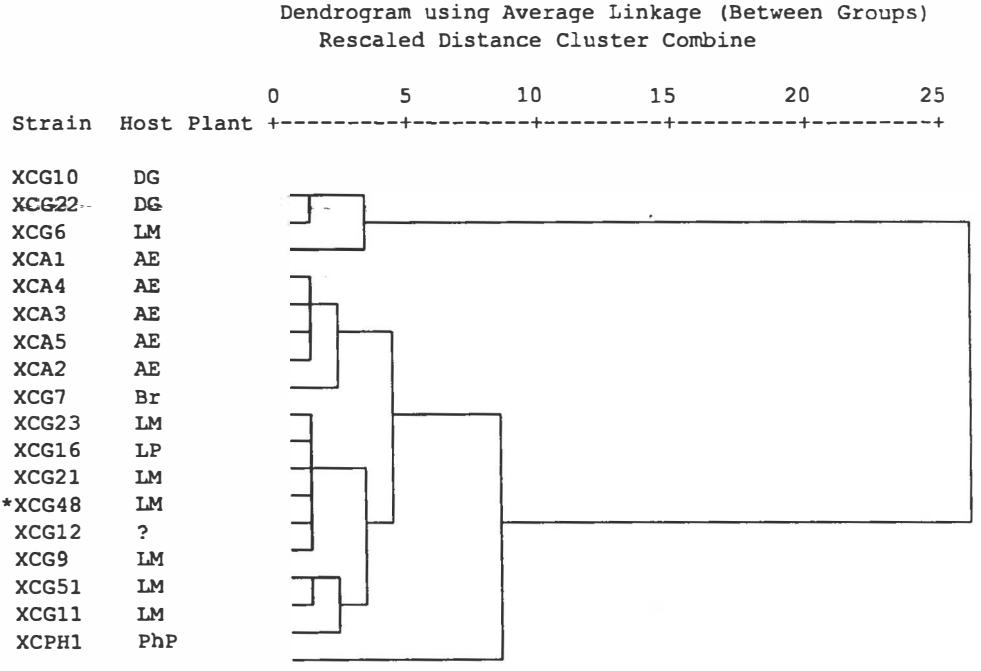
In summary two dendrograms were produced from all data collected so far. In Figure 11 all experiments (plant trials, biochemical tests and fatty acid analysis) including the trial with cutting and pricking were computed with SPSS hierarchical cluster analysis.

Fig. 11: Numerical analysis of 96 phenotypic and phytopathological features (growth and special conditions, plant trials and fatty acid patterns)



The resulting dendrogram allows a clear differentiation between *Arrhenatherum* and *Phleum* isolates on the one side and on the other side a group of isolates derived from *Lolium, Bromus, Poa* and *Dactylis,* but because of limited data this has to be . As described above the mechanical injuries due to the infection method of cutting and pricking had an influence on scores and we excluded this trial from the general analysis.

Fig. 12: Numerical analysis of phenotypic and phytpathological features excluding plant trials with cutting and pricking



* Pathovar Reference Strain NCPPB 2700

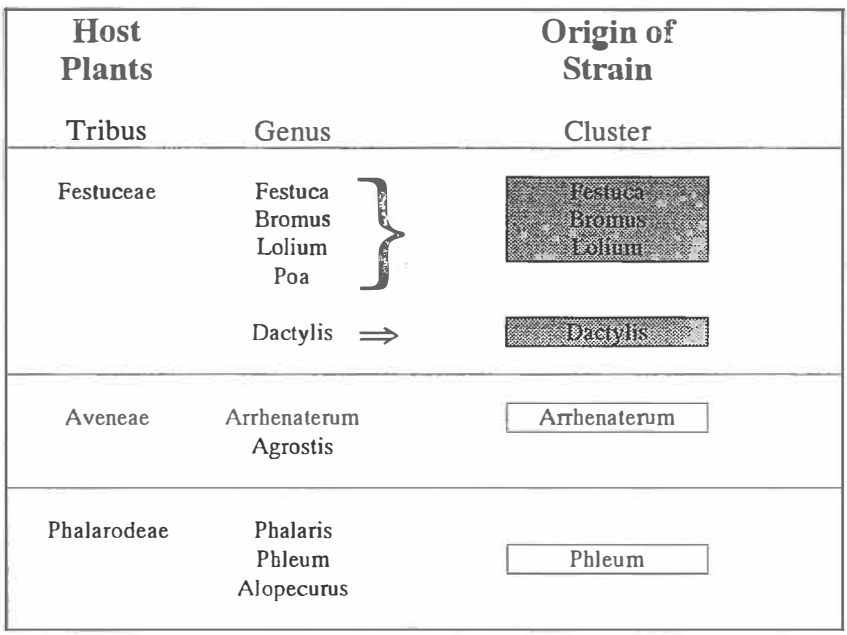
After computing the dendrogram as presented in Figure 12 we found a distinct grouping of the two *Dactylis* isolates and one *Lolium* isolate. Beside these

the group of *Lolium, Bromus* and *Poa* and the groups of *Arrhenatherum* and *Phleum* isolates can again be found.

A possible explanation of the separation of isolate groups lies in the relationship between grass species, and some evidence to support this was found (Fig. 13).

Further analysis of the taxonomical relationships between the host plants and the groups of isolates, which can be found after cluster analysis, showed the reasons for these findings.

Fig. 13: Comparison of the host plant relationship and grouping of the strain origin grouping



Egli & Schmidt (1982) named the *Xanthomonas campestris* pathovars isolated from plants belonging to the Tribus *Festuceae Xanthomonas campestris* pv. *graminis*. For the Tribus *Aveneae* the name *X. campestris* pv. *arrhenatherum* and for the Tribus *Phalarodeae* the name *X. campestris* pv. *phlei* was given. Our results correspond with these designations, but furthermore we can find a distinct grouping of the dactylis isolates. Further taxonomic investigations on bacterial wilt should consider these aspects.

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Incidence of *Fusaria* on Italian and Perennial Ryegrass (*Lolium multiflorum* Lam. and *Lolium perenne* L.)

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Keywords: *Fusaria*, occurrence, identification, toxin production, rygrass

Abstract

The presence of *Fusaria* in samples of *Lolium* cultivars, grown in 1991 at three different locations in the North Rhine region of Germany was examined by plating necrotic leaves on Potato Dextrose Agar. Plant pathogenic as well as saprophytic species of *Fusarium* could be isolated with this method. Depending on sampling date, location and cultivar, 41 to 100 % (average 81 %) of the samples were *Fusarium* positive. Differences in infestation with *Fusarium* between cultivars of *Lolium perenne* were dependent on location and did not correlate with yield. The three species of *Fusarium* pathogenic to *Lolium* spp. (*F. graminearum*, *F. culmorum* and *F. acuminatum*) accounted for 34.4 % of the isolated strains. 14 species could be isolated from *Lolium* samples. In descending frequency, these were: *F. culmorum*, *F. sambucinum*, *F. equiseti*, *F. acuminatum*, *F. semitectum*, *F. oxysporum*, *F. subglutinans*, *F. avenaceum*, *F. sporotrichioides*, *F. proliferatum*, *F. tricinctum*, *F. anthophilum*, *F. dimerum* and *F. graminearum*.

Introduction

Some *Fusaria* are phytopathogenic and most *Fusaria* produce mycotoxins. The influence of *Fusaria* on the yield of forage grasses, especially *Lolium*-cultivars is difficult to estimate and probably not very significant in most cases. Most *Fusarium*-species are able to produce mycotoxins in the field, and in the period until harvest they have enough time to grow and produce secondary metabolites. Recording of the incidence of *Fusaria* in grass is thus important to assess risks to animal health potential and food contamination.

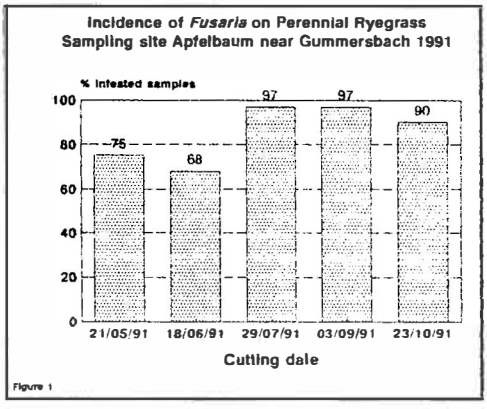
Material and methods

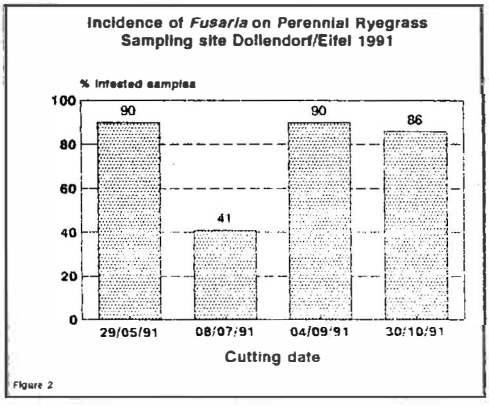
In 1991, 832 samples were taken from all plots of two trials with 17 cultivars of *Lolium perenne* (Perennial ryegrass) and one trial with 11 cultivars of *Lolium multiflorum* (Italian ryegrass) at every sampling date. The former trials were located in Apfelbaum near Gummersbach and Dollendorf/Eifel, the latter in Horbach near Aachen (all sites in the Federal County of North Rhine Westfalia). 2486 strains of *Fusarium* were isolated by

plating 5 necrotic leaves (not surface sterilised) out of every sample on Potato Dextrose Agar. Plant pathogenic as well as saprophytic species of *Fusarium* could be isolated with this method. Suspicious outgrowing fungi were isolated, single spore cultures were made, and pure cultures were identified to genus level, as far as possible. 250 randomly selected strains previously identified as *Fusarium* were identified to species level according to Nelson et al. (1984), in combination with Nierenberg (1989).

Results and Discussion

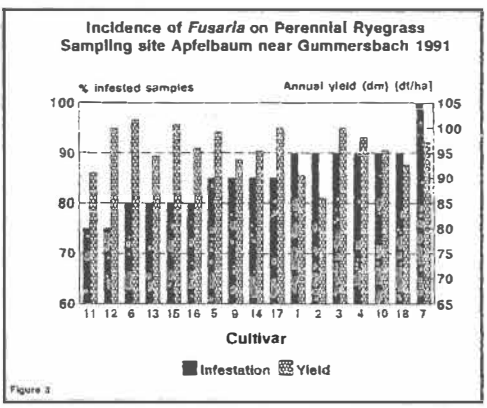
Depending on sampling date, location and cultivar, 41 to 100 % (average 81 %) of the samples were *Fusarium* positive.

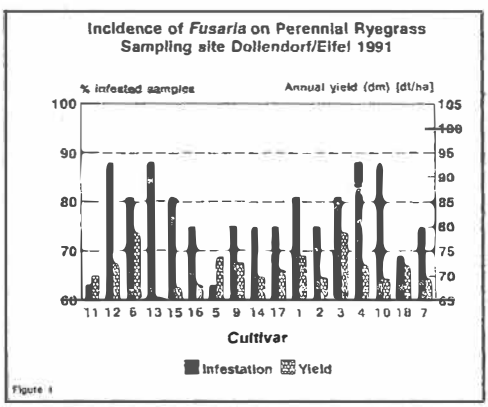




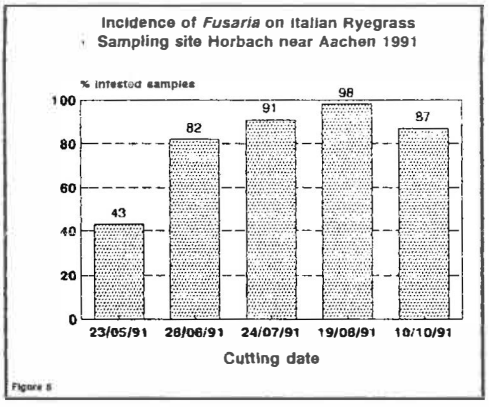
Figures 1 and 2 show the incidence of Fusaria for all sampling dates of the two *Lolium perenne* trials. The sampling sites differ mostly by precipitation. There is no clear seasonal pattern in the incidence of *Fusaria*, which seems to be more dependent on the

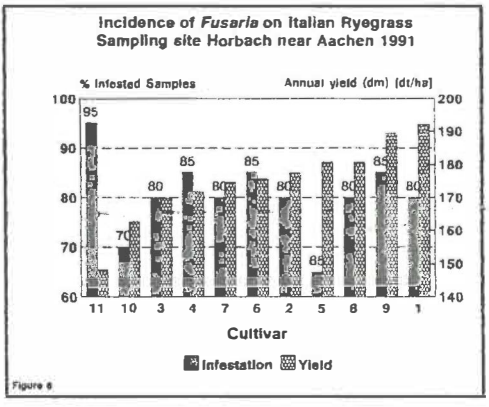
age of the cut leaves. The site with the lower precipitation per year (Dollendorf/Eifel) had as a tendency to a lower incidence of fusion.





Figures 3 and 4 show the variation of the incidence of *Fusaria* by cultivar and site. Cultivars are sorted according to the infestation with Fusaria in Apfelbaum. At both sites, there is no correlation between infestation and annual yield. The ranking of cultivars by infestation also differs significantly between sites. These results may be attributed to the lack of differentiation between phytopathogenic and saprophytic *Fusarium* species with the isolation method used.





Figures 5 and 6 show the results of the incidence of *Fusaria* for *Lolium multiflorum*. In this trial, the incidence of *Fusaria* shows a seasonal fluctuation which has to be confirmed. Infestation and yield are not correlated.

Section	Species	%	Zea.	Tri. A	Tri. B	Mon.	Other toxins
Eupionnotes	*F. dimerum* PENZIG	0.8					
Sporotrichiella	*F. tricinctum* (CORDA) SACC.	1.6					x
"	*F. sporotrichioides* SHERB.	4.8	x	x			x
Roseum	*F. avenaceum* (FR.) SACC.	4.8				x	x
Arthrosporiella	*F. semitectum* BERK. & RAV.	6.4	x			x	
Gibbosum	*F. equiseti* (CORDA) SACC. sensu Gordon	**8.0**[2]	x	x			
"	***F. acuminatum* ELL. & EV. sensu Gordon**[1]	6.4		x		x	x
Discolor	*F. sambucinum* FUCKEL	**25.6**[2]		x			x
"	***F. graminearum* SCHWABE**[1]	0.8	x		x		x
"	***F. culmorum* (W. G. SMITH) SACC.**[1]	**27.2**[2]	x		x		x
Liseola	*F. proliferatum* (MATSUSHIMA) NIERENBERG	1.6				x	
"	*F. subglutinans* (WOLLENW. & REINKING) NELSON, TOUSSOUN & MARASAS	5.6				x	
"	*F. anthophilum* (A. BRAUN) WOLLENW.	0.8				x	
Elegans	*F. oxysporum* SCHLECHT. emend. SNYD. & HANS.	5.6				x	x

Table 1: Incidence of *Fusaria* in *Lolium*-samples and theoretical ability to produce toxins (according to Marasas et al. (1984) and Thrane (1989)).

%	=	Percentage of isolates from *Lolium*-samples
Zea.	=	Zearalenone and derivatives
Tri. A	=	Trichothecenes type A (e.g. T-2 toxin, Diacetoxyscirpenol)
Tri. B	=	Trichothecenes type B (e.g. Deoxynivalenol, Nivalenol)
Mon.	=	Moniliformin
other toxins	=	Fusarin C, Butenolide, Fusaric acid or Fumonisine
1	=	Phytotoxic to *Lolium*
2	=	Highest incidences

Table 1 shows the results of the identification of the 250 *Fusarium* strains. The three species of *Fusarium* pathogenic to *Lolium* spp. (*F. graminearum*, *F. culmorum* and *F. acuminatum*) accounted for 34.4 % of the isolated strains. The three most frequent species (F. culmorum, F. sambucinum and F. equiseti) accounted for 60.8 % of the isolated strains. 5 of the 14 isolated species are able to produce Zearalenone, 6 are able to produce Trichothecenes and 13 of the 14 isolated species are reported to be toxigenic. These results show the relevance of *Fusaria* as potential health risk for animals fed with infected grass.

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Tokyo.

Study on the occurrence of leaf spot fungi in seed production of timothy (*Phleum pratense*)

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ABSTRACT

During 1989 - 1991 leaf samples were collected from 20 seed crops of timothy. The samples (flag leaf F, F-1, F-2) were analysed for leaf disease fungi using an agar plate test. *Cladosporium phlei* and *Drechslera phlei* were found in all locations.

A more detailed study in nine of the fields showed that the percentage of leaves attacked by a fungus generally increased with plant age. Among the true leaf spot fungi, *Cladosporium phlei* and *Drechslera phlei* occurred most frequently. However, *Cladosporium phlei* spread faster in the fields than the Drechslera species. At the flowering stage, with the exception of two fields, more than 90 % of the leaves were infected by the Cladosporium fungus, with Drechslera such a high percentage of infected leaves was found only at the dough stage. Other pathogenic fungi e.g. species of Ascochyta, Fusarium, Leptosphaeria, Septoria occurred in all fields but much less frequently.

Keywords: *Cladosporium phlei*, *Drechslera phlei*, leaf spot fungi, *Phleum pratense*, seed production, spread of disease.

INTRODUCTION

During the work at the Federal Biological Research Centre on the occurrence and importance of fungal pathogens of grasses some research was carried out in seed production. Among fungal pathogens, the leaf spot fungi e.g. Drechslera spp., Mastigosporium spp., Rhynchosporium spp. can cause considerable damage to grass species (O'Rourke 1976, Teuteberg 1983). This paper reports some investigations on the occurrence of leaf spot fungi in timothy (*Phleum pratense* L.) during 1989 - 1991. In particular a more detailed study in 1991 including nine seed growing fields is discussed. Most of the results of this detailed study are part of a master thesis of Mrs. Barbara Fischer (Technical University Braunschweig, unpublished).

METHODS

The leaf samples were collected in 1989 - 1991 from 20 locations in the eastern part of Niedersachsen (Lower Saxonia, Germany). At different growth stages (beginning and end of heading, flowering, dough stage) 10 5-culm samples were taken on a diagonal line through the fields at a distance of about 10 m each. In most cases the three upper leaves (flag leaf F, F-1, F-2) of the culms were stored in a freezer. For investigation pieces of 1 cm length of the surface sterilized leaves (sodium hypochlorite, available chlorine 1,5 %) from the tip, base and the upper and lower part of the leaf blade were incubated on SNA-agar, a low-nutrient medium (Nirenberg 1976) at a temperature of 16 - 19° C. The plastic Petri plates were irridiated with near ultraviolet light (black light) for 12 hours per day. From each leaf stage sample 50 leaves in 50 plates (4 pieces/plate) were prepared. Generally after 14 days the outgrowing fungi were identified under a microscope, in addition microscopic preparations were made from the fungi in lactophenol blue solution for measuring conidia. Isolations of fungi were made on SNA- and malt peptone-agar to verify its identification.

A leaf was recorded as infected or colonized by a fungus, if the fungus was detected on one of the four sections of the plate. The experiments investigated only the occurrence of a fungus on leaves but not the extent of colonization. Percentage infection values are based on 150 leaves/sample in 1991 (more than 3800 leaves investigated) and on 50 leaves in 1989 - 90. As a result of the observations in the preceeding years and for technical reasons in 1991 samples were taken at beginning and end of heading only from three and six fields respectively.

Leaf damage (leaf spots and tissue browning) were rated by a simplified rating scale from 1 (no damage) to 5 (more than 75 % leaf damaged).

RESULTS

In 1989 and 1990 the investigation of leaf samples from 11 locations showed a frequent occurrence of the leaf spot fungi *Cladosporium phlei* (C.T. Gregory) de Vries (syn.: *Heterosporium phlei* C.T. Gregory) and *Drechslera phlei* (Graham) Shoem. in all fields. In 1989 on almost every leaf of each sample at milkripe/dough stage the Cladosporium species could be detected, 94 to 100 % of the leaves were infected. *Drechslera phlei* was also occurring in all fields but less frequently (range 16 to 76 %). Results obtained in 1990 were similar.

In 1991, with a more detailed study at nine locations, the results showed again that both fungi were prevailent. However, a different rate of spread of the two fungi was observed: *Cladosporium phlei* spread faster in the fields than the Drechslera species. While at the end of heading the percentage of leaves infected by *Cladosporium phlei* varied from 69 to 91 % in the individual fields, those infected by Drechslera were found

less abundantly (range 7 to 31 %). At the flowering stage, with the exception of two fields, more than 90 % of the leaves were infected by the Cladosporium fungus, the corresponding data for Drechslera ranged between 15 and 81 %. Only at the dough stage Drechslera reached the level of Cladosporium. This tendency was clearly established in all the fields tested. An overall view of the average values of all the samples investigated shows the different degree of spread of the two pathogens in the upper leaf region of the seed plants and increasing leaf damage with ripening of the crop (Fig. 1). Age of plant stand (years since sowing) did not have any effect on the incidence and relative importance of pathogens (Fig. 2). Even in the sixth harvest year at flowering time *Drechslera phlei* was found to a lesser extent than *Cladosporium phlei*.

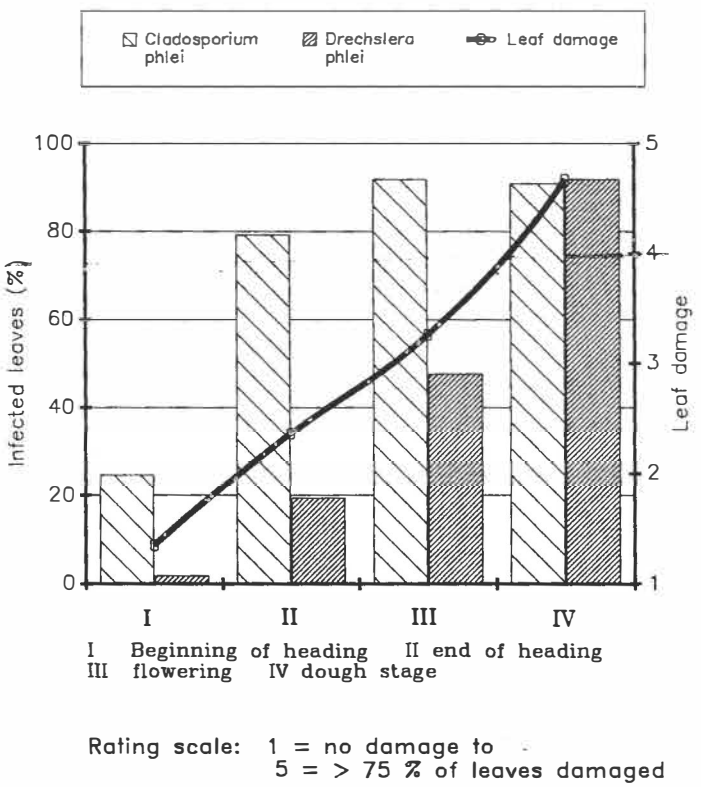

Fig. 1: Percentage of leaves infected by *Cladosporium phlei* and/or *Drechslera phlei* at different growth stages of the plants in relation to the rating values for leaf damage, average values of locations tested in 1991

Besides the two main pathogens, other fungi occurred and altogether 28 taxa (genera or species) were determined. Among these fungi, several genera pathogenic to grasses were found (Table 1). The data show that with the exception of Ascochyta sp. (not yet finally determined) the percentage of infected leaves reached a maximum level at dough stage. Of the genus Drechslera in addition to *Drechslera phlei* the species *D. biseptata* (Sacc. et Roum.) Richardson et Fraser, *D. siccans* (Drechsl.) Shoem. and *D. sorokiniana* (Sacc.) Subram. et Jain were found but rarely only at dough stage. Five Fusarium species were determined: *F. avenaceum* (Fr.:Fr) Sacc., *F. culmorum* (W. G. Sm.) Sacc., *F. merismoides* Corda, *F. poae* (Peck) Wollenw. and *F. sporotrichioides* Sherb.

Naturally high percentages of several other fungi often widespread on weakened or dead leaves were found particularly at dough stage e.g. species of Alternaria, Acremonium, Cladosporium, Epicoccum, Torula.

Table 1: Percentage of infected leaves by different genera of pathogens at flowering and dough stage in 1991

Genera	Flowering	Dough stage
Ascochyta	13,5	4,7
Drechslera *	0	0,9
Fusarium	7,9	16,9
Leptosphaeria	14,8	58,9
Phoma	2,5	3,6
Septoria	20,5	32,7
Stemphylium	10,8	23,4

* excluding *D. phlei*
Average values of 8 (flowering) and 9 (dough stage) locations

DISCUSSION

In all of the 20 investigated seed crops of timothy the leaf spot fungi *Cladosporium phlei* and *Drechslera phlei* were detected most frequently. This result confirms earlier observations obtained by the examination of leaf samples from trial fields, seed plots and turf in West Germany (Teuteberg 1975). Burhenne et al (1990) also report on the common occurrence of the two pathogens in our region. Both fungi have been known to occur in other countries for many years (Teuteberg 1975, O'Rourke 1976). The observed increase of the percentages of these fungi with age of the plants is known generally for many pathogens and saprophytes and this is also true for grasses (Mäkelä 1972). Both fungi are

spread by wind and rain. Factors affecting spread of both fungi in the fields are for example the rate of sporulation, liberation of spores and spore size (Ingold 1978).

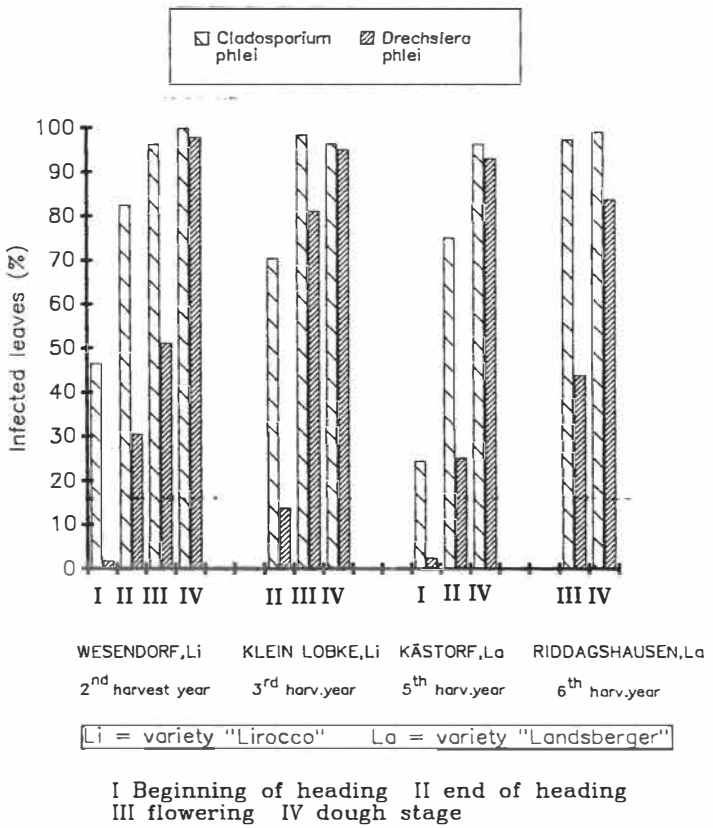

Fig. 2: Percentage of leaves infected by *Cladosporium phlei* and/or *Drechslera phlei* at different growth stages of the plants in 1991, four locations, seed fields several years in use

A considerable loss of yield is expected to occur only if the upper leaves of the culms are infected at an early stage of growth. In our investigation at the flowering time in particular *Cladosporium phlei* was already widespread on the upper leaves. However, the Drechslera species was detectable in some of the fields at a high level as well. All the other leaf spot fungi were found to occur much less frequently. To investigate the

importance of the two pathogens further experiments on the loss of seed should be conducted.

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IMPORTANT DISEASES OF TURFGRASSES IN FRANCE

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ABSTRACT

From survey data collected over the last 15 years, the main fungal diseases of turfgrasses were ranked in the following order of importance :

1) Red thread disease (*Laetisaria fuciformis*) and pink patch disease (*Limonomyces roseipellis*).

2) Rusts (*Puccinia coronata, P. poarum, P. striiformis, P. graminis*).

3) Snow mould (*Microdochium nivale*).

4) Helminthosporium leaf spot diseases (*Drechslera* spp., *Bipolaris* spp., *Curvularia* spp.).

5) Fairy rings (*Marasmius oreades, Lycoperdon* spp., *Agaricus* spp. and other basidiomycetes)

In addition, some pathogens (*Gaeumannomyces graminis, Rhizoctonia* spp., *Pythium* spp.) were particularly damaging to *Agrostis* spp. in golf course greens.

Keywords : France, fungal diseases, surveys, turfgrasses, amenity grasses.

INTRODUCTION

In 1993, the amenity grass area in France was ca 7000 km^2 including domestic lawns, soccer and rugby fields, race tracks, motorways verges, communal gardens, ski runs, and golf courses.

Turfgrass seed sold in 1992 reached 17,000 t valued at 310 m F.fr ($55m). This market has increased 80 % since 1981. Although national seed production has increased annually, it still meets only 25 % of the demand.

Our objective was to identify the important lawn diseases in France to allow plant breeders, seed producers and merchants to produce and supply the most appropriate cultivars for particular uses.

METHODS

Surveys

During the last 15 years, 11 surveys were made throughout France, except Corsica, with the effort concentrated around the cities of Paris and Poitiers. We observed soccer fields, race tracks, amenity turfgrasses in gardens of the cities of Lille, Arras, Paris, Versailles, Rennes, Angers, Poitiers, Bordeaux and Nîmes and we also surveyed motorway verges between these towns. Diseased plant samples were diagnosed and isolates of fungi were made in the laboratory.

In addition, samples of diseased grasses from golf courses around Paris were assessed by one of us (MC).

For 21 diseases, the percent occurence out of 975 observed diseased fields was calculated.

Interpretation

The relative importance of the identified fungal diseases was estimated by taking into account disease prevalence and severity and their potential for spread based on the biology of the pathogen, the type of soil and the management (cuts, fertilizers etc.) of the grass.

RESULTS

The fungal diseases were ranked in the following order of importance :

1) Red thread and pink patch (*Laetisaria fuciformis* and *Limonomyces roseipellis*).

These diseases were detected in 40 % of the surveyed locations. The disease intensity increased with factors such as the number of cuts (Bahuon, 1986), the presence of sand in soil and the lack of nitrogen.

2) The rusts (*Puccinia coronata, P. poarum, P. striiformis, P. graminis, P. brachypodii* var. *nemoralis*).

Rust diseases were present at 19 % of the sites but were considered less important as cutting removes and destroys infected tissues in many managed turfgrass situations.

3) Snow mold (*Microdochium nivale*)

This disease is particularly important on *Agrostis* spp. on golf courses. It was identified in 12 % of our survey sites. Because of the high cost of seed and golf course management and the consequent economic importance of the disease, we have ranked it as third in importance.

4) "*Helminthosporium*" diseases (*Drechslera* spp., *Bipolaris* spp., *Curvularia* spp).

These leaf spotting fungi were diagnosed in 15 % of the samples. *Drechslera poae* was harmful to *Poa pratensis*, but this grass species is little used for lawns in France. In *Lolium perenne* and *Festuca* spp., *Drechslera dictyoides* was often prevalent, but in well managed turfgrasses, the damage was low. Sometimes *D. poae* and *D. dictyoides* induced root and crown rots.

5) Fairy rings

These occured at 9 % of survey sites. The most prevalent pathogen was *Marasmius oreades*. Its damage was significant and control is difficult, although an effective method has been developed by Chatard (see Courtillot, 1990) which is based on soil injection with the systemic fungicide oxycarboxin. This method is used in Europe in USA military cemeteries

6) *Gaeumannomyces graminis, Rhizoctonia cerealis, R. solani, R. zeae, Pythium* spp.

These fungi were only scored in 5 % of observations, although in samples of *Agrostis* in golf course greens, approximately 35 % were diseased.
Other diseases, although detected, were not sufficiently prevalent to be included in the list of major diseases. Those that deserve mention include :
a) *Sclerotinia homeocarpa* : This was mostly observed in the summer time in *Agrostis* spp. from diseased golf courses greens. The current practice of chemical control has been effective in reducing the disease and it appears to be less prevalent than 20 years ago.
b) *Fusarium culmorum* : This was often isolated in the laboratory from decaying plants after the infection by another fungus or after other stresses.
c) *Colletotrichum graminicola* : This pathogen was prevalent in late autumn in *Poa annua* but damage was slight.
d) *Athelia rolfsii* : This pathogen was damaging to golf courses in south-west France.

DISCUSSION

A list of the most important pathogenic fungi on turfgrasses in France has been compiled based on survey date of disease incidence and on potential contributing factors of environment and management. The most important were *Laetisaria fuciformis* and *Limonomyces roseipellis* responsible for the red thread/pink patch disease complex. Bahuon (1986), Courtillot (1990), Smiley (1980), Smith (1980), Smith et al. (1989) confirm this. The spread of these fungi is increased by environmental factors including sandy soil, cut frequency, and surface wetness. The common grasses *Festuca rubra, F. ovina, Lolium perenne, F. arundinacea* and *Poa pratensis* were susceptible to *Laetisaria fuciformis* and to *Limonomyces roseipellis*. In our opinion these fungi could be responsible for the most important disease of lawns in the temperate zones.

In colder countries, *Microdochium nivale* is important (Winter, 1986) and in France it causes damage on golf course greens.

Gaeumannomyces graminis was only damaging on *Agrostis* spp. in golf course greens and this result is in accordance with the observations of Lucas et al. (1992).

Some pathogenic fungi (*Rhizoctonia* spp., *Fusarium culmorum*, *Athelia rolfsii*) are increasing in golf course greens. Reasons for this may include pH variation due to application of sulphur or lime fertilizer, especially on widely occuring sandy soils. In addition, erratic water supply may also have an effect (Courtillot, 1991).

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First results on detection of Mastigosporium on Dactylis

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Keywords: Mastigosporium species, cocksfoot , identification, screening

Mastigosporium spp. are widespread fungi on cocksfoot (Dactylis spp.) causing leaf spotting and yellowing.

First reports of the disease were given by Schneider and Meyer (1963), were described a striking disease on cocksfoot in Germany. Two years later, Buhl and Lange reported on eyespots on cocksfoot leaves in monocultures which infection resulted in high yield losses. In 1988 Huss et al. detected Mastigosporium muticum in meadows in Austria, where yield reduction reached 40 %.

A collection of Dactylis genotypes from different German plant breeding stations was analysd for occurrence of leaf spots that are typical of infections with fungi of the genus Mastigosporium. A number of isolates was selected from following breeding stations:

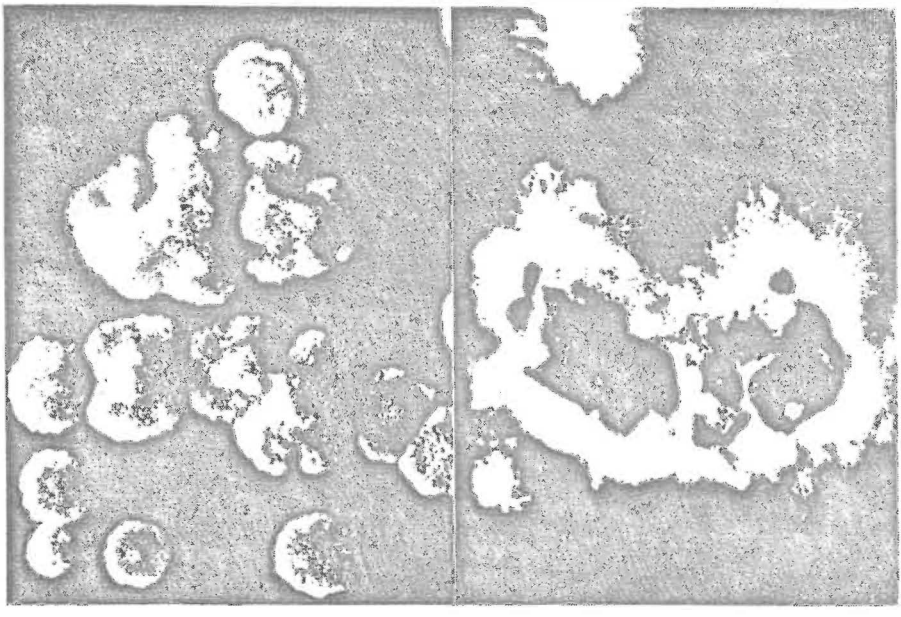
A: Norddeutsche Pflanzenzucht, Hans-Georg Lembke KG, Hohenlieth, Germany, from Dactylis
B: UNI-Gesamthochschule Hohenheim, Landessaatzuchtanstalt Hohenheim, Germany, from Dactylis
C: DSV Lippstadt, Saatzuchtstation Hof Steimke, Germanay, from Dactylis.

Leaves showing symptoms, were surface sterilised and cultivated on malt peptone agar (MPA) at 20 °C under black light illumination (30 W, UV: 300 - 420 nm).

The morphology of two isolates was characterized by means of light and scanning electron microscopy. Figure 1, 2 and 3 show difference in mycelium growth, size and shape of conidia (Fig.3). The latter were formed on short conidiophores (Fig.4) which were either arranged in groups or singly.

According to these data the two isolates could be classified as Mastigosporium muticum (Sacc.) Gunnerb. and Mastigosporium kitzebergense Schlöss.

A simple and reliable method was developed for cultivation of the fungi and an efficient production of inoculum.



A B

Fig. 1 : type of mycelia of : A - Mastigosporium muticum
B - Mastigosporium kitzebergense

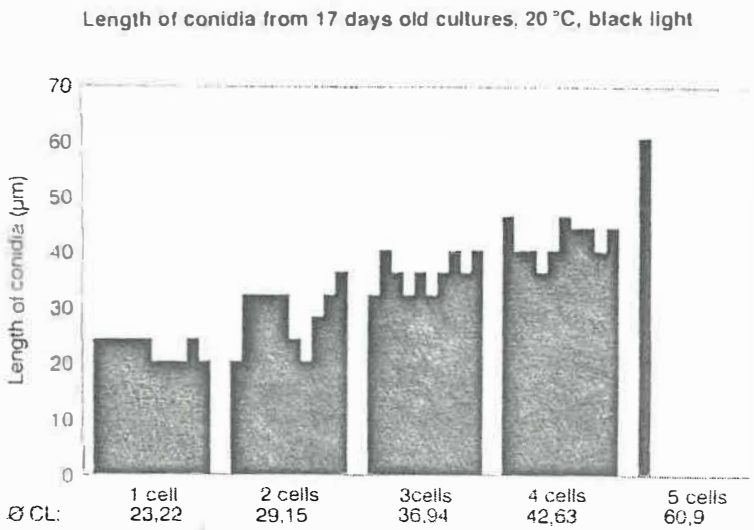
(Fig. 5). On this basis a spectrum of host plants was infected and the time course of infection (Fig.6), symptomatology (Fig.7) and susceptibilty of different grass species (Tab.1) have been investigated.

Table 1 : Susceptibility of different grass species to Mastigosporium species

grass species	M. muticum	M. kitzebergense
Dactylis glomerata	+ + +	+ +
Dactylis aschersoonia	+ + +	-
Phleum pratense	-	+ +
Agrostis gigantea	-	-
Agrostis stolonifera	-	-
Agrostis alba	-	-

The results obtained from these experiments are currently used for development of a method for screening Dactylis plants for resistance to Mastigosporium spp.

Mastigosporium muticum



Mastigosporium kilzebergense

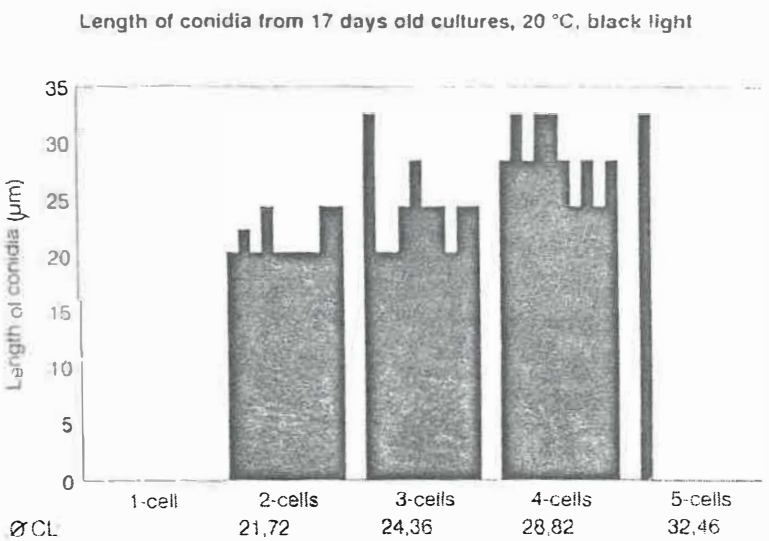
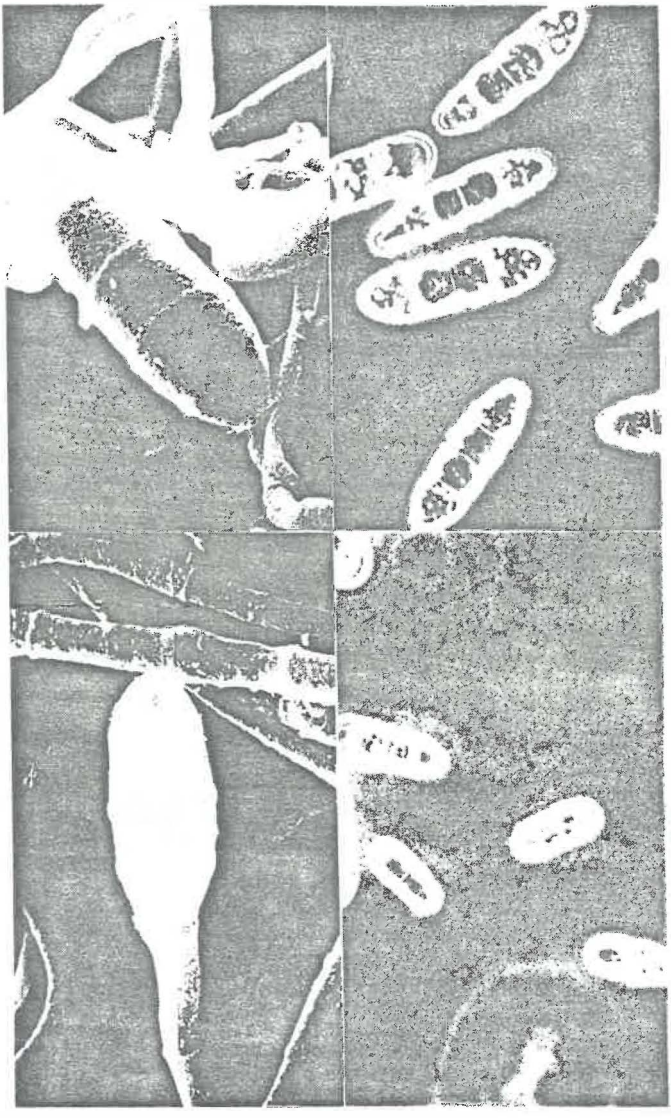


Fig. 2 : Length of Conidia of Mastigosporium spp.

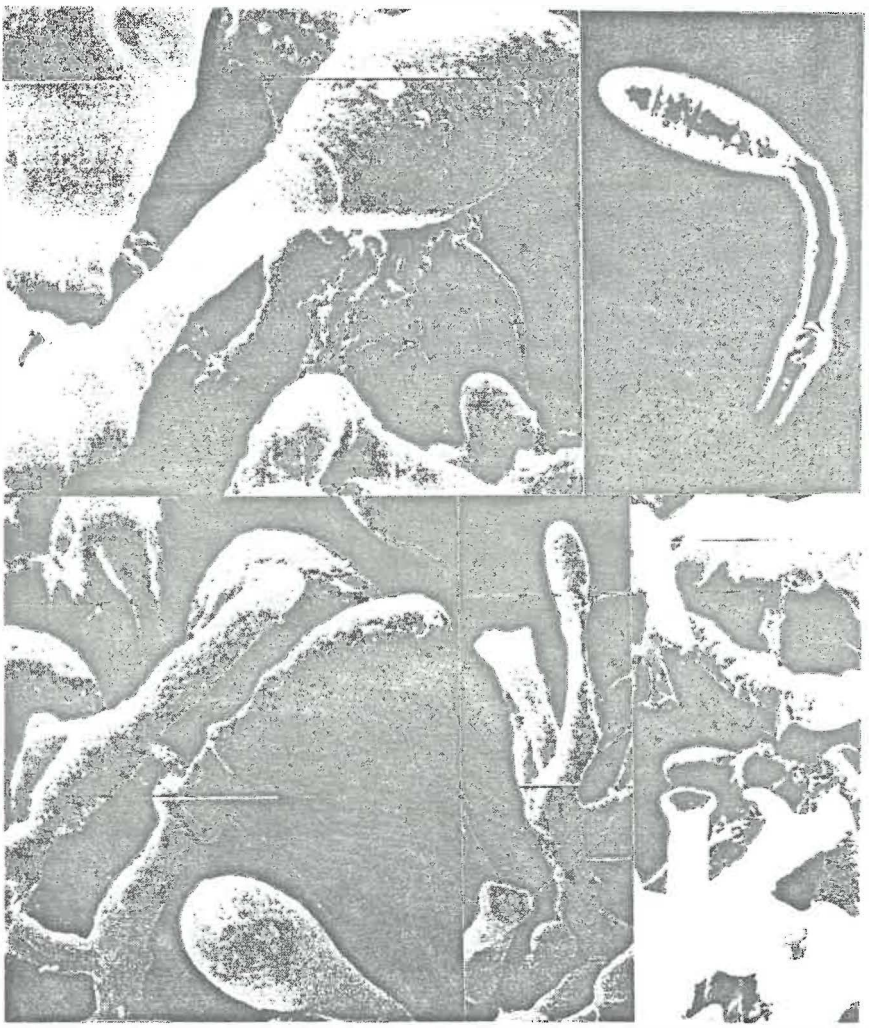
A B



C D

*Fig. 3 : typs of conidia:
A, B : *M. muticum* (2500 . 1 ; 1000 x)
C, D : *M. kitzebergense* (4500 : 1 ; 1000 x)

A B



C D E

Fig. 4 : conidiophores of *Mastigosporium muticum*

A, C, D, E - scanning electron miroscopy picture from conidiophores, arranged in groups or solitary (A - 8000 : 1, C - 7400 :1, D - 5200 :1, E - 4800 : 1)

B - conidiophor with conidia in a light microscopy picture (1000 x)

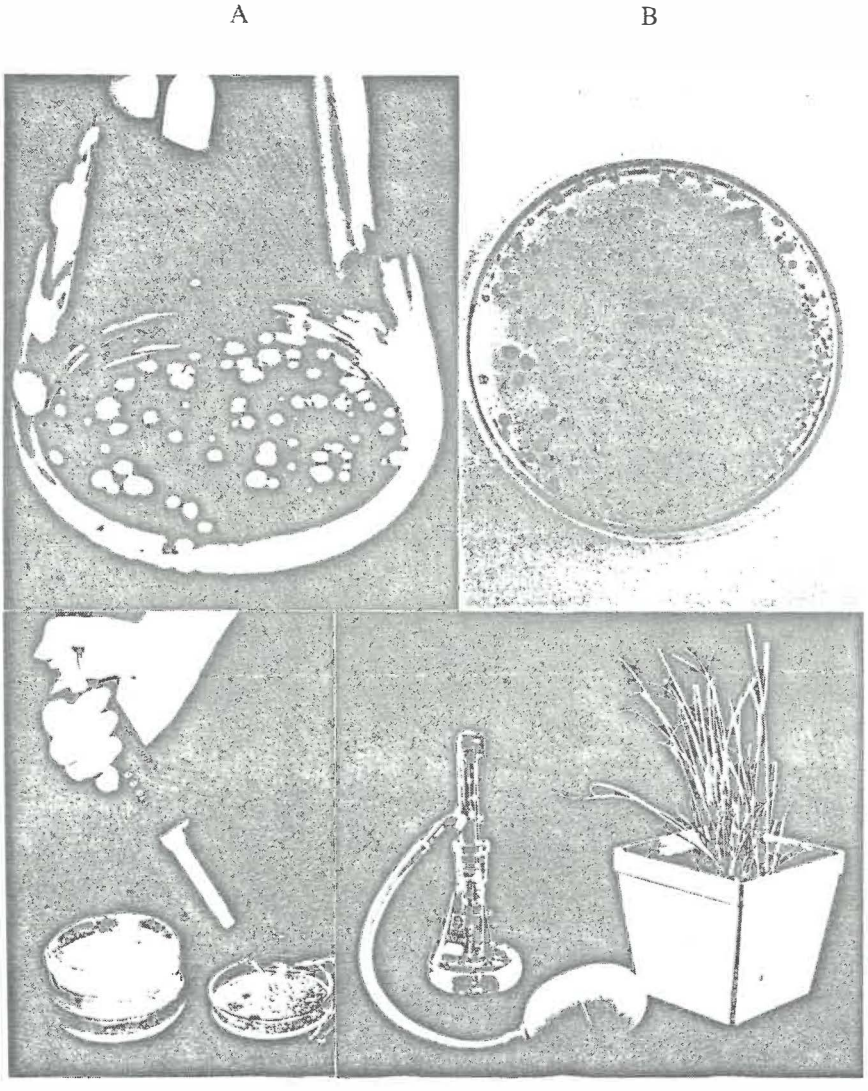


Fig. 5: cultivation, production of inoculum and method of infection of *Mastigosporium* spp.

A - cultivation of mycelia on MP - broth
B - formation of conidia on MPA after 2 - 3 weeks
C - production of conidia suspension in tap water with Tween 20
D - spray inoculation of plants

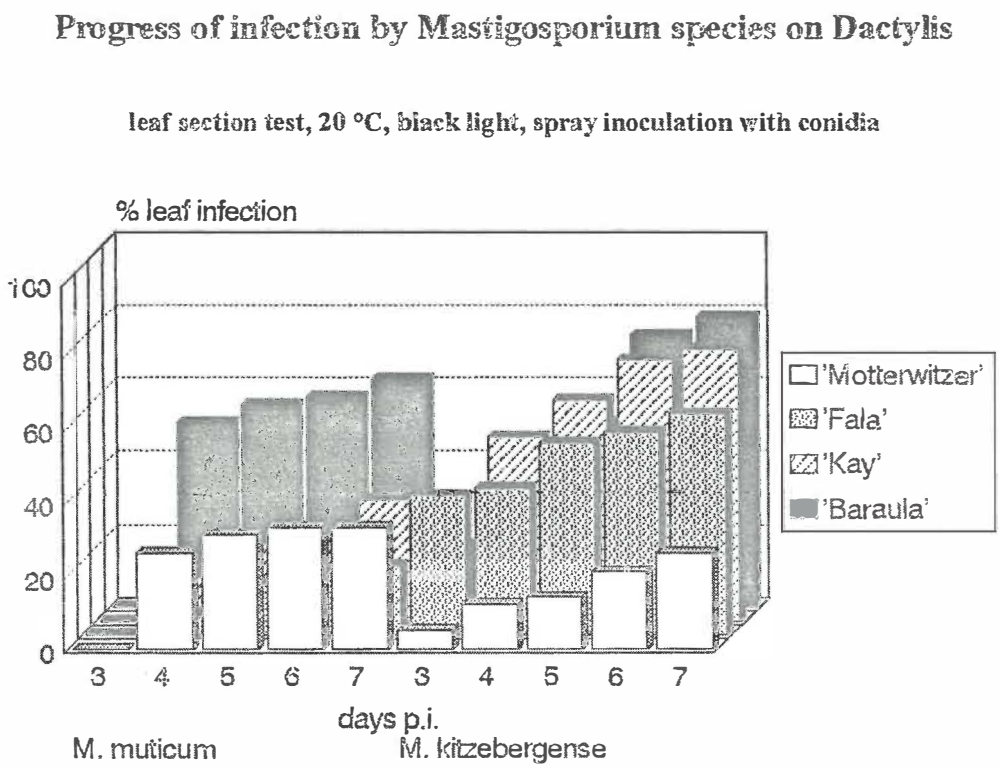
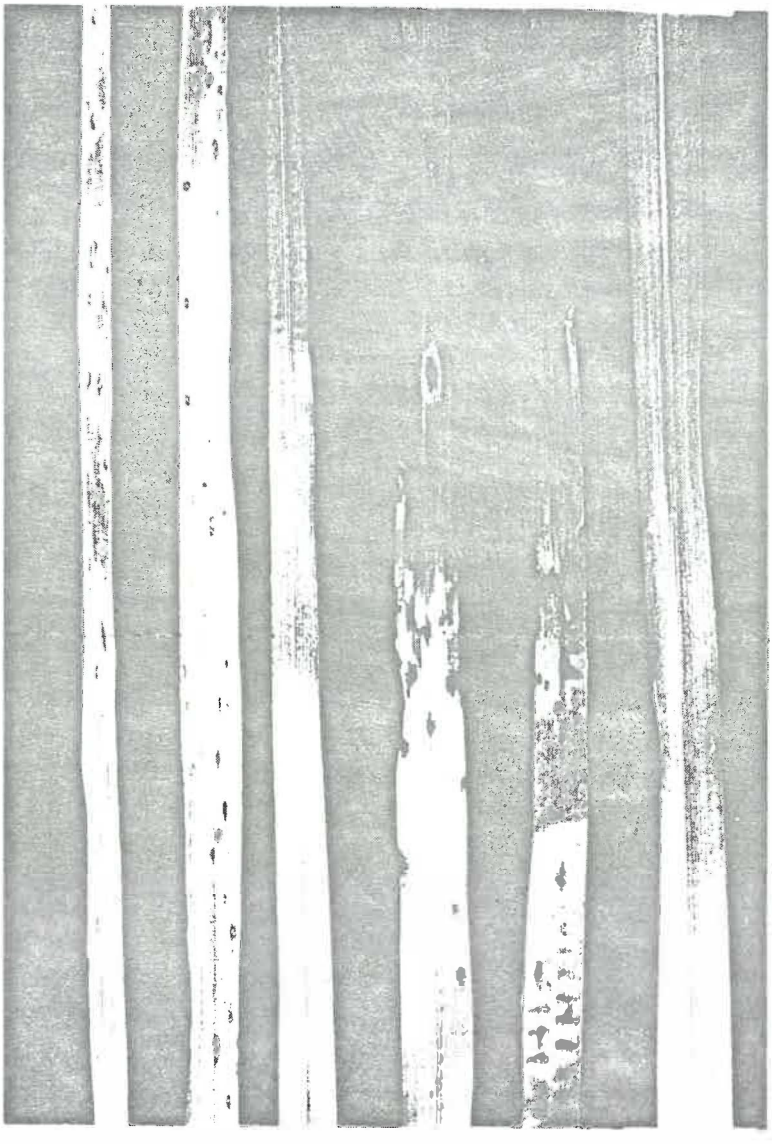


Fig. 6 : The time course of infection with Mastigosporium spp.



1 2 3 4 5 6

Fig. 7 : symptomatology of *Mastigosporium* spp.
1, 2 - M. *kitzebergense* on Phleum
3, 6 - healthy control
4, 5 - M. *muticum* on *Dactylis*

Rapid identification of graminaceous *Drechslera* species by isozyme analysis

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ABSTRACT

Leaf spot diseases, caused by several *Drechslera* species are common throughout the world and are important fungal pathogens on pasture, turf's and grassland. *D. siccans* (Drechsl.)Shoem., *D. andersenii* (Scharif)Lam and *D. dictyoides* infect perennial and Italian ryegrass, but only *D. siccans* and *D. andersenii* affect quality and yield. Identification of the pathogens symptoms alone on the leaves is uncertain and difficult. An easier method is required to distinguish aggressive and harmless species on *Lolium* sp. The conidium-morphology is a specific distinguishing feature. Thus, much effort was taken to elaborate specific artificial conditions in order to produce sporulating cultures of *Drechslera* species. However distinguishing *D. andersenii* and *D. dictyoides* by their conidia is still difficult.

Isoenzyme analysis, performed with polyacrylamide gel electrophoresis, was used as a taxonomic tool to distinguish *D. siccans*, *D. dictyoides*, and *D. andersenii* by banding patterns. 15 isolates of these three pathogens, all identified by their conidia-morphology and culture behaviour in a large-scale conventional identification, were separated by isozymes of eight different enzymes. By analysing mycelium, prepared in liquid cultures, it was possible to reproduce results obtained in the conventional way. In addition it was possible to study variation within different species.

Malate dehydrogenase, NADH-diaphorase, phosphoglucomutase, phosphogluconate dehydrogenase and glucose phosphate isomerase proved to be isozymes with inter-specific variation, whereas α-esterase and acid phosphatase showed intra-specific bands. The results were subjected to cluster analysis from similarity coefficients and illustrated by a phylogenetic tree.

KEYWORDS: *DRECHSLERA*, ELECTROPHORESIS, IDENTIFICATION, ISOZYMES, TAXONOMY

INTRODUCTION

Leaf spot diseases, caused by several *Drechslera* species are common throughout the world and are important fungal pathogens on pasture, turf's and grassland. In pasture and meadows these fungi have a world-wide distribution but are usually not able to cause severe yield losses, but the disease in breeding nurseries is much more harmful. The pathogen can be seed-borne.

In Germany there are three different *Drechslera* species, harmful to ryegrass (*Lolium* sp.): *Drechslera siccans, D. dictyoides and D. andersenii.* The severity of infection differs according to the species involved. Hence an easy and rapid identification method is required. Phenotypic and physiological methods are uncertain. Identification of *Drechslera* species based on symptoms on leaves is not possible, and experience and expertise is essential to distinguish the fungi by conidial morphology.

MATERIALS AND METHODS

ABBREVIATIONS:

IEF	isoelectric focusing
PAGE	polyacrylamide gel electrophoresis
MDH	malate dehydrogenase, E.C. 1.1.1.37
PGD	phosphogluconate dehydrogenase, E.C. 1.1.1.44
GPI	glucose phosphate isomerase, E.C. 5.3.1.9
ACP	acid phosphatase, E.C. 3.1.3.2
DIA	NADH-diaphorase, E.C. 1.6.2.2
α-EST	α-esterase, E.C. 3.1.1.1
PGM	phosphoglucomutase, E.C. 2.7.5.1
GD	glucose-6-phosphate dehydrogenase, E.C. 1.1.1.49
TEMED	N,N,N´,N´-tetramethylethylenediamine

COLLECTING AND CULTURING OF ISOLATES

Single spore isolates of *Drechslera* were obtained from infested plants at different locations throughout Germany. 15 different isolates of three *Drechslera* species were chosen to determine the amount of genetic variation among the fungi by biochemical markers (Table 1).

Table 1: Number and origin of the Drechslera samples

No.	Species	Origin	Year	Host
DS04	*D. siccans*	Berlin	?	*Lolium perenne*
DS09	*D. siccans*	Soest	1987	*Poa pratense*
DS36	*D. siccans*	Thüle	1987	*L. perenne*
DS42	*D. siccans*	Hohenheim	1987	*L. perenne*
DS69	*D. siccans*	Freising	1988	*L. perenne*
DD30	*D. dictyoides*	Freising	1987	*Festuca pratense*
DD49	*D. dictyoides*	Asendorf	1987	*F. pratense*
DD64	*D. dictyoides*	Steinach	1988	*F. pratense*
DD68	*D. dictyoides*	Freising	1988	*F. pratense*
DD74	*D. dictyoides*	Eichhof	1989	*F. pratense*
DA28	*D. andersenii*	Scharnhorst	1987	*L. perenne*
DA40	*D. andersenii*	Asendorf	1987	*L. perenne*
DA46	*D. andersenii*	Asendorf	1987	*L. multiflorum*
DA78	*D. andersenii*	Schweiz	1989	*L. perenne*
DA81	*D. andersenii*	Steinach	1989	*L. perenne*

The fungi were maintained on a V8 juice agar (Miller 1955), subjected to near UV light at 16°C for four weeks and 12 h per day. For the production of proteins extracted from mycelium, the fungi were cultured on a liquid medium (Burhenne 1992). Uniform conditions of light, temperature, and nutrition were used to minimise the differential expression of induced enzymes. To obtain mycelium, from each fungus a pre-culture was prepared by inoculating a 250 ml Erlenmeyer flask (50 ml of substrate) with five pieces (8 mm Ø) of a mature agar culture. The flasks were agitated for five days (120 rpm) on an orbital shaker at 20°C. 0.5 % of the pre-culture fresh mass was transferred aseptically to the main-culture (500 ml flasks with 100 ml substratum) and agitated for three days.

EXTRACTION OF PROTEIN

Mycelium from liquid medium was washed with de ionised water and centrifuged twice. Lyophilised mycelium was homogenised under liquid nitrogen, suspended in extraction buffer (0.1 m Tris/HCl/0.5 m saccharose, pH 7.1) for 3 min and centrifuged for 5 min at 10 000 rpm. Supernatant was applied immediately to the gels.

GEL COMPOSITION

4.9 % (w/v) acrylamide, 0.15 %(w/v) bisacrylamide - based on final content acrylamide, 2% (v/v) carrier ampholyte (Servalyt® or Pharmalyt®), 0.05 % TEMED, 0.2 % (w/v) ammonium persulfate.
Self prepared PAGE gels were used with a pH-area from 3 to 6, 3 to 9 and 4 to 6.5 respectively. PAGE-Gels (thickness 300 μm) were cast on Gel-Fix film using a flap technique according to the manufacturers manual (Pharmacia, Freiburg).

ELECTRODE BUFFERS

Anode: 0.25 M acetic acid, cathode: 0.25 M potassium hydrochloride. Filter stripes soaked in electrode solution were used as anodic and cathodic electrodes.

SEPARATION

Samples (1 μl) were applied directly onto the prefocussed gel with a silicone application mask. For optimal separation of each isozyme the best ampholytic combination and application print were chosen (Table 2).

Table. 2: Carrier ampholyte composition and and run parameters of IEF

Proportion of	Servalyte	Pharmalyte		Position from
Enzym pH:	3-6	3-9	4-6,5	cathode [cm]
MDH		X		1.9
α-Est			X	1.9
DIA			X	1.9
ACP	X			0.9
GPI	X			0.9
PGM			X	0.9
PGD	X			0.9

The separation took place in a PhastSystem®. Each gel was run for 2 kV at a constant current of 2.5 mA (5.0 mA for pH 4 to 6.5) and cooled at 15°C. Gels (4.5 x 5.0 cm) were prefocused for 10 min. After sample application they were focused until the limit volt-hour-product (410 Vh) was reached in about 45 min.

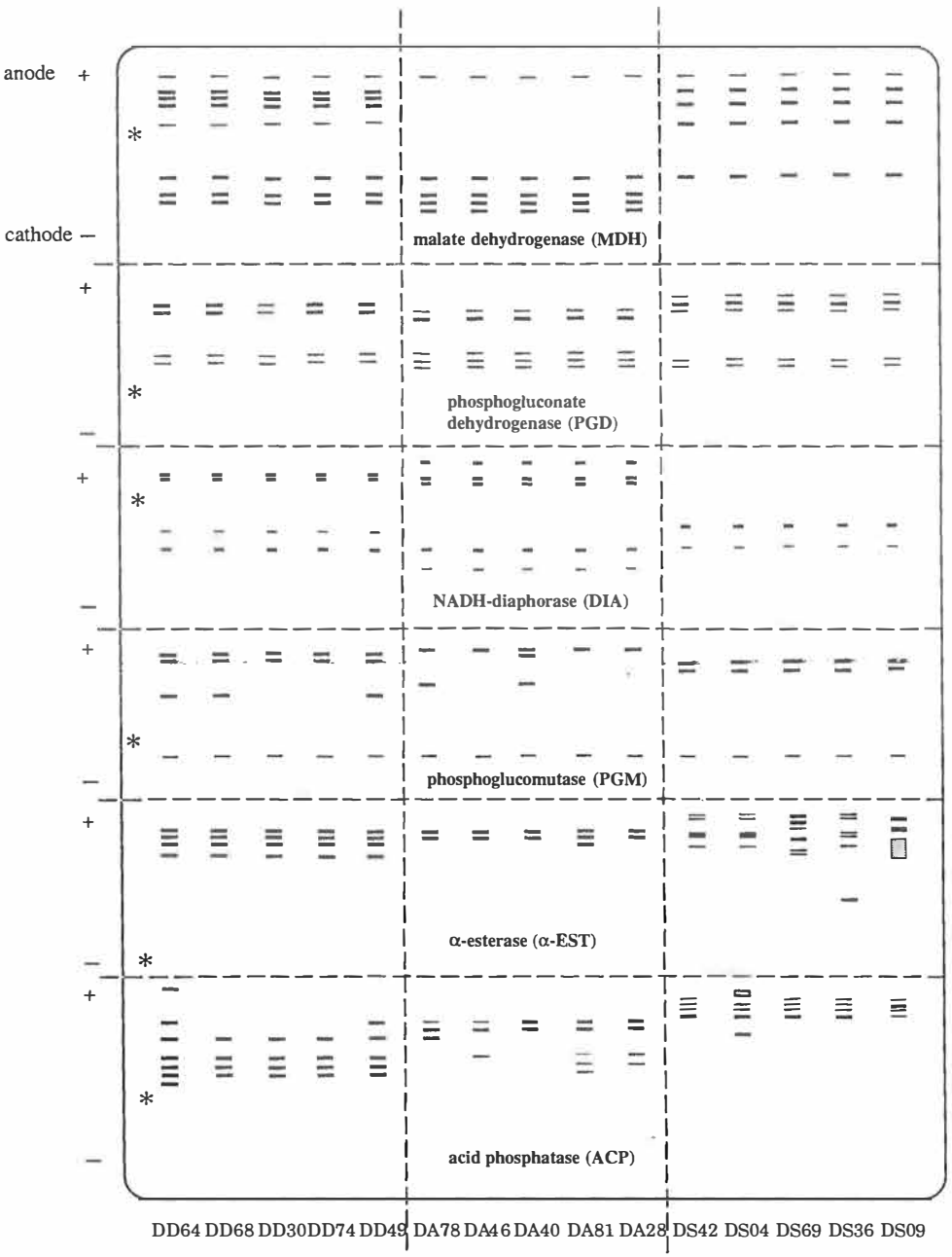

Fig. 1: Schematic representation of the isozyme pattern of 8 enzymes from 15 isolates of three *Drechslera* species (DD = *D. dictyoides*; DA = *D. andersenii*; DS = *D. siccans;* * = position of the sample application mask.

STAINING PROCEDURES

The isozyme staining procedures of Harris and Hopkins (1976) were used. Some of which were slightly modified. All of the enzymes belong to the intracellular proteins. The enzymes PGD, GD, GPI and PGM were visualised on gels by the agar overlay technique. Stained gels were estimated by visual counting and comparison of bands.

CLUSTER ANALYSIS

The genetic relationship of the isolates was estimated by the distance of the similarity (S) and the dissimilarity coefficient (D) with the isozyme data. S is based on the number of shared bands. It ranges from 0 (complete divergent) to 1 (complete uniform). D considers only comparable electrophoretic bands. D is always greater or equal to zero (squared euclidean distance).

Dissimilarity coefficient (D) $\quad \text{distance}(x,y) = \sum_{[i]}(x[i]-y[i])^2$

Similarity coefficient (D) $\quad \text{distance}(x,y)=\dfrac{(\sum_{[i]}x[i]-y[i])}{\sqrt{(\sum_{[i]}x[i]^2)\times(\sum_{[i]}y[i]^2)}}$

i = number of comparable results in each isolate
x, y = the two isolates being compared

RESULTS

For stained isozymes only distinct parts of the protein spectrum is visualised. Enzymes on different alleles dispose of different electric charges. This is substantiated in the distinct composition of amino acids (Micales *et. al.* 1965). Not all isozymes were stainable after IEF and for some isozymes not all bands could be resolved because of the short separation distance.

All eight enzymes showed good activity, with 7 isozymes a good differentiation was achieved. In total 69 isozyme bands of *Drechslera* species were applied (Fig. 1).

After staining IEF gels for MDH 9 isozymes were indicated. The enzyme pattern illustrated that this isozyme can not be used for a clear differentiation between the three species. Similar results were found for the PGD enzyme. All single spore isolates within the three groups formed common bands. 7 different isozymes showed good activity. There was also genetic variation between but not within the species; this is shown by the different isozyme pattern of the different

species but the same pattern within one species. The same results were obtained with DIA. All isolates shared the same pattern within a species.

Gels stained for phosphoglucomutase (PGM) showed 6 different isozymes. *D. siccans* shared the same bands but *D. dictyoides* and *D. andersenii* revealed genetic variations within the species. As compared with α-EST no variation within the *D. dictyoides* species occurred, but great genetic variation within the 5 isolates of *D. siccans* was apparent. The isozyme patterns were specific for just one individual fungus. Thus the genetic distance was clearly higher than with *D. dictyoides* and *D. andersenii*. The enzyme acid phosphatase (ACP) revealed a distinct genetic variation between and within the three *Drechslera* species. 14 isozymes were visible.

Apart from the characteristic differences between *D. siccans*, *D. andersenii* and *D. dictyoides*, several genotypes are probably identifiable within the species. The resolution of GD did not allow a clear differentiation between the fungi. Only one individual band for all isolates was obvious.

A high level of similarity within a species was achieved when all isozyme patterns were used to calculate the similarity, S. All single spore isolates within a species were closely related with S = 89 % to 94 % (Tab. 3). As expected *D. andersenii* revealed a better relationship with *D. dictyoides* (S: 63 %) than with *D. siccans*. The highest distance was obtained with *D. andersenii* and *D. siccans* with 43 % similarity. The same results showed the dissimilarity coefficients D. For *D. andersenii* the highest distance values were calculated with *D. siccans* (D: 27.9) and lowest value with *D. dictyoides* (D: 18.0).

Table. 3: Isozyme dissimilarity coefficients (D) and similarity coefficiants (S) of Drechslera spp..

	D. dictyoides	*D. andersenii*	*D. siccans*
D			
D. dictyoides	3.4 (0-8)		
D. andersenii	18.0 (14-22)	2.4 (1-5)	
D. siccans	25.2 (21-30)	27.9 (26-31)	6.6 (3-9)
S			
D. dictyoides	0.943		
D. andersenii	0.629	0.942	
D. siccans	0.552	0.425	0.889

( ) greatest and smallest distance

D: 0 = completely uniform S: 0 = divergent, 1 = completely uniform

To find out which isolates can be combined in a phylogenetic tree a cluster analysis based on the dissimilarity coefficient was performed (Fig. 2). The phylogenetic tree of the 15 isolates constructed from the dissimilarity coefficient separated the three species and showed the relationship within the *Drechslera* species and reveals a clear differentiation between *D. dictyoides* and *D. andersenii*. In all cases the single spore isolates of the species were grouped together. A high level of variation existed within the *D. siccans* group. The degree of relatedness between the isolates was lower than with the *D. dictyoides* or the *D. andersenii* group.

DISCUSSION

Leaf spot diseases caused by *Drechslera* spp. are not easily to identified. Sporulating cultures of Drechslera special are difficult to produce *in vitro*, and in any case, two species, *D. andersenii* and *D. dictyoides* are particularly difficult to separate on the basis of conidial morphology (Burhenne 1992). These two fungi have been identified as a single species, separated into two formae specialis *dictyoides,* found mainly on *Festuca* spp., and *perenne,* found on *Lolium* spp.. In 1985, Lam described *D. andersenii* distinguishing it from *D. dictyoides* on the basis of numerical taxonomy (Lam & Chapman 1985).

The purpose of the study was the identification of characteristic zones in the isozyme patterns of *D. andersenii*, *D. dictyoides*, and other *Drechslera* fungi that may occur on *Lolium spp.*.

IEF techniques do not require large quantities of spores . By protein extraction from lyophilized mycelia grown on liquid medium, the three *Drechslera* species could easily be differentiated. However, the present study revealed that determination of *Drechslera* species by separation of isozymes with gel electrophoresis is a practicable technique. Electrophoresis using a PhastSystem® is known as a rapid and easy technique, but the separation way is short because of the small gels used. But the definition of 69 Isozymes by this technique confirmed, that the resolution of the isozyme patterns were sufficient.

In summary it can be stated that the identification of *D. siccans* and especially *D. andersenii* and *D. dictyoides* by their isozymes is easy to achieve. For the technique of isozyme electrophoresis to be of taxonomic value interspecific differences must be greater than intraspecific differences.

At least 3 proteins were specific for the different *Drechslera* species. MDH was an easy and rapid enzyme to determine the genetic variation between the *Drechslera* species and there was no variation within the species. It was identified in all isolates and it can be used for taxonomic classification. DIA and PGD also proved to be good enzymes for the determination of variation between the species, α-EST and PGM showed variation within the species. Studies of ACP revealed that this isozyme could not be used for differentiation between species but could be used

to differentiate isolates within a species. The isolates of *D. siccans* are phenotypically very uniform and easy to separate from the other species, but as seen from the phylogenetic tree there is a great genetic variation within this group. Within the *D. andersenii* and *D dictyoides* species there were subspecies with very small genetic variations between them.

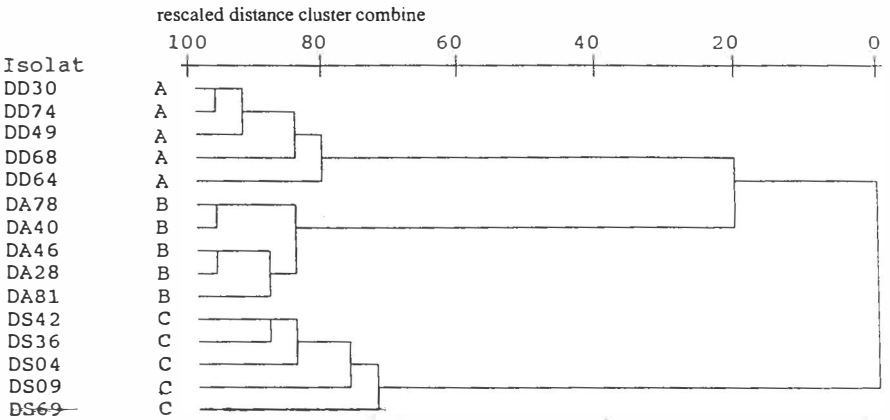

Fig. 2: Phylogenetic trees of 15 *Drechslera* species constructed from isozyme dissimilarity coefficiant D

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Microbial populations in the rhizosphere of various grass species

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ABSTRACT

The following groups of microorganisms were examined in soil samples taken from sods of 17 grass species: Phialophora-like fungi, vesicular-arbuscular mycorrhizal (VAM) fungi and bacteria (Azotobac- ter, Bacillus, Pseudomonas). Phialophora graminicola occurred under all grass species tested but at different population levels. Low populations of this fungus were found under Bromus erectus and B. inermis, while Festuca pratensis, F. rubra, F. arundinacea, Dactylis glomerata and Arrhenatherum elatius harboured high populations of P. graminicola. Phialophora sp.(lobed hyphopodia) was detected at low population levels under 7 grasses only.

The number of spores of VAM fungi ranged from about 1350 propagules per 100 g of soil in the rhizosphere of Agrostis gigantea to 2550 and 2800 spores under D. glomerata and Phalaris arundinacea, respectively., Azotobacter cells occurred under 7 of 17 grass species tested being most numerous in the rhizosphere of F. arundinacea, F. rubra, F. ovina and A. gigantea. The highest numbers of bacteria from the genus Pseudomonas were found in soil under Poa palustris, Alopecurus pratensis, B. erectus and F. rubra but the lowest numbers under F. ovina and Poa pratensis. Isolates of Pseudomonas spp. antagonistic to Gaeumannomyces graminis v. tritici (take-all fungus) amounted to 80-90% of the total populations of these bacteria in soil under F. arundinacea, F. rubra, Phleum pratense and P. pratensis but only to 6% in the rhizosphere of A. pratensis. The total numbers of bacteria from the genus Bacillus and their antagonistic activity were generally lower than those of Pseudomonas spp..

KEYWORDS: anatagonism, Azotobacter, Bacillus, beneficial microorganism, Gaeumannomyces, grasses, Phialophora, Pseudomonas, rhizosphere, soil

INTRODUCTION

Grasses are known not only as important fodder crops, particularly for ruminants, but also they constitute main components of amenity turfs and greens. While there is plenty of information concerning effects of different agrotechnical practices on the growth of grasses as well as on the quantity and quality of their biomass, considerably less attention has been given to soil microflora occurring under grasses, especially with respect to non-pathogenic, beneficial groups of microorganisms (Deacon 1976; Falkowski and Nowak 1975; Goral and Goral , 1987; Martyniuk 1991). Composition of microbial

communities in the rhizosphere of grasses are particularly important when grasses are grown as field crops rotated with other plants (Deacon 1976; Martyniuk 1986; Prew 1981).

Several years ago a research project has been initiated in the De-, partment of Microbiology of the Institute of Soil Science and Plant Cultivation in Pulawy to study the occurrence of some groups of beneficial microorganisms in the rhizosphere of various grass species.

This article summarizes the results of these studies.

METHODS

Replicated cores (1,7x10 cm) were sampled from sods of different grass species grown for 3-4 years on a alluvial soil at the Experimantal Farm in Pulawy. Coarse roots were removed from the collected samples by sieving them through a screen with 2 mm openings and the soils analysed for: 1) Phialophora-like fungi(Phialophora graminicola /telem. Gaeumannomyces cylindrosporus/ and Phialophora sp./lobed hyphopodia/) (Deacon 1976; Martyniuk 1987); 2) numbers of spores of vesicular-arbuscular (VAM) fungi (Allen et al 1979); and 3) numbers of bacteria: Azotobacter, Bacillus, Pseudomonas. Randomly selected isolates of Bacillus and Pseudomonas were also tested for their antagonistic activity to Gaeumannomyces graminis v. tritici (Martyniuk 1991).

RESULTS AND DISCUSSION

Low populations of Phialophora sp. with lobed hyphopodia were found only under 7 of 15 grasses tested. On the other hand, Phialophora graminicola occurred in soil under all grass species, but at different populations levels (Tab. 1.). The lowest populations of this fungus were found under two species of Bromus and relatively low populations under Agrostis gigantea and Festuca ovina. Other examined grasses harboured high populations of P. graminicola, with Festuca pratensis, F. rubra, F. arundinacea, Dactylis glomerata and Arrhenatherum elatius as the best hosts for this fungus. Table 1 shows also that the grasses studied differed significantly with respect to the numbers of spores of VAM fungi found in their rhizosphere. The highest numbers of spores were detected in soil under Phalaris arundinacea, Dactylis glomerata, Bromus inermis and Agropyron cristatum but the lowest under Agrostis gigantea, Festuca rubra and Poa palustris. The presented results seem to be interesting not only from an ecological point of view but they may have a practical aspect, as well. For example, it has been shown in G. Britain that cereals generally suffered less from take-all when sown after grass leys than after other crops, mainly due to biological control of G. graminis by P. graminicola which was abundant in grass leys (Deacon 1976; Prew 1981). In such crop sequences it would be the best to sow grasses known to harbour high populations of P. graminicola and other beneficial groups of microorganisms.

Table 1. The occurrence of Phialophora-like fungi and spores of vesicular-arbuscular mycorrhizal (VAM) fungi in soil under grasses

Grasses	% wheat roots infected		Numbers of VAM pores/100g of soil
	Phialophora graminicola	Phialophora (lobed hyphopodia)	
Agropyron cristatum	68 abc*	15	2484 ab
Agropyron sibiricum	-	-2018 bc	
Agrostis gigantea	24 de	13	1352 a
Agrostis vulgaris	-	-	1961 c
Alopecurus pratensis	63 bcd	6	-
Arrhenatherum elatius	95 ab	2	1842 c
Bromus erectus	4 c	0	-
Bromus inermis	10 c	0	2584 a
Festuca arundinacea	92 ab	0	1948 c
Festuca ovina	27 de	0	1896 c
Festuca pratensis	100 a	0	-
Festuca rubra	96 ab	5	1763 cd
Phalaris arundinacea	38 cde	8	2795 a
Phleum pratense	65 abc	2	-
Poa pratensis	88 ab	0	-

*Numbers followed by the same letter are not \ signif. different P=0.05\,

Bacteria from the genus Azotobacter capable of fixing atmospheric nitrogen occurred in soil under 7 grasses only (Fig.1). The order of the grasses with Azotobacter from the highest to the lowest population of these bacteria was as follows: F. arundinacea > F. rubra > A. gigantea > F. ovina > A. vulgaris > P. pratensis > P. palustris. Azotobacter cells were found only under those grasses which maintained a rhizosphere soil pH greater than 6. Sensitivity of Azotobacter to low soil pH is well documented (Alexander 1965, Pfeiffer-Maliszewska 1975). It is interesting to note that the pH of the soil under the studied grasses differed markedly. For example, the soil pH under A. elatius and B. erectus was more than by one unit lower than that under F. arundinacea and F. rubra., Numbers of bacteria from the genus Pseudomonas in the soil under the tested grasses ranged from 7 x 10^5 to 8.6 x 10^6 cfu (colony forming units) per 1 g of soil dry matter. The highest numbers of these bacteria were found in the rhizosphere soil of P. palustris, A. pratensis, B. erectus and F. rubra, and the lowest numbers in the soil under F. ovina P. pratensis, A. elatius and A. sibiricum (Fig. 1)., Numbers of Bacillus spp. in the examined soils were generally lower than those of Pseudomonas spp., and ranged

from 5.5 x 10^5 cfu under F. ovina to 2.6 x 10^6 cfu under F. rubra. Randomly selected isolates of Pseudomonas spp. and Bacillus spp. were tested for their antagonistic activity against G. graminis v. tritici (Ggt) on PDA plates (Tab. 2). Allmost all (90-94%) the tested strains of Pseudomonas spp. isolated from the rhizosphere soil of F. rubra, F. arundinacea, P. pratensis and P. pratense were inhibitory to Ggt. Moreover, most of these strains were proved to be strong inhibitors of Ggt on PDA plates. On the contrary, only 6% and 22% of Pseudomonas isolates from the rhizosphere of A. pratensis and P. palustris, respectively, were inhibitory to Ggt and they appeared to be poor antagonists of the pathogen used. Many members of bacteria belonging to the genera Pseudomonas and Bacillus are known as strong antagonists of plant pathogens and have been shown to be potent biocontrol agents of soilborne plant diseases under field conditions (Weller 1988)., The results of the presented studies have shown that microbial communities occurring in the rhizosphere of various grass species can differ significantly, both quantitatively and qualitatively. The way grasses influence soil biological and chemical properties seems to be important, both from phytosanitary and soil fertility point of view., Further work is needed with more grass species and different soil types for better understanding the effect of grass crops on soil environment.

Table 2. Antagonisms of Pseudomonas and Bacillus strains isolated from rhizosphere of grasses to Gaeumannomyces graminis v. tritici

Grasses	Pseudomonas		Bacillus	
	% inhibitory strains	rate of antagonism	% inhibitory strains	rate of antagonism
A. cristatum	61	1.4	11	0.2
A. sibiricum	83	2.3	17	0.3
A. gigantea	72	1.6	17	0.17
A. vulgaris	78	1.8	6	0.17
A. pratensis	6	0.06	11	0.3
A. elatius	72	1.4	25	0.4
B. erectus	83	2.2	0	0
B. inermis	78	2.1	11	0.17
D. glomerata	61	1.6	33	0.5
F. arundinacea	94	2.4	0	0
F. ovina	64	1.5	11	0.2
F. rubra	94	2.8	17	0.2
Ph. arundinacea	61	1.2	170.2	
Ph. pratense	89	1.9	0	0
P. angustifolia	72	1.9	6	0
P. palustris	22	0.6	0	0
P. pratensis	89	2.1	15	0.3

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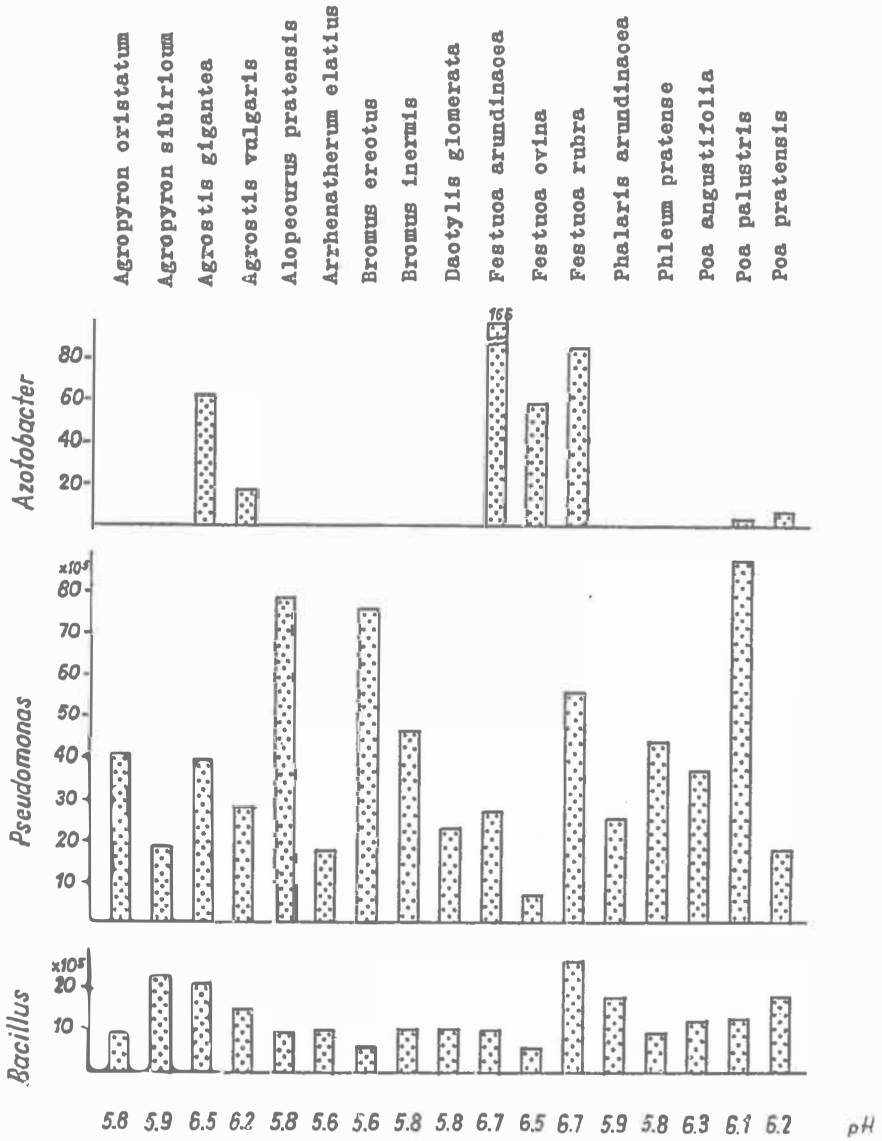

Fig. 1. Numbers of bacteria /in 1 g of soil d.m./ and pH of the rhizosphere soil of various grasses

Occurrence of fungal endophytes in *Lolium perenne*

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ABSTRACT

Populations of *Lolium perenne* ecotypes originating from various grasslands of Northern, Central and Southern Germany were examined for infection with endophytic fungi. The endophytes were detected in the leaf sheath of the grasses by staining the internal fungal hyphae and examination under the microscope.

In most of the screened populations *Lolium perenne* individuals were found to contain endophytic fungi, only some populations proved to be completely negative. The frequency of infected plants mainly varied between 1 to 30%, but several populations showed higher infection levels up to about 80%. The extent of endophyte-positive biomass of infected original grasslands was estimated to range between 1 and 34% and appeared to be independent from the nitrogen fertilisation management.

A wide geographic distribution of endophyte-infected *Lolium perenne* on German grassland can be concluded. Further research on the beneficial or detrimental effects of these grass-fungus associations is regarded to be necessary.

Keywords: endophytic fungi, occurrence, *Lolium perenne*, grassland

INTRODUCTION

Little information is available so far on the occurrence of endophytic fungi in forage grasses of Europe, although perennial ryegrass and tall fescue endophytes which are of great importance in the USA and New Zealand are suggested to be of European origin (Siegel et al. 1985).

Surveys on ryegrass endophytes have been done in the United Kingdom and in France, where a wide geographic distribution of fungal endophytes in *Lolium perenne* was observed (Lewis and Clements 1986; Grand-Ravel et al. 1993). A better knowledge about the incidence and performance of endophyte-infected grasses in European grassland is needed to understand the role of these symbiotic relationships under temperate climatical conditions.

To get information on the occurrence of grass endophytes in Germany, a study was carried out screening ecotypes of perennial ryegrass originating from various German grasslands for the presence of endophytic fungi.

METHODS

Plant material

A collection of 38 populations of *Lolium perenne* ecotypes, each comprising 180 single plants cultivated on an experimental field in Braunschweig (FAL), was used in the study. Fifteen populations originated from grassland locations of Lower Saxony and three from the island of Poel, the altitude of these sites ranged between -2 to +5 m above sea level. Ten populations derived from mountainous regions (140 to 700 m altitude) of Hesse, and ten populations derived from swards situated at 580 up to 1000 m in South Bavaria. Only grassland not re-seeded since 1975 and showing a considerable ratio of *Lolium perenne* was included in the selection of plant material. The proportion of *Lolium perenne* in the biomass at the sampling area was estimated according to Klapp and Stählin (1936).

At each original grassland location *Lolium perenne* was sampled from three places, each covering a circular area of about 250 m^2. Within these areas the plants were picked from the centre and along four concentric circles with stepwise increasing radii up to a maximum of 8-9 m.

Detection of fungal endophytes

Of each population a subgroup of 30 single plants was analysed for endophyte infection. To detect endophytic fungi the interior epidermis of leaf sheath was stained with dye solution (50 ml of lactic acid and 100 ml of 0,1% aqueous aniline blue) according to Bacon et al. (1977) and examined by microscope at 320 x magnification. When at least two of eight investigated leaf sheaths per single plant contained typical intercellular hyphae the plant was defined to be endophyte infected.

RESULTS

In most of the investigated populations of *Lolium perenne* originating from German grassland, individuals infected with fungal endophytes were found (Table 1). Only 13 % of the populations were free of endophytic fungi. In most populations, about 1 to 30% of the single plants proved to be infected by endophytes. Infection levels exeeding 30% were observed in 18 % of the populations.

Table 1: Endophyte infection of Lolium perenne from German grassland

Infection level of the populations %	Number of populations n	Frequency of infected populations %
0	5	13
1-30	15	40
11-30	11	29
>30	7	18

Table 2: Endophyte infection of Lolium perenne in relation to geographic region

Region	Number of swards n	Sward biomass Proportion of Lolium perenne %		Infection level of the populations %		Proportion of infected sward biomass %	
		$\bar{x}$	range	$\bar{x}$	range	$\bar{x}$	range
Lower Saxony	15	60	37-90	13	0-50	8	0-30
Island of Poel	3	56	22-78	28	3-50	14	2-31
Hesse	10	41	8-81	24	0-77	9	0-34
Bavaria	10	54	35-72	15	0-47	9	0-31

A wide geographic distribution of endophyte-positive grasses of *Lolium perenne* was observed (Table 2). Average infection levels of the populations from all sampled regions varied at moderate values of between 13 to 28%, but some populations were highly infected, up to nearly 80 %. The extent of endophyte-infected biomass of the swards, calculated by relating the infection values of the populations to the proportions of *Lolium perenne* estimated at the original locations, appeared to be low (average values: 8

to 14%) in most cases (Table 2). However, values of about 30 to 34 % infected biomass were estimated for some individual locations at all geographic regions.

Table 3: Endophyte infection of Lolium perenne in relation to grassland fertilisation

N fertiliser rate	Number of swards	Sward biomass Proportion of Lolium perenne		Infection level of the populations		Proportion of infected sward biomass	
kg N/ha.a	n	%		%		%	
		$\bar{x}$	range	$\bar{x}$	range	$\bar{x}$	range
>200	11	61	27-87	18	3-43	10	2-25
100-200	14	54	28-90	20	0-77	10	0-34
<100	13	45	8-78	15	0-50	6	0-31

The infection frequencies of the *Lolium perenne* ecotypes were unrelated to the nitrogen fertilisation of the swards (Table 3). At fertiliser rates of more than 200 down to less than 100 kg N/ha and year no evident differences in infection levels of the ryegrass populations and the biomass of the swards were observed.

DISCUSSION

The widespread occurrence of endophytic fungi in *Lolium perenne* ecotypes is in agreement with surveys of ryegrass endophytes in the United Kingdom and in France (Lewis and Clements 1986; Grand-Ravel et al. 1993). No geographic region appeared to be favourable or unfavorable for the grass endophytes.

In most cases the infection levels varied from low to moderate levels, but at some individual swards, higher degrees of infection may occured. However, these values have to be regarded as a minimum estimation, as coexisting grasses of other genera, in particular Festuca species, might also be infected by endophytic fungi (White et al. 1992; Schmidt 1989; Leuchtmann 1992). The specific circumstances leading to different infection levels in grassland remain to be elucidated.

No obvious differences in infection frequencies were observed in relation to the level of the nitrogen supply at each sites although there might be effects on the yield of endophyte-positive plants at different nutrient availabilities as described by Cheplick et al. (1989), who revealed more biomass production of infected perennial ryegrass compared to

endophyte-free grasses at high and intermediate nutrient levels, but less at low nutrient supply.

Further research on the beneficial or detrimental effects of the grass-fungus associations on grassland performance and the grazing animal is regarded to determine the biological and ecological role as well as the agronomic implication of grass endophytes under the temperate climatical conditions of Central Europe.

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ENDOPHYTES IN EUROPEAN VARIETIES OF FESTUCA SPECIES

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ABSTRACT

Despite the fact that fescue endophytes have been dealt with for some years in the USA and New Zealand, no work about the infestation level of *Festuca* species in Germany appears to have been published.

In the present work a survey of European varieties (mostly from the Netherlands and from Germany) of *Festuca pratensis, F.rubra, F.arundinacea* and *F.ovina* has been compiled to detect fungal hyphae in *Acremonium* species. A hundred seeds per variety were stained with rose bengal and examined microscopically. The fungus was detected in 17 of the examined 135 fescue varieties.

In addition to that several clones and samples from different propagation plots of *F.pratensis* varieties were screened. There was wide variation of the infection level between different seed lots of the same variety.

The endophyte from a meadow fescue and a tall fescue variety was successsfully cultured *in vitro*. Small pieces from the leaf sheaths were placed in Petri dishes on PDA. After 4 weeks Acremonium-like hyphae could be detected. The slow growth of the colonies was characteristically for *Acremonium.* It remains to discover what species the fungus belongs to, as no conidia were found.

Keywords: endophytes, fescue species, Europe, survey.

INTRODUCTION

Endophytes have been examined on a large scale in the USA and New Zealand for some years. Little work, however, has been done in Europe.

In the U.K. 61 swards were checked for the presence of endo-phytes in *Lolium perenne* (Lewis and Clements 1986). *Lolium perenne* families from breeding programmes were examined to evaluate the occurrence of *Acremonium lolii* in France (Grand-Ravel, Charmet and Balfourier 1993). In Switzerland an *Acremonium* species was isolated from plants of *Festuca pratensis* growing in the wild (Gams, Petrini and Schmidt 1990). Cagas (1991) examined the occurrence of *Acremonium coenophialum* in 56 tall fescue cultivars

from the genebank of the Grassland Research Station in Roznov and in 42 tall fescue ecotypes originating from various regions in the former USSR.
No work about the infestation level in fescue species appears to have been published in Germany so far. In the present work a survey of European varieties of *Festuca pratensis*, *F.rubra*, *F. arundinacea* and *F.ovina* has been made to detect fungal hyphae of *Acremonium* species.

MATERIALS AND METHODS

Seeds were stained with rose bengal (Saha, Jackson and Johnson-Cicalese 1988). Two staining solutions were prepared: 1) alka-line solution, 0.2 % rose bengal dissolved in 2.5 % sodium hydroxide and 2) alcoholic solution, 0.5 % rose bengal dis-solved in 5 % aqueous ethyl alcohol. Seeds were soaked overnight (16 h) in alkaline staining solution, rinsed under tapwater and soaked for an additional 3-4 h in alcoholic staining solution.

100 seeds per variety were checked for the presence of endophytic fungi. The aleurone layer was placed on glass slides, covered with cover slips and slightly crushed. Under the microscope (250-400 magnification) the endophyte was detected as a coarse, mostly unbranched, convoluted intercellular mycelium (Siegel, Latch and Johnson 1985).

In a preliminary survey breeders were asked to send us seed samples of certified European varieties. We received 135 fescue cultivars which included 73 *Festuca rubra*, 33 *F.arundinacea*, 21 *F.pratensis*, and 8 *F.ovina* varieties without any specification concerning the propagation site. After these first general investigations we screened new seed material from those samples that were found to harbour the fungus. This time propagation site and harvest year of the seed lots were taken into consideration. Thus, three German varieties FP 7, FP 8, FP 10 were examined more carefully, depending on the site of propagation and year of harvest. In addition seeds from several clones of FP 7 were included into our research.

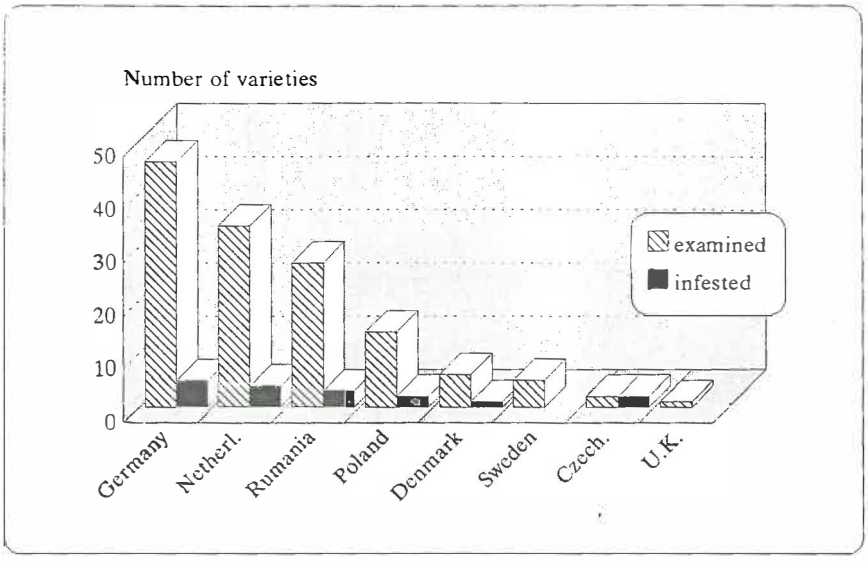
In a second experiment the fungus from two meadow fescue varieties (FP 17 and FP 24) and a tall fescue variety (FA 32) was succesfully cultured in vitro. Small pieces from the leaf sheaths were placed in Petri dishes on PDA after sterilization in ethanol (10 s) followed by NaOCl (10 min) (Latch and Christensen 1985). Growth rates of the colonies were compared to those of species already described (White and Cole 1986; White 1987).

RESULTS

Experiment 1: The investigations revealed that 17 of the recently examined 135 varieties contained a fungus, probably of the genus *Acremonium* (Table 1). In F.rubra we found 3 infected varieties with only a low infection rate, from 1 to 5 %. In *F.pratensis* 9 of the 21 varieties were infected with Acremonium hyphae. Three samples had a high

infection rate. There was only one very highly infected variety in *F.ovina*. In *F.arundinacea* 4 of the 33 varieties showed a medium infection range from 7-32 %. Figure 1 shows the origin of the fescue varieties.

Figure 1: Origin of the examined and infested fescue varieties



Results of the second survey revealed large differences in the endophyte level within the same varieties (Table 2 and 3). In the first survey a seed sample from variety FP 7 (FP 7/0) had an infestation rate of 23 %, but the new seed samples from different propagation plots (they are named FP 7/1-5) contained endophytes from 0-78 % in 1992, and from 5-73 % in 1993, thus emphasizing the differences among seed lots from one variety. From FP 8 we examined seed lots from different years of harvest and sites of propagation. In our first survey this variety had an endophyte level of 1 % (FP 8/0). The endophyte content of FP 8/2 was 0 % in 1992 and in 1993 4 %. FP 8/4 samples from 1991, 1992 and 1993 varied only from 65 to 81 %. In another seed lot FP 8/3 endophyte hyphae were detected in 34 of 100 seeds, which is clearly different from the FP 8/2 and FP 8/4 figures. The sample FP 10/1 did not contain any fungus, FP 10/3 on the other hand showed a high level of infestation over three years with 65, 59 and 74 % at the same site of propagation. In the first survey FP 10/0 was found to have an endophyte content of 2 % (Table 2). In addition to that we got several clones from the variety FP 7 from the harvest 1992 and 1993. All these clones had a very high level of infection, reaching from 91 to 100 % (Table 3).

Experiment 2: Four weeks after placing small pieces of sterilised leaf sheaths in Petri dishes on PDA *Acremonium*-like hyphae were detected. After another 28 days in

culture the fungus had reached a diameter of 10-15 mm, the slow growth being characteristic of *Acremonium*.

The growth rates of the endophytes isolated from a meadow fescue (FP24) and from the tall fescue (FP32) were slightly different, but corresponded with those of other Acremonium species. The tall fescue endophyte appeared to grow a little faster than the meadow fescue endophyte (Table 4).

We have not detected conidia so far. Further investigations are necessary to find out what species the fungi belong to.

DISCUSSION

In this paper European *Festuca* varieties were screened for the presence of endophytes. It is the first time that German fescue varieties were reported to be infected with Acremonium endophytes.

This study revealed that meadow fescue had the highest number of infected varieties (9 of 21). Within certain varieties of meadow fescue a great variation of the infestation level of samples from different propagation plots was observed. The endophyte infection of red fescue was surprisingly low. *F.arundinacea* took an intermediate position. The percentage of infestation was higher than that of *F.rubra*, but does not reach the figure of

F.pratensis. Only 1 *F.ovina* variety was found to contain the endophyte, but only 9 varieties have been examined so far.

The endophyte content depends to a large degree on the time of storage which can differ among the single sites of propagation (and therefore among several seed lots) of the same variety. Storage of seed-samples for more than one or two years, depending on the storage conditions (mainly temperature and humidity) may affect the ability of the fungus to propagate from seed to plant in future generations, thus influencing the percentage of endophyte infestation of certain samples in future generations (Maddaloni, Sala and Carletti 1989; Cerna, Bumerl, Hofbauer 1989; Hare, Rolston, Christensen and Moore 1990). Therefore, samples from one variety were found to have endophyte levels ranging from 0 to 100 % (for example FP 7). A variety described as 'endophyte-free' because of no detection of the fungus in one special seed sample might be actually infected if the examined sample originates from a plot with an interrupted propagation cycle. It is possible that the real infestation level in European varieties is higher than investigated.

The results of this study suggest that one variety cannot possibly be labeled as having a high or a low endophyte level.

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Tab.1 Endophyte infestation of European fescue varieties

	F.rubra	F.pratensis	F.ovina	F.arundinacea
Number of examined varieties	73	21	8	33
Number of infected varieties	3	9	1	4
Intensity of infestation (per 100 seeds)	FR 69/0 - 1%	FP 8/0 - 1%	FO 1/0 - 98%	FA 1/0 - 7%
	FR 111/0 - 2%	FP 10/0 - 2%		FA 15/0 - 8%
	FR 16/0 - 5%	FP 22/0 - 2%		FA 19/0 - 22%
		FP 11/0 - 4%		FA 32/0 - 32%
		FP 17/0 - 10%		
		FP 7/0 - 23%		
		FP 9/0 - 50%		
		FP 21/0 - 59%		
		FP 24/0 - 70%		

FP = Festuca pratensis
7/0 = Variety number 7 with no specification concerning site of propagation (0)

Tab.2 Variation of the endophyte infestation level of meadow fescue within different propagation plots and years of harvest

Variety	Endophyte content of meadow fescue varieties from different seed samples	
FP 7	FP 7/1; H'92 - 0 % FP 7/2; H'92 - 0 % FP 7/3; H'92 - 9 % FP 7/4; H'92 - 57 %	FP 7/5; H'92 - 78 % FP 7/6; H'93 - 36 % FP 7/7; H'93 - 5 % FP 7/8; H'93 - 73 %
FP 8	FP 8/2; H'92 - 0 % FP 8/2; H'93 - 4 % FP 8/3; H'92 - 34 %	FP 8/4; H'91 - 76 % FP 8/4; H'92 - 65 % FP 8/4; H'93 - 81 %
FP 10	FP10/1; H'92 - 0 %	FP10/3; H'91 - 65 % FP10/3; H'92 - 59 % FP10/3; H'93 - 74 %

FP = Festuca pratensis
7/1 = Variety number 7/ propagation plot 1
H'92 = Year of harvest

Tab.3 Endophyte infestation of several meadow fescue clones, variety FP 7

	clone 1	clone 2	clone 3	clone 4	clone 5
Harvest '92	96 %	97 %	100 %	-	91 %
Harvest '93	96 %	96 %	98 %	98 %	96 %

Tab.4 Growth rate of endophytic fungi

Endophyte species	Growth rate	Temperatur
own cultures (from F. pratensis)	10-17 mm / 33 d	23°C
own cultures (from F. arundinacea)	11-23 mm / 33 d	23°C
A.chisosum	11 mm / 40 d	20°C
A.loliae	12,3 mm / 23 d	25°C
A.coenophialum	14 mm / 23 d	25°C

Transmission of *Acremonium* spp. infection

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ABSTRACT

Seed disseminated *Acremonium* endophytes are predominantly transmitted via the maternal parent. However, unexpected results recorded in progenies of interspecific hybrids involving *Lolium perenne* L. (Lp), *L. multiflorum* Lam. (Lm) and *Festuca arundinacea* Schreb. (Fa) suggest a transmission via the pollen parent.

Research on transmission of infection of *Acremonium lolii* and *A. coenophialum* via the pollen parent was initiated in 1990 and is based on the study of progenies of E- x E+ pair crosses made at Oak Park Research Centre. Plants of Lp, Lm and Fa free of infection (E-) were the maternal parents and E+ plants of Grasslands Nui (Lp) and Kentucky 31 (Fa) highly infected by *A. lolii* and *A. coenophialum* respectively, were the male parents. The presence of endophytes in the F_1 progenies was tested by staining for the mycelium and microscopic examination (MSM), enzyme-linked immunosorbent assay (ELISA), protein-A sandwich enzyme-linked immunosorbent assay (PAS-ELISA), tissue print immunoblot (TPIB) and loline alkaloid analysis.

Seed MSM showed a fungal infection in some progenies of the 1991 crosses. Non-convoluted septate hyphae were lying between the testa and the aleurone layer, but in contrast with infected seeds of Grasslands Nui and Kentucky 31, only a few pieces of hyphae were found in the intercellular spaces of the aleurone layer of the hybrid seeds. Although the mycelium detected in the hybrid seeds showed some resemblance with that observed in Grasslands Nui and Kentucky 31, discrepancies recorded among TPIB reactions caused the conclusion that the hyphae were of an *Acremonium* spp. to be equivocal. The results of leaf sheath MSM and PAS-ELISA (seedlings) demonstrate that the fungal mycelium present in the seeds did not reach the embryo or did not survive. Consequently, all hybrid plants analysed were free of infection. Some factors which possibly were responsible for impeding the growth of the mycelium were considered.

Although the results showed that the F_1 plants tested were free of infection, some relevant information was collected in this study which demonstrates that the research on the

possible non-maternal transmission of both *A. lolii* and *A. coenophialum* should be pursued.

Keywords: *Acremonium coenophialum, A. lolii,* endophyte detection, *Festuca arundinacea*, infection transmission, loline alkaloids, *Lolium spp.*

INTRODUCTION

The first report of the transmission of grass fungal endophytes via the maternal parent was given by Sampson (1935). She studied the infection of *Lolium temulentum* L. and *L. perenne* L. by an endophyte and observed the presence of infected (E+) and uninfected (E-) plants in both species. Reciprocal crossing of E- and E+ plants showed that the *Lolium* endophyte invades the ovule and is vegetatively transferred from the female parent.

Although seed disseminated *Acremonium* endophytes are predominantly transmitted via the maternal parent, unexpected results recorded in progenies of interspecific hybrids involving *Lolium perenne* L. (Lp) or *L. multiflorum* Lam. (Lm) as female parents and *Festuca arundinacea* Schreb. (Fa) suggest a possible transmission through the pollen parent. Thus, Buckner *et al.* (1981, 1985), Bush *et al.* (1982) and Jones *et al.* (1985) studied the accumulation of pyrrolizidine alkaloids (N-formyl loline and N-acetyl loline), which are positively associated with the presence of the endophyte *Acremonium coenophialum* Morgan-Jones and W. Gams, in Lm and Lp x Fa hybrid derivatives. These alkaloids were also detected in *Acremonium* infected plants from progenies of hybrids produced at Oak Park in which the initial mother parents were either *L. multiflorum* or *L. perenne* (Do Valle Ribeiro and Bush, 1980; Do Valle Ribeiro, 1993).

This paper describes research to investigate the possible transmission of infection of both *Acremonium lolii* Latch, Christensen and Samuels and *A. coenophialum* Morgan-Jones and W. Gams via the pollen parent. This work has been conducted since 1990 and is based on the study of progenies of E- x E+ pair crosses made at Oak Park in 1990 and 1991.

MATERIALS AND METHODS

Parental material and crosses

Seventeen and thirteen pair crosses were made respectively in summer 1990 and 1991 in which plants of *L. perenne*, *L. multiflorum* and *F. arundinacea* completely free of endophyte infection (E-) were the maternal parents and E+ plants of Grasslands Nui (Lp) and Kentucky 31 (Fa) highly infected by *A. lolii* and *A. coenophialum* respectively were male parents. The type and number of crosses made in 1990 and 1991 are presented below

	Number of crosses	
Type of cross	1990	1991
Lp (-) x Lp (+)	3	7
Lm (-) x Lp (+)	6	-
Fa (-) x Fa (+)	4	6
Lm (-) x Fa (+)	4	-

In 1990 the Lp (E-) plants were of cultivar Frances and Lm (E-) Weldra. The Lp (E-) plants used as mother parents in 1991 crosses were of cultivars Frances, Barkate and Liprior. In both years all Fa (E-) plants were of ecotype Bn 1180 with the exception of one cross in 1990 in which a plant of ecotype CH5/11/4 was used. Plants selected as either female or male parents were thoroughly searched for endophytes using the leaf sheath MSM of 6-8 week old seedlings.

Parental material was grown as single plants in pots filled with compost and kept outside the glasshouse up to the crossing time i.e. near anthesis. For each pair cross, at near anthesis, inflorescences of both parents were enclosed together in an appropriate pollination bag and the isolation maintained until seed matured.

Hybrid seeds were germinated in petri dishes in October and F_1 seedlings planted individually in 35.2 x 21.5 x 6.3 cm plastic trays with 12-15 small pot inserts which were kept in the glasshouse for 8-10 weeks for seedling tests or moved outside in February and sampled for MSM in May.

Detection of endophytes

Four techniques were used to detect the presence or absence of *A. lolii* in progenies of Lp and Lm (E-) x Lp (E+) and *A. coenophialum* in Fa (E-) x Fa (E+) and Lm (E-) x Fa (E+): Staining of mycelium and microscopic examination (MSM), tissue print-immunoblot (TPIB), protein-A sandwich enzyme linked immunosorbent assay (PAS-ELISA) and enzyme linked immunosorbent assay (ELISA).

MSM adopted was as described by di Menna and Waller (1986) and Welty et al. (1986 a,b). This method was used to detect endophyte in both plants and seeds. Twelve to 20 plants were tested per hybrid progeny and five vegetative actively growing tillers were collected in each plant in May 1991 and 1992. One leaf sheath was taken from each tiller, stained with lactophenol-cotton blue and examined under a compound microscope at 400 magnification. Seeds of each cross made in 1991 were soaked overnight in a 5% sodium hydroxide solution containing 0.1% trypan blue and after being gently washed in running tap water were stained and 50 were microscopically observed at 400 magnification.

TPIB assay, as reported in Gwinn et al (1991), was used to search for *Acremonium* endophytes in F_1 seeds of the hybrids made in 1990 and 1991. As the seed available in

the 1990 crosses was small only 10-20 seeds were assayed per progeny while 50 seeds were analysed for each 1991 hybrid. In the analysis carried out in Lexington in 1993, the softened seeds soaked in sterile water overnight were individually ground in 50 ul of water with a glass rod and 20 ul portions were used in the TPIB assay.

PAS-ELISA, described by Reddick and Collins (1988) and considered as more reliable than ELISA in predicting infection levels, was applied to find the percentage of *Acremonium* infection in hybrid seedlings. Fifty seedlings (8-10 weeks old) were assayed in the progenies of the 1991 crosses whose seeds were also submitted to TPIB.

ELISA, as outlined by Johnson et al. (1982), was only used to detect the endophytes in herbage samples taken in July 1991 from plants of two 1990 hybrids which were previously MSM tested.

N-acetyl and N-formyl loline alkaloids were determined using a modified capillary gas liquid chromatographic method with phenylmorphiline as the internal standard (Kennedy and Bush, 1983).

RESULTS

Progenies of 1990 crosses

Table 1 shows results of the individual seed TPIB assay of samples of the progeny of each cross tested in September 1990 and June 1993. Fifteen progenies had a negative TPIB and only two, Frances (G) x Grasslands Nui (16) and Bn1180 (4) x Kentucky 31 (7), had a number of seeds with positive reaction. These two crosses were assayed again in June 1993 and seeds with positive reaction were detected. Seeds of these two hybrids, picked from the same lots that were TPIB assayed, were germinated in petri dishes with moistured filter paper in an incubator in October 1990 and seedlings were single planted in trays and kept in the glasshouse up to February 1991. Then the plants were moved outside and 14 of the progeny of the first cross and 24 of the second were MSM (leaf sheath) tested in May 1991 and found to be free of *Acremonium* infection. Herbage samples were taken in July 1991 from each hybrid plant MSM analysed and assayed with ELISA for the presence of *Acremonium*. All plants had a negative ELISA reaction which agrees with the MSM results, that the endophyte was not transmitted via the pollen parent. The herbage samples of Bn1180 (4) x Kentucky 31 (7) plants were also analysed for loline alkaloids and the results were all negatives.

Progenies of 1991 crosses

Seed of each hybrid progeny produced in summer 1991 was stored at -15^{o}C and used in the tests carried out in 1992 and 1993 at Oak Park, Knoxville and Lexington.

Table 2 shows that a fungal infection was detected in seeds of 10 hybrids which were MSM tested in 1992. Non-convoluted septate hyphae were lying between the testa

and aleurone layer in infected seeds. In the hybrid seeds only a few pieces of hyphae were found in the aleurone layer. This is in contrast with what was observed in Grasslands Nui and Kentucky 31 where the hyphae were abundantly seen in the intercellular spaces of the aleurone layer. The hyphae observed in the hybrids where the maternal parents were plants of cultivars Barkate and Liprior showed some resemblance with those detected between the testa and aleurone cells of infected seeds of Grasslands Nui (Figs. 1 and 2).

Table 2 indicates also that the TPIB reactions of the hybrid seeds assayed at Knoxville were all negative but a higher than normal background assay reaction was found in the perennial ryegrass hybrids. One seed with positive reaction was found in three hybrid progenies by the TPIB in Lexington. This discrepancy between the results of Knoxville and Lexington assays will be considered in the discussion.

Seedlings from the same seed lots which were TPIB analysed were assayed for PAS-ELISA at Knoxville when they were 8-10 weeks old and the reactions were all negative (Table 2). These results are in good agreement with those of the MSM (leaf sheath) tests carried out in May 1993 at Oak Park where all plants analysed were completely free of *Acremonium* infection.

DISCUSSION

Figure 1 Mycelium of *Acremonium lolii* in the testa of a Grassland Nui seed. Bar = 10 um.

Figure 2 Endophytic hyphae in the testa of a seed of Liprior (E-) x Grasslands Nui (E+). Bar = 10 um

The results reported show that all F_1 plants of progenies of E- x E+ crosses were free of *Acremonium* infection. However, some relevant information was collected in this research which deserves to be considered. In certain progenies of 1991 crosses the presence of fungal hyphae lying between the testa and aleurone layer was revealed by MSM. Although these hyphae show some resemblance with those of *Acremonium* spp. observed in infected seeds of the male parents, as shown in Figs. 1 to 4, there is no proof that they are an *Acremonium* endophyte.

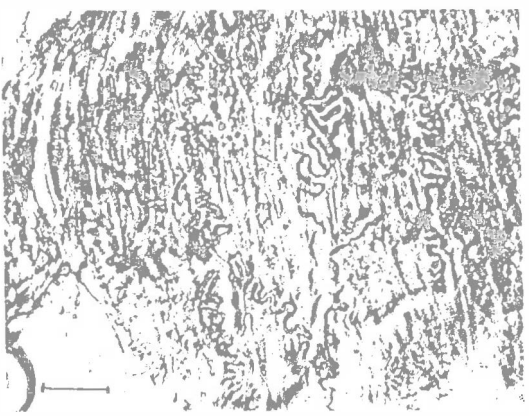

Figure 3 Mycelium of *Acremonium coenophialum* in the testa of a Kentucky 31 seed. Bar = 10 um

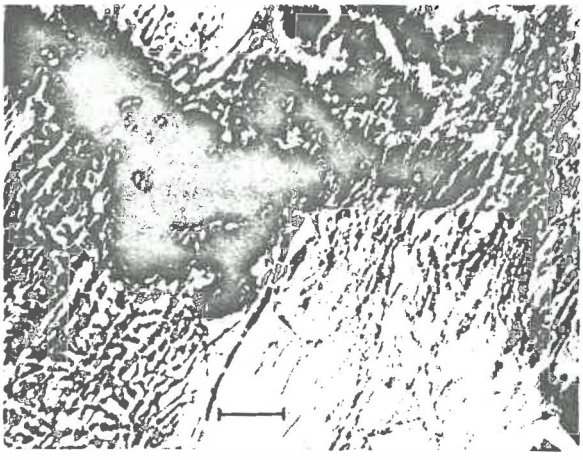

Figure 4 Endophytic hyphae between the testa and aleurone layer of a seed of Bn 1180 (E-) x Kentucky 31 (E+). Bar = 10 um

The fact that no infected plants originated from seeds of these crosses demonstrates that even if the hyphae were an *Acremonium* spp they were too distant from the embryo to cause infection. Philipson and Christey (1986) showed in their electron microscope investigations the endophyte was present within both *Lolium* and *Festuca* embryos before the scutellum had been differentiated. They also stated that it is likely that the endophytic mycelium lying beneath the testa and outside the aleurone layer is subject to eventual degradation.

The interpretation of TPIB data is difficult due to discrepancies between results obtained in the two laboratories. It is possible that the positive reactions recorded at Lexington are false positives.

Tests done at Knoxville had a higher than normal background reaction in seeds of progeny of *Lolium* crosses and this is explained as the effect of interfering proteins which is greater in ryegrasses. Thus, it is possible that the false positives suggested for the Lexington tests were caused by contaminating plant proteins.

Also, it is possible that the antiserum used at Lexington is more sensitive and may have allowed detection of endophyte at low frequency and low density levels. If this is true it means that the false positives are a true indication of endophyte hyphae detected between the testa and aleurone layer. Studies are in progress at Knoxville and Lexington comparing the two antiserum using seed halves and ground seed. If the question cannot be resolved using the TPIB assay then the only way to identify the endophyte from the crosses that gave TPIB positive reactions would be to recover it from seed plated on agar.

If the mycelium detected between the testa and the aleurone layer in seed of specific hybrids is an *Acremonium* endophyte then there is a need to find why all the

hybrid plants assessed were free of infection. It is possible, as stated above, that the mycelium did not reach the embryo and therefore the seedling was not infected. What would be the factors that stop the growth of fungal hyphae? Low density and the incompatibility between the fungus and host are among the possible causes of arresting of hyphae growth. The lack of lolines in the F_1 plants of Bn1180 (4) x Kentucky 31 (7) is another indicator of the possibility that infection in the seeds does not carry through to the plant due to incompatibility or some other factor. However, abiotic factors such as environmental conditions, particularly air temperature and relative humidity (RH), at the time of pollination and during the seed formation may have an important role in the growth of an endophytic mycelium. Thus in the glasshouse, pollination and subsequent seed development occurred under very high temperatures and low RH i.e. under conditions different from those prevalent in the field. Once the transfer of the endophyte via the pollen parent takes place in an adverse environment (very hot and dry) the growth of the fungus could certainly be affected.

Pair crosses to breed interspecific Lp and Lm x Fa hybrids were made at Oak Park by bagging together the inflorescences of both parents which were growing in the field. Leaf sheath MSM of a number of F_1 hybrid plants from Lm x Fa crosses showed, as reported by Do Valle Ribeiro (1993), that in 4 of 19 plants examined an endophyte was present and GC analysis detected loline alkaloids at levels between 111 to 827 ug/g. Bush et al. (1982) found that the accumulation of loline alkaloids in tall fescue is positively associated with the presence of *A. coenophialum*, therefore the endophyte present in the four hybrid plants was introduced into the hybrid seed from the *Festuca* parent. Siegel (M. Siegel, unpublished, 1993) found that in a Lm "Marshall" (E-) x Kentucky 31 (E+) cross, the F_1 plants were infected by an endophytic fungus with an ELISA positive reaction and when isolated from seed planted onto agar was morphologically very similar to *A. coenophialum*. However, it should be noted that while the seed was infected via the male parent, without growing the plants it cannot be said that the endophyte in the hybrid was a stable and compatible association.

In conclusion the research just described is a first step in the investigation of possible non-maternal transmission of both *A. coenophialum* and *A. lolii*. The study should be pursued including as suggested by Philipson (M. Philipson, personal communication, 1993) electron microscope examination of stamens and anthers from infected plants to determine whether these tissues are infected. The occurrence of *Acremonium* infection via the pollen parent, if proved, will have serious implications in grass breeding.

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Table 1: Individual seed reaction of hybrids assayed at Lexington in September 1990 and June 1993 (1990 crosses)

Hybrids	TPIB seed (+)/total	
	1990 (a)	1993 (b)
Frances (F) x Grasslands Nui (3)	0/10	
Frances (G) x Grasslands Nui (16)	2/8	2/20
Frances x Grasslands Nui (19)	0/10	
Weldra (2) x Grasslands Nui (14)	0/10	
Weldra (3) x Grasslands Nui (7)	0/10	
Weldra (5) x Grasslands Nui (9)	0/10	
Weldra (9) x Grasslands Nui (1)	0/10	
Weldra (10) x Grasslands Nui (15)	0/10	
Weldra (8) x Grasslands Nui (10)	0/10	
Weldra (1) x Kentucky 31 (3-7)	0/10	
Weldra (2) x Kentucky 31 (13)	0/8	
Weldra (4) x Kentucky 31 (9)	0/8	
Weldra (7) x Kentucky 31 (4)	0/10	
Bn1180 (4) x Kentucky 31 (7)	5/10	3/27
Bn1180 (4) x Kentucky 31 (13)	0/10	
Bn1180 (2) x Kentucky 31 (5)	0/10	
CH5/11/4 (14) x Kentucky 31 (11)	0/10	

(a) = TPIB of seed halves
(b) = TPIB of whole ground seed

Table 2: Results of MSM (seed and leaf sheath), TPIB and PAS-ELISA tests of progeny of E- x E+ crosses made in 1991

Hybrids and Lp cultivar	MSM infected seeds/total	TPIB seeds (+)/total		PAS-ELISA (+) seedlings %	MSM (leaf sheath) infected plants %
		Knoxville	Lexington (b)		
Barkate (1) x G. Nui (9)	5/50	0/50	1/10	0.0	0.0
Barkate (2) x G. Nui (7)	15/50	0/50	0/10	0.0	0.0
Barkate (3) x G. Nui (19)	2/50	0/50	0/10	0.0	0.0
Liprior (1) x G. Nui (18)	11/50	0/50		0.0	0.0
Liprior (3) x G. Nui (17)	20/50	0/50	1/10	0.0	0.0
Frances (G) x G. Nui (16)	2/50	0/50		0.0	0.0
Frances (G) x G. Nui (1)	0/50	0/50			
Grasslands Nui (19)	50/50 (a)			100.0	93.8
Bn1180 (4) x Ky. 31 (7)	8/50	0/50	1/19	0.0	0.0
Bn1180 (4) x Ky. 31 (14)	23/50	0/50		0.0	0.0
Bn1180 (4) x Ky. 31 (7A)	0/50				
Bn1180 (4) x Ky. 31 (7B)	2/50				
Bn1180 (4) x Ky. 31 (17)	0/50				
Bn1180 (4) x Ky. 31 (20)	10/50				

(a) = *Acremonium lolii*
(b) = TPIB of whole ground seed

ARTIFICIAL INFECTION OF TILLERS FROM PERENNIAL RYEGRASS MATURE PLANTS WITH *ACREMONIUM* ENDOPHYTES

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Key words: *Acremonium* endophytes, artificial infection, *Lolium perenne*.

Introduction:

Inoculation is of the utmost importance in studies of the influence of *Acremonium* endophytes on its host.The seedling inoculation reported by LATCH and CHRISTENSEN (1985) is relatively easy but does not permit the inoculation of the same genotype with several strains of endophyte. Thus other inoculation methods without this disadvantage are reported by JOHNSON et al (1987), O'SULLIVAN and LATCH (1993) to study the efficiency of different strains using respectively callus and meristem cultures. Both methods theoretically allow inoculation of the same grass genotype with several strains of *Acremonium*. However, they are more time consuming and have some disadvantages : somaclonal variations can occur by means of callus culture and obtaining regenerated plants by means of meristem culture is not easy.

Therefore, due to the lack of effective inoculation methods of mature plants to obtain a single grass genotype inoculated by several strains of endophyte, this paper deals with an attempt to inoculate tillers in axenic conditions.

Materials and methods :

Tillers were removed from the base of mature plants of perennial ryegrass. The basal part (1-2 cm) of the tiller was excised and the roots were removed. The explants were sterilized by soaking in 70% ethanol for 5 minutes followed by rinsing in sterile water, soaking for 15 minutes in chlorine bleach (0.5 % available chlorine) and again rincing in sterile water. Finally, they were soaked in a bayrolchlore solution for 20 minutes and rinced 3 times in sterile water. The explants were air-dried on sterile filter paper. We used only healthy tillers in order to reduce contamination which was very high with tillers from mature plants).

The explants were inoculated by 2 methods : 1) a small piece of mycelium on P.D.A. medium was placed in a longitudinal slit (5 mm) made at the base of the explant;

2) a solution of crushed mycelium in sterile water was injected at the base of the explant.

The inoculated tillers were placed upright in Petri dishes containing Miller medium, Fugi vitamins and hormones (0.5 g/l IAA and 0.5 g/L NAA). The dishes were maintained in the dark for 3 days and in a growth chamber (22°C, 12 hours light cycle) until the tillers had produced enough roots and leaves. Then, they were transplanted into compost in a greenhouse and one month later texamined for signs of infection.

In this experiment we used the cultivar Vigor and one strain of *Acremonium* able to sporulate and originating from the South of France. Endophyte infected plants had alreday been obtained with this material by means of sterile plants, showing that this association was possible.

Results and discussion :

22 plants out of 360 were successfully inoculated by either method. Altough this result is not very optimistic. The methods show that inoculating tillers from mature plants is possible. The success rate is about 6%. Similar results are reported by LATCH and CHRISTENSEN (1985).

The most serious problem was the level of infection by saprophytic fungi or bacteria and achieving sterile tillers was very difficult. This depended on the age of the mature plant used (contamination increased with age) and its origin (field or greenhouse).

Checking for the endophyte presence after inoculation could be difficult. One month after transplanting 14 infected plants were found, with a further 8 detected after two months. Thus, time of examination is critical if carried out too early, association may not be sufficiently developed, if too late, incompatible reactions may have occurred.

Whatever the inoculation method used (a small piece of mycelium on PDA or a drop of crushed mycelium in sterile water), the percentages of infected tillers obtained were similar. Thus we now use the less time consuming method ie.introducing a drop of crushed mycelium at the meristem level with a needle.

In order to show that the method described in this paper is not dependant on genotype or endophyte strain, we used it to infect tillers coming from another European endophyte free cultivar (Parcour) and another strain isolated from a wild perennial ryegrass population originating from Spain.

With Parcour inoculated with the French strain, we obtained 7 endophyte infected tillers and 41 endophyte free tillers. Unfortunately, we failed to obtain endophyte infected tillers with the Spanish strain using both cultivars. We failed also to obtain these combinations despite inoculating 300 seedlings of each cultivar. Similar results were reported by LATCH and CHRISTENSEN (1985) who concluded that the percentage of

inoculated seedlings which became infected varied greatly in different experiments using different inoculation methods and host endophytic associations. LEUCHTMANN and CLAY (1993) also suggested significant differences in host range, as measured by seedlings inoculation, between several isolates of *Epichloë typhina* which would be related to *Acremonium* endophytes (CLAY, 1993). HUMES (1993) also mentioned problems encountered by CHRISTENSEN (unpublished data) with artificial endophyte infected material mainly concerning the compatibility and stability of some novel endophyte strain/plant genotype associations.

To conclude, we are now able to inoculate several mature plant genotypes with *Acremonium* endophyte and we also note the high level of specific adaptation of endophytes for particular hosts as suggested by CLAY (1993).

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ENDOPHYTIC FUNGI IN TALL FESCUE IN ITALY

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KEYWODS: Endophyte, incidence characteristics , tall fescue, Italian cultivar register

ABSTRACT

Tall fescue is a host of the endophytic fungus Acremonium coenophyalum Morgan-Jones and Gams. Seed samples of sixteen cultivars registered in Italy and five additional foreign cultivars of tall fescue were analyzed. Endophyte hyphae characteristic of Acremonium spp were found in registered cvs Tanit (40%), Titan (57%), Vera (6%), and Clarine (9%).

From seeds and sheaths of young plants of Tanit, one fungus differing from A. coenophyalum, mainly in the conidial size (4,5-7 mm), has been isolated.

This isolate could be accommodated within a new taxonomic group of Acremonium endophytes of tall fescue with shorter conidia. As reported by other researchers, this new group seems to be associated with the southern populations (South Europe and North Africa) of tall fescue, and the Sardinian (Italy) origin of Tanit confirms this hypothesis.

INTRODUCTION

Tall fescue (Festuca arundinacea Schreb.) is a grass of minor importance in Italy, and only few local varieties have been registered in the official list.
Like other grasses it is host to mutualistc seed-born endophytic fungi of the genus Acremonium Link sect. Albo-lanosa Morgan-Jones and Gams. In Italy the level of the contamination by these fungi is unknown. With this preliminary study we present information regarding the occurrence of endophytic fungi in commercial tall fescue verieties in our Country.

MATERIALS AND METHODS

Twenty one cultivars grown in Italy - sixteen of which are registered in the Italian Variety List - were analysed; one hundred seeds per sample were stained with 0.1% cotton blue in lacto-phenol after digestion in a 3% NaOH solution, and observed under the microscope. The seeds were surface sterilised by soaking in 50% sulphuric acid for 20 min followed by rinsing with sterile water, soaking in 10% sodium hypochlorite for 20 min and rinsing again with sterile water. Leaf sheath tissues of plants grown from seed from the same sample, were surface sterilised by dipping in 90% ethanol, then shaken in a

solution of 10% sodium hypochlorite. Sterilised seeds and sheaths were immersed in potato dextrose agar (PDA) in Petri dishes and incubated at 22°C in the dark.

Marginal fragments of the colonies were transferred onto PDA plates, grown at 22°C in the dark, and compared to Acremonium coenophyalum TF 31 isolated from tall fescue in New Zealand, and to Acremonium sp. TF 33 isolated from tall fescue cv Triumph in the U.S.A. (1).

Mycelial plugs of endophyte cultures were placed on the centre of Petri dishes containing PDA, and grown for four weeks in the dark at 22°C. Sub-cultures from the margins of colonies of Drechslera erythrospila (Drechsler) Shoemaker and D. graminea (Rabenh, ex Schlecht) Shoemaker were placed at the edge of the plates. The amount of antibiosis was assessed after one week by measuring the width of the zone of growth inhibition surrounding the Acremonium plug.

RESULTS

Seeds of the cultivars Tanit, Titan, Vera and Clarine showed characteristic hyphae of the Acremonium endophyte, with an incidence of 40%, 57%, 6% and 9% respectively (Tab. 1). An Acremonium isolate belonging to the section Albo-lanosa Morgan-Jones was isolated from Tanit. Isolations attempted from other cultivars were unsuccessful. Aerial mycelium appeared white, brain-like and bearing abundant conidia. There was no variation in colony morphology among different isolates. Nevertheless, they showed different growth rates (0.37 mm/d) compared to A. coenophyalum TF31 (0.55 mm/d) and Acremonium sp. TF33 (0.80 mm/d), and the conidia were shorter (4.5-7 μm) than in isolates TF31 (9-12 μm) but similar to those of TF33 (6-8 μm).

The three isolates compared showed different inhibitory activity, but there was no difference between the test fungi Drechslera erythrospila and D. graminea (Tab. 2).

CONCLUSIONS

Until recently A. coenophyalum was the only endophytic fungus found in F. arundinacea. Recent studies (Christensen et al., 1991, Christensen et al., 1993) reported the presence of other Acremonium endophytes, with shorter conidia, not belonging to A. coenophyalum. All fungi isolated from F. arundinacea were accommodated within three taxonomic groups (A. coenophyalum, FaTG2 and FaTG3) based on the morphological characters of colonies and conidia, as well as on the isozyme phenotypes and the production of alkaloids by the plant. Before our findings, Acremonium with shorter conidia was found only once in a North American cultivar, Triumph, and it was accommodated within FaTG2.

Tab. 1 - Incidence of the endophyte Acremonium in tall fescue cultivars.

CV	Origin	% of infected seeds
1) ARPA	I	0
2) ASTICO	I	0
3) BARVETIA	NL	0
4) CELONE	I	0
5) CLARINE	F	9
6) LINCE	I	0
7) MAGNO	I	0
8) ONDINE	F	0
9) PENNA	I	0
10) SAMANTHA	I	0
11) SIBILLA	I	0
12) TANGAROA	I	0
13) TANIT	I	40
14) TITAN	USA	57
15) VERA	I	6
*16) BARCEL	NL	0
*17) M. KASBA	GB	0
*18) M. JEBEL	GB	0
*19) DOVEY	GB	0
*20) SOPLINE	F	0

cultivars not registered in the Italian Variety List.

The Acremonium sp isolate from Tanit was different from A. coenophyalum and Acremonium sp described in Triumph. Based on the above classification, but lacking at present information regarding the isozymes pattern and the alkaloids, our isolate seems to belong either to FaTG2 or FaTG3.

Other similar isolates were always found on ecotypes or populations originating from Spain, Portugal and North Africa. The origin of Tanit - a synthetic variety derived almost exclusively from parentals selected from natural populations collected in Sardinia, Italy - confirms the hypothesis of the existence of a southern population of F. arundinacea infected by Acremonium with shorter conidia, compared to a northern population infected by A. coenophyalum.

(1) The Authors wish to thank Garry Latch and Mike Christensen, AgResearch Grassland, Palmerston North, New Zealand, who supplied the two Acremonium isolates.

Tab. 2 - Range of variation among Acremonium isolates.

Isolate	Growth rate (1)	Lenght of conidia(mm)		Antifungal activity(2)	
		Range	Mean	D.e.	D.g.
A.coenophyalum sp TF 31	0.55	9-12	10.3	-	-
Acremonium sp TF 33	0.80	6-8	7.1	++	++
Acremonium sp Tanit	0.33	4.5-7	5.9	+	+

1) Radial extension (mm/d) at 22°C in the dark after 15 days;

2) - : no inhibition;
+ : slight inhibition (zone of inhibition < 10 mm);
++ : moderate inhibition (zone of inhibition 10-20 mm);
D.e.: Drechslera erythrospila;
D.g.: Drechslera graminea.

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A SURVEY ON THE OCCURRENCE OF ENDOPHYTIC FUNGI IN EUROPEAN SEED LOTS OF *LOLIUM PERENNE*

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Key words: endophytic fungi, seed lots, Europe, *Lolium perenne*

Abstract

88 European seed lots of different ryegrass cultivars (*Lolium perenne* L.) were examined for the presence of endophytes by staining with lactophenol/aniline blue. Endophyte mycelium was found in eight cultivars.

In contrast to Australian and New Zealand ryegrass cultivars which are often between 80 - 100 % infected with endophytes of the genus *Acremonium,* the European cultivars had only a maximum of 28 % infection. Endophyte was found in Dutch, British, and French cultivars. Though the incidence of infection was highest in Dutch cultivars levels only reached a maximum of 15 %. The highest infection level was found in one of two British cultivars with 28 %. No infected German cultivar has been found, yet.

To estimate the level of vital endophytes in the seed lots tested, plants were grown from the infected seed samples in a climate chamber under controlled conditions. Endophyte infection was tested by staining leaf sheaths with aniline blue. Endophytic mycelium was found in only three of the eight cultivars. The level of infection was 3.7, 4.2 and 6 %, respectively, but never reached the level of the corresponding seed lots.

In order to identify the endophytes isolations were made from tillers of the seedlings found to be endophyte infected. Isolated mycelium was cultivated on four different media. Identification of the isolates is still in progress.

Introduction

No reports on the significance and effects of endophytic fungi in grasses are available in Germany although intensive research has been carried out in Australia, New Zealand and the USA for over 15 years. In Germany as yet there is no information on where these endophytes occur, to what extent, and in which grass hosts they can be found. Also there are no reports on the effects of secondary metabolites of this symbiosis on their hosts or on pests or grazing animals, as

described by BACON (1977). This is despite the fact that in other European countries like the United Kingdom, Italy, France and The Netherlands endophytic fungi of the species *Acremonium* were detected and reported many years ago. Intensive work has since been carried out and results indicate that the *Acremonium* endophyte seems to be quite common in these countries (LEWIS, 1986; Grand-Ravel et al., 1992), not only in perennial ryegrass (*Lolium perenne*). There are even reports of toxic effects (ryegrass stagger), from the United Kingdom (LEWIS et al. 1989) and the Netherlands (VERMEULEN, 1986,) where eczema solare has been reported, indicating the importance of research on the significance and effects of these endophytes in Germany as well.

Apart from negative effects, there has been no research related to the resistance effects that endophytes may confer against various insect pests (JOHNSON et al., 1985) and fungal diseases (WHITE & COLE, 1985). It is unknown for instance whether aphid resistance occurs in Europe as reported by PRESTIDGE et al. (1982) in New Zealand, or if there are any effects on plant growth such as better drought stress resistance and enhanced biomass production.

To investigate the ocurrence of *Acremonium* endophytes in Germany, we started to examine a range of German and European seed lots of perennial ryegrass (*L. perenne*) on the occurrence of endophytic fungi. From the seed lots found to be endophyte positive, plants were grown on and the endophytes were isolated and cultured on four media under five conditions in vitro to characterise and identify the endophyte.

First results of this survey are presented in this paper.

Materials and Methods

As material we used 88 seed lots of partly certified perennial ryegrass cultivars (*L. perenne*) from 7 European countries, including 26 German cultivars, kindly provided to us by Dr. Feuerstein.

100 seeds of each cultivar were tested using the aniline blue staining method based on the technique described by WELTY (1986). The microscopic examination was carried out on squashed seeds with a Zeiss light microscope at x 160 to x 400 fold magnification.

The solutions 1 and 2 used for the staining of seeds are listed in Table 1.

From the seed lots found to contain endophytic mycelium, plants were grown in a climate chamber with 12 hours day/night cycles at 17°C / 12°C, respectively, to estimate the fungal vitality. After six to eight weeks, depending on plant growth, 100 plants of each cultivar were tested for the presence of endophytic mycelium by

isolating a leaf sheath and staining with aniline blue as described by WELTY (1986) (Table 1, solution no. 3).

Table 1. Solutions for staining endophytic fungi in seeds and plant material of grasses

Solution no.	Ingredients	Application
1	5 % NaOH 1 % aniline blue	soaking of seeds
2	100 ml lactic acid 100 ml glicerine 100 ml liquified phenole 800 mg aniline blue 500 ml demin. water	staining of the soaked and water washed seeds
3	1 part lactic acid (85 %) 2 parts aniline blue (1 %)	staining of endophytes in leaf sheaths

Endophytes were isolated from tillers of plants containing endophytic mycelium according to the technique described by BACON (1977). This technique involves surface sterilisation with 1% sodium hypochloride for 20 minutes and placing piecesof about 5 mm long of the basal end of tillers on potato dextrose agar (PDA) under sterile conditions and incubating at 25°C in the dark for four to six weeks.

To characterise and identify the endophytes isolates were cultured on four different media under various conditions. Media and conditions are listed in table 2.

Results and Discussion

In eight of the 88 seed lots tested we found endophytic mycelium.
Infection levels varied from 0 to 28,2 % (Table 3).

In seed samples from Germany, Ireland, Sweden and Denmark no endophyte infection was found, but infection did occur in seeds from the United Kingdom, France, and The Netherlands. In Dutch cultivars infection levels reached a maximum of 15 % and in one of two British cultivars 28 % of the seeds were found to be infected. We observed a difference in the location of endophytic mycelium.

Table 2 Culture media and conditions used for the in vitro differentiation of endophyte isolates

Media*
1) Potato dextrose agar (PDA, Merck)
2) Potato dextrose broth (Difco) and 1% agar (Merck)
3) 2% Malt extract (Difco) and 1% agar (Merck)
4) Cornmeal agar (Difco)
Culture conditions
1) 17°C dark
2) 17°C nUV (12h nUV, 12h dark)
3) 22°C dark
4) 22°C nUV (12h nUV, 12h dark)
5) 22°C 12 h light

*every medium was used under all five conditions

Table 3 Origin and number of endophyte containing seed lots of *L.perenne* tested

country of origin	total number of cultivars	number of endophyte containing cultivars
Denmark	8	0
Sweden	1	0
France	3	1
United Kingdom	2	2
Ireland	1	0
The Netherlands	27	5
Germany	26	0

In most cases the majority of endophytic mycelium was found between the cells of the aleuron layer of the seeds (Plate 1), but we also found many seeds with almost no mycelium in the aleuron layer but a dense mat of mycelium lying between the aleuron layer and the inner seed sheath.

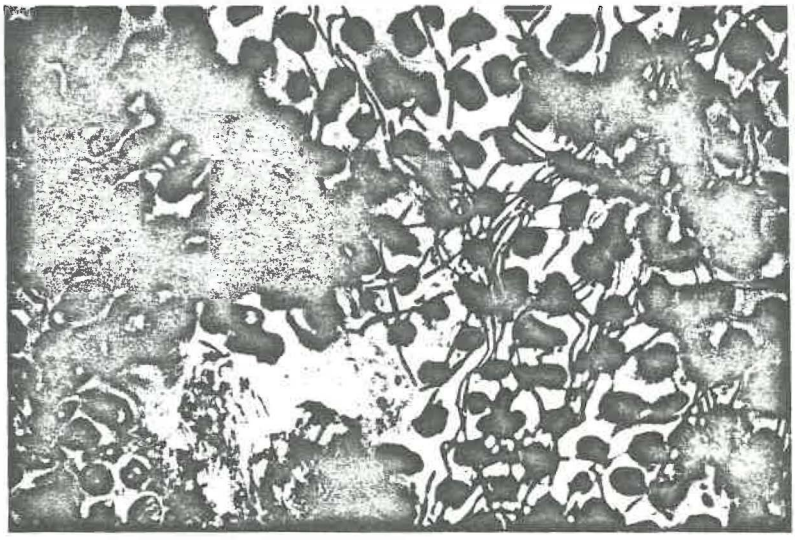

Plate 1 Endophytic mycelium between the cells of the aleuron layer stained with aniline blue

This situation was found in several seeds of most of the lots tested. Whether the location of endophytes in the seeds is due to different endophyte spcies or perhaps due to the culture conditions seeds were grown in has not yet been determined.

The assessment of endophyte vitality by detecting them in seedlings grown from infected seed samples showed that in no case did the infection level of the seedlings reach the infection of the corresponding seed lots (Table 4). This may be due to the storage and/or culture conditions the seeds were germinated in. Unfortunately the origin, age, and storage record of several seed lots is unknown.

The reason for the loss of the endophyte vitality in seeds is probably due to a too long and / or too dry and warm storage of the seeds (SIEGEL 1981). Maybe during such a suboptimal storage of their host seeds the endophyte dies or is weakened and not capable of surviving in its host for more than several weeks.

Table 4 Endophyte contents of the *L.perenne* seed lots and seedlings tested

country of origin	no. of endophyte containing cultivars	endophyte containing seeds in %	endophyte containing seedlings in %
Denmark	0	0	0
Sweden	0	0	0
France	1	1	0
United Kingdom	2	1 / 28,2	0 / 5,2
Ireland	0	0	0
The Netherlands	5	3 / 4 / 6,3 / 15 / 26	0 / 0 / 1 / 0 / 3,7
Germany	0	0	0

As an identification of endophyte species by microscope is not possible in vitro growth of the endophyte is required. Endophytes were thus isolated from infected seedlings on PDA-medium and cultured on the media and conditions shown in table 2. Though clear differences in *in vitro* growth were apparent, identification of the isolates is still in progress.

When endophytes have been identified we hope to be able to decide whether the differences in location of endophytes in seeds are due to different types of endophytes or due to other, yet unknown, growth conditions. First results of our investigation indicate, that the differences in location of endophytic mycelium in seeds may be due to different species, or different strains.

We found clear differences in the contents of endophytic mycelium in leaf sheaths varying from very few, short hyphae near the base of the leaf sheath to numerous hyphae running through the entire length of the samples excised. From earlier experiments we know that endophytes sometimes die in host plants growing in the greenhouse without any known reason, but up to now this observation did not appear with the weak presence of the fungus in the seedlings or the seeds.

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A NOVEL AND RAPID STAINING METHOD FOR THE DETECTION OF VITAL ENDOPHYTES IN SEEDS AND LEAF SHEATHS OF *LOLIUM PERENNE*

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Key words: endophyte, rapid detection, vital staining technique

Abstract

To estimate the level of vital endophyte in infected seed lots it is common to let the seeds germinate and grow for three to six weeks. A leaf sheath is then removed and tested by staining with aniline blue for endophyte presence. Up to now this is the only effective method besides isolating the endophyte from the seeds in vitro, to determine whether the endophyte is viable or not. These procedures are very time and space consuming.

In our experiments we developed a method to estimate the endophyte´s vitality within three days with a minimum of labour and space.
This method is based on staining with Fluoresceine diacetate (FDA), a common method to estimate vitality of plant cell cultures. Although a microscope with an ultraviolet light source and a special filter system is necessary, the rapid preparation procedures and the low cost materials are advantageous.

To check the reliability of this detection method, the common approaches using seedlings and direct isolation from the seeds were carried out in parallel.
Compared with the results from seedlings grown from endophyte infected seeds, and tested for endophyte presence, the FDA-method resulted in significantly higher levels of vital endophytes. These findings were confirmed by data from the isolation of endophytes from seeds in vitro.

Our investigations indicate that testing seedlings is not a reliable method to estimate the level of vital endophyte in seed lots of ryegrass (*Lolium perenne* L.). We also found that the germination- and culture-temperature has an important effect on the ability of the endophyte to grow into the developing plant.
Further investigations to determine the growth temperature which results in maximum endophyte infection of seedlings are in progress.

Introduction

In view of the biochemical effects which are induced by the presence of endophytic fungi in grasses (BACON et al., 1986), it is most important for farmers and plant breeders to be able to determine rapidly the level of vital endophyte in a seed lot. Up to now, the only methods to test for this are to check germinated seedlings for the presence of endophytic hyphae or to isolate the endophyte directly from the seeds. This method is very time and space consuming. The number of samples that can be tested is limited by laboratory and greenhouse space and results are availiable at the earliest within three to six weeks. In addition, these results depend on growing conditions because vital endophytes do not always grow into host plants. The aim of this work was to develop a quick and efficient method which gives information not only about the presence of endophytes in seed lots and in plants but to obtain information about the level of vital endophyte.

Based upon the fact that flourescein diacetate (FDA) is commonly used in plant cell cultures to test the contents of living cells in a suspension culture (WINDHOLM, 1972) and according to DHINGRA & SINCLAIR (1985) can also be used for the detection of vital fungi in soil samples, too, we checked its ability to stain endophytic mycelium.

With this method we are now able to test the endophytes vitality within three days in seed lots instead of weeks and within about two minutes in leaf sheaths.

Materials and methods

For the staining procedure we used a 5% (w/v) FDA stock in acetone, diluted in cold water 1 : 200 (v/v) as a staining solution. This staining solution was prepared freshly before use and kept cool (3 - 6°C) to avoid precipitation of the FDA.

As endophyte material we used suspension cultures of *Acremonium lolii*, *A. coenophialum* and *Epichloe typhina*. As plant material we used seed lots of 9 different *Lolium perenne* and two *Festuca pratensis* cultivars all infected with endophyte. We also used two 100% infected seed lots of *L. perenne* from New Zealand (cultivars `Coronet´ and `Ellet´) which had been heated at 57°C in water for 20 minutes and dried at 30°C in an oven for 3 h as described by LATCH & CHRISTENSEN (1982) in an attempt to kill the endophyte. In addition we used six week old seedlings grown from all of these seed lots in a climate chamber (12 h day/night cycle; 23°C / 17°C temperature scheme, respectively).

To check the ability of FDA to stain endophytic mycelium we poured 1 ml of a four week old suspension culture (cultured in 200 ml potato dextrose broth (PDB,

Difco)-medium in a 500 ml Erlenmeyer flask covered with an aluminium cap placed on an orbital shaker at 120 rpm at room temperature and daylight) in 10 ml of the staining solution. After two minutes, microscopic analysis with ultra violet light (UV, filter system 510-520 nm) was carried out.
The same procedure was carried out with four week old cultures heated for one hour at 60°C in an oven and with cultures of the same age standing still for two days with the flasks sealed with an aluminium cap and parafilm to eliminate gaseous exchange.

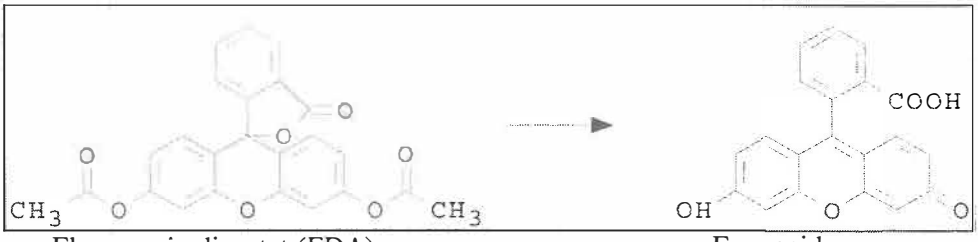
Seeds of each cultivar were soaked for 12 h at room temperature in water and subsequently soaked after removing the water in the FDA staining solution over night at room temperature. After removing the FDA solution seeds were soaked for at least 12 h in a 5% sodium hydroxide (NaOH) solution at room temperature. After washing with running tap water, the seeds were squashed and examined microscopically under UV (filter system 510-520 nm).

To stain plant material a leaf sheath was removed and the inner epidermal layer was isolated and put with the adaxial side up in a drop of staining solution on a slide. After two minutes microscopical examination was carried out as described for the seeds.

Isolation of endophytes were made on potato dextrose agar (PDA, Merck) and on cornmeal malt agar (CMM, Difco) with seeds of another sample of the *L.perenne* cultivar 'Ellet'. This seed lot was known to be 77,5% endophyte infected. Both media were supplemented with each 50 mg/l streptomycin and chloramphenicol to suppress bacterial growth. After surface sterilisation of 20 min in 5% sodium hypochloride (NaOCl) seeds were soaked in sterile water over night and again surface sterilised in 1% NaOCl for 10 minutes. Subsequently 300 seeds were placed on each medium, 30 seeds per 9 cm plastic Petri dish. Seeds were incubated at 25°C in the dark for six weeks.

Results and discussion

Our experiments show that staining endophytic mycelium with FDA is a very rapid and effective method of estimating fungal vitality. The FDA is a free cell wall permeable non fluorescent substance. In a living cell esterases split off the two acetate residues and the resulting fluorescein is fluorescent but no longer cell wall permeable (Figure 1). This molecule is not transported out of the cell, so an accumulation of the stain in the cells results.

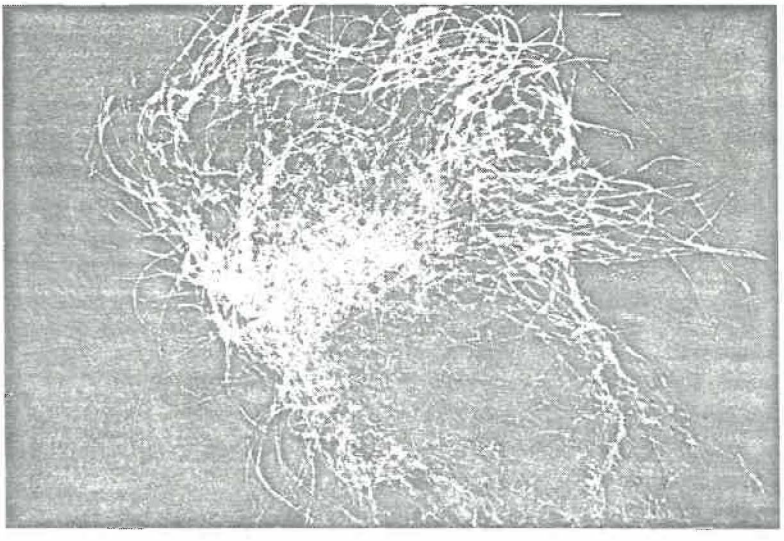


Fluorescein diacetat (FDA) Free acid

Figure 1 Structure formula of the fluoresceine diacetate molecule and its free acid

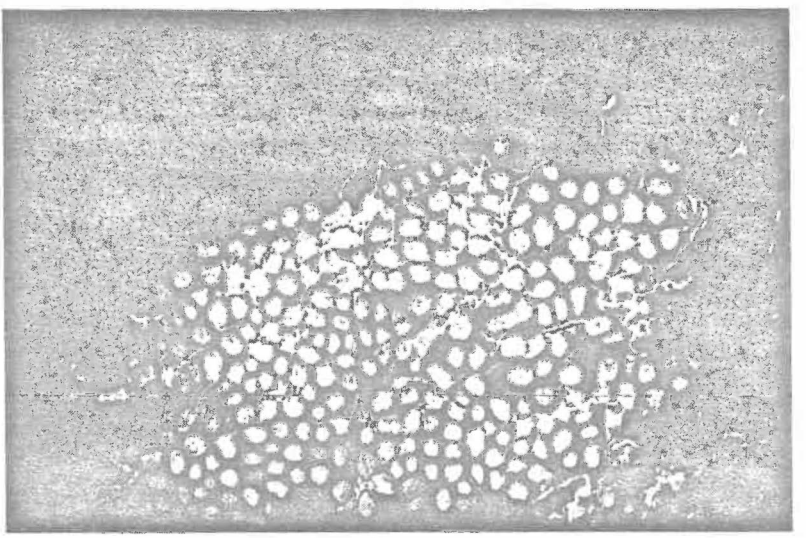
Mycelia from suspension cultures readily took up the stain and were clearly visible under a microscope in UV (Plate 1). Mycelia from heat treated colonies, or those grown under conditions of limited gaseous exchange, were not visible under UV microscopy.

Plate 1 A colony of *Acremonium lolii* from a suspension culture stained with FDA



When staining seeds with FDA, aleuron cells, and pollen grains are visible under UV . The endophyte is clearly visible between the aleuron cells and as a dense mat between the aleuron layer and the innner seed sheath (Plate 2).

Plate 2 A squashed seed of *Lolium perenne* stained with FDA showing cells from the aleuron layer and endophytic mycelium



Compared with staining seeds with aniline blue the total number of endophyte infected seeds was lower due to the fact that only vital endophytes were visible in UV. When seedlings were stained, there was no difference between the two methods besides the fact that staining with FDA was faster and easier and required no heating stage (Table 1).

The staining of seeds heated as described by LATCH & CHRISTENSEN (1982) showed a surprising result. Though both cultivars were 100 % endophyte infected according to staining with aniline blue, staining with FDA resulted in 100% infection for 'Ellet' and only 90,6% infection for 'Coronet'. After heating, 83% remained FDA positive in the cultivar `Coronet´ and 90,6% in `Ellet´. Seedlings grown from these treated seeds and tested for endophyte presence after six weeks were totally endophyte free (Table 2).

Isolation of endophytes from seeds gave two different results. On PDA the seeds with endophytic fungi reached the level measured by staining with FDA. We found 72,3% of the seeds to be endophyte containing. In contrast on CMM we only

found 33,3% of the seeds to be endophyte containing in the same incubation time (Table 3).

Table 1 Endophyte contents of the seed lots of *L.perenne* and seedlings stained with aniline blue and FDA

cultivar	endophyte containing seeds in %		endophyte containing seedlings in %	
	aniline blue	FDA	aniline blue	FDA
Barlenna	15	4,2	0	
Barmaco	26	10,6	3,7	3,7
Martlett	100	63	17,8	17,8
Tasdale	70,8	25	5,5	5,5
Coronet	100	90,6	13,7	13,7
Trophy	100	100	15,7	15,7
Nui	88,5	82,8	0	0
Ellet	100	100	0	0
Numen	6,3	6	0	0

Table 2 Contents of vital endophytes in the *L.perenne* seed lots of the cultivars 'Ellet' and 'Coronet' after heating according to LATCH & CHRISTENSEN (1982) and staining with aniline blue and FDA

cultivar	endophyte containing seeds in %		endophyte containing seeds after heating in %	endophyte containing seedlings after heating (%)
	aniline blue	FDA	FDA	FDA / aniline blue
Ellet	100	100	90,6	0
Coronet	100	90,6	83	0

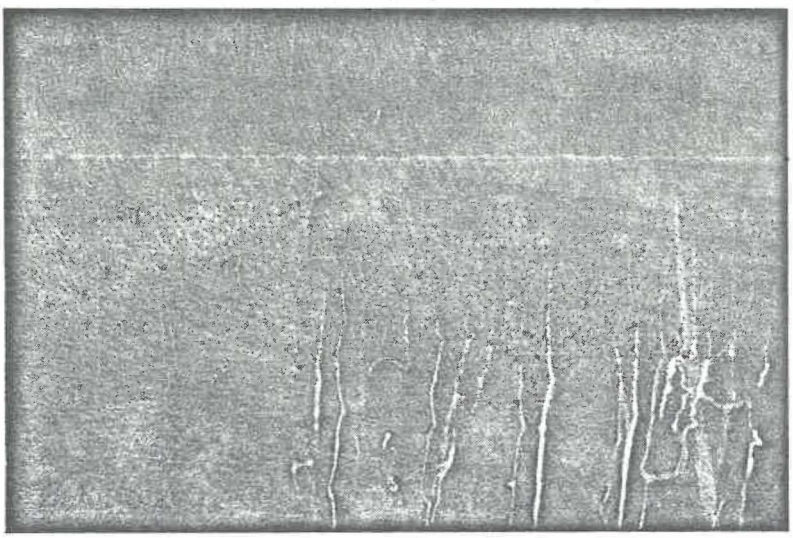
Table 3 Endophyte contents of the *L.perenne* cultivar 'Ellet' measured with three different methods

% of seeds containing	Staining with aniline blue	FAD	seedlings stained with aniline blue	direct isolation from PDA	seeds on CMM
no endophytes	22,5	23,5	24	27,7	66,7
endophyte	77,5	76,5	76	72,3	33,3
vital endophyte	?	73,1	76	72,3	33,3
dead endophyte	?	26,9	24	27,7	66,7

Endophyte detection on six week old seedlings was carried out with both staining methods. Here no difference between the staining methods was recorded. Endophytic mycelium was clearly visible between the cells in most cases.

However, FDA stained mycelium was easier to recognise since, in carefully prepared material, only the dead, non fluorescent cells of the epidermal layer are excised, and thus only the hyphae of the endophyte are visible under UV (Plate 3)

Plate 3 Leaf sheath of *Lolium perenne* containing endophytic mycelium stained with FDA



To conclude, in our opinion staining with FDA is the most efficient method for estimating the endophyte content of a seed lot and even of plants. Compared with other methods it is rapid, less critical, requires little time and space and results in direct information about the level of vital endophyte. However it must be noted that the level of vital endophyte as measured by FDA does not necessarily result in the same level of endophyte containing plants.

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Production, Product Quality, and Environment

The microflora of short rotation ryegrass and westerwolths ryegrass fertilised with cattle slurry, Part I. Bacterial flora

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ABSTRACT

Two grass species, short rotation ryegrass and westerwolths ryegrass cultivated as pure stands and in mixtures with persian clover, were fertilised with crude cattle slurry and with mineral fertilisers. The fertilised soil and fresh and dried plants were analysed for presence of bacteria. Significant contamination with bacteria was found in both the soil and plant material. Numerous pathogenic bacteria were isolated from both kinds of samples. Drying of plants reduced the number of bacteria, including pathogens, but this treatment did not kill all of the microbes. Feeding animals with plants fertilised with crude cattle slurry can affect their health.

Keywords: bacteria, clover, contamination, fertilisation, microflofa, pathogenic, ryegrass, slurry

INTRODUCTION

Fertilisation with crude cattle slurry introduces numerous microorganisms to soil, surface and ground waters, and air. Some of them are harmful to health (Zurawski et al. 1978, Kluczek 1986). Kluczek and Skinder (1988) found 3-4 fold increase of number of bacteria in stubble aftercrops fertilised with slurry. Microorganisms have a relatively high survival rate (Slizak 1984) and because of this feeding animals with plants fertilised in this way can be dangerous. The aim of the study was to determine the degree of contamination of soil and plants fertilised with crude cattle slurry with bacterial flora, as compared with mineral fertilisation.

MATERIALS AND METHODS

Plants of two grass species: westerwolths ryegrass (*Lolium multiflorum* var. vestervoldicum) and short rotation ryagrass (*Lolium multiflorum* var. oldenburgicum), cultivated in pure stands and in mixtures with persian clover (*Trifolium resupinatum*), were fertilised with crude cattle slurry. Control plots were fertilised with minerals. The doses of mineral fertilisation were: P_2O_5 - 120 kg/ha, K_2O - 2 x 80 kg/ha, N - 3 x 80 kg/ha and 3 x 30 kg/ha, in the case of pure stands and mixtures, respectively. Doses of slurry were determined after analysis of components, and fertilisation equivalents used under pure grasses were 3 x 54.2 m^3/ha and 3 x 20.5 m^3/ha for the mixtures with persian clover. The experiment was conducted in four replications of randomised sub-blocks of 20

m^2 plots each for three years. Three cuts were collected. Samples of slurry, fertilised soil and fresh and dried plants were analysed for presence of microorganisms. Slurry samples were diluted with sterilised physiological saline solution in the ratio 1 : 4 and then applied to culture media. Soil samples were taken with a sampling stick from depths up to 30 cm. Prior to microbial analysis they were shaken in sterilised saline solution with glass beads. Plants were crumbled, suspended in saline solution and shaken before application to culture media. Agar, agar with blood, SS and McConkey media were used. After 24 - 48 h of incubation at 37°C numbers of bacteria were determined and the microorganisms were identified on comparative tests API - 20E, API - 20 Staph, API - 20 Strep (26,17,23,35).

RESULTS

Microbiological analysis of crude slurry proved the presence of numerous bacteria. Their number varied in particular samples and, among others, depended on storage conditions of the samples, season of the year and temperature. Numbers varied from 3.61×10^9 to 12.46×10^{10} in 1g of slurry (Table 1). In 1g samples of soil fertilised with slurry from 3.27×10^4 to 5.62×10^{10} bacterial colonies were found, whereas corresponding samples of soil fertilised with minerals developed from 4.53×10^3 to 5.66×10^6 colonies. Bacterial counts in soil with slurry increased together with a subsequent fertilisation. Mean numbers of colonies were 4.66×10^4, 3.59×10^9, and 3.87×10^9, after application of first, second and third dose of slurry, respectively, while corresponding control samples maintained almost the same bacterial count: 3.94×10^5, 3.87×10^6 and 3.92×10^6 colonies, respectively.

Table 1. The bacteria identified in cattle slurry

Achromobacter spp., Acinetobacter calcoaceticus var. anitratus, A.calcoaceticus var. lwoffi, Alcaligenes spp., Arizona hinshawii, Chromobacterium spp., Citrobacter diversus, C.freundii, Dicrococcus spp., Edwardsiella tarda, Enterobacter aerogenes, E.agglomerans, E.cloacae, E.hafniae, E.sakazakii, E.sergoviae, Escherichia coli, Flavobacterium multivorum, Klebsiella oxytoca, K.ozaenae, K.pneumoniae, Micrococcus spp., Neisseria spp., Pasteurella multocida, P.spp., Proteus mirabilis, P.rettgeri, P.stuartii, P.vulgaris, Providencia alcalifaciens, P.stuartii, Pseudomonas aeruginosa, P.cepacia, P.fluorescens, P.maltophilia, P.putida, Salmonella arizonae, S.typhi, S.spp, Serratia fonticola, S.liquefaciens, S.marcescens, S.odorifera, S.plymuthica, S.rubidaea, S.spp., Shigella spp., Staphylococcus saprophiticus, S.xylosus, Streptococcus agalactiae, S.bovis, S.faecium, S.lactis, S.uberis, S.spp. Yersinia enterocolitica, Y.intermedia, Y.pseudotuberculosis
Colony count in 1 g 10^9 - 10^{10}

Numerous pathogenic bacteria were isolated from samples of soil fertilised with slurry, namely: *Edwardsiella tarda*, *Klebsiella pneumoniae*, *Pasteurella multeciola*, *Salmonella typhi*, *Streptomyces bovis*, *Streptomyces agalactiae*, *Staphylococcus saprofiticus*.

Fertilisation with slurry significantly increased the number of bacteria in plant roots (Table 2). The increase depended on dose of slurry, plant species and atmospheric conditions. Numbers of bacterial colonies varied from 2.82×10^5 to 4.65×10^6, however there were much less bacteria in roots sampled from plant mixtures.

Table 2. The bacteria identified from roots of plants fertilised with cattle slurry and mineral fertilisers

Cattleslurry	Mineral fertilisers
Achromobacter spp., Acinetobacter calcoaceticus var. anitratus, A.calcoaceticusvar lwoffi, Alcaligenes spp., Arizona hinshawii, Citrobacter diversus, C.freundii, Dicrococcus spp. Edwardsiella tarda, Enterobacter aerogenes, E.agglomerans, E.cloacae, E.hafniae, E.sakazakii, E.sergoviae, Escherichia coli, Flavobacterium multivorum, Klebsiella oxytoca, K.ozaenae, K.pneumoniae, Micrococcus spp., Neisseria spp., Pasteurella multocida, Proteus mirabilis, P.rettgeri, P.stuartii, P.vulgaris, P.spp., Providencia alcalifacies, P.stuartii, Pseudomonas cepacia, P.maltophiia, P.putida, P.spp., Salmonella arizonae, S.typhi, S.spp., Serratia liquefaciens, S.marcescens, S.odorifera, S.plymuthica, S.rubidaea, S.spp., Shigella flexnerii, S.spp., Staphylococcus saprophiticus, S.xylosus, Streptococcus agalactiae, S.bovis, S.faecium, S.lactis, S.uberis, Yersinia enterocolitica, Y.intermedia, Y.pseudotuberculosis	*Citrobacter diversus, C.freundii, Enterobacter aergenes, E.cloacae, E.kazakii, Escherichia coli, Seratia liquefaciens, S.odorifera, S.plymuthica*
Colony count in 1 g	
$2,82 \times 10^5$- $4,65 \times 10^6$	$3,70 \times 10^3$- $4,03 \times 10^4$

Root samples of plants fertilised with minerals contained fewer bacteria than the other samples, i.e. from 3.13×10^3 to 4.03×10^4 colonies and fewer special were present. Similar results were noted during the whole experimental period.

Large numbers of bacteria and fungi introduced with slurry caused a disturbance of the microbial balance in soil, and thus also the microflora of root nodules changed. Both

the number of bacteria on the nodules as well as the species composition changed. Pathogenic bacteria were isolated from many samples (Table 3).

Table 3. The bacteria identified from root nodules of plants fertilised with cattle slurry and mineral fertilisers

Cattle slurry	Mineral fertilisers
Achromobacter spp., Acinetobacter calcoaceticus var. anitratus, A.calcoaceticus var. lwoffi, Alcaligenes spp., Arizona hinshawii, Citrobacter diversus, C.freundii, Dicrococcus spp., Edwardsiella tarda, Enterobacter aerogenes, E.agglomerans, E.cloacae, E.hafniae, E.sakazakii, E.sergoviae, Escherichia coli, Flavobacterium multivorum, F.spp., Klebsiella oxytosa, Micrococcus spp., Neisseria spp., Proteus mirabilis, P.rettgeri, P.spp., Providencia alcalifaciens, P.stuartii, Pseudomonas cepacia, P.maltophilia, Salmonella arizonae, S.spp., Serratia liquefaciens, S.marcescens, S.odorifera, S.plymuthica, S.rubidaea, Shigella spp., Staphylococcus saprophiticus, S.xylosus, Streptococcus faecium, Yersinia intermedia	*Enterobacter cloacae, E. sakazakii, Escherichia coli, Citrobacter diversus, C.freundii, Serratia odorifera, S.plymuthica*
Colony count in 1 g	
$2{,}63 \times 10^4$ $3{,}30 \times 10^5$	$3{,}41 \times 10^2$ $3{,}86 \times 10^2$

The results of analysis of fresh plant material are given in Table 4.
Fertilisation with slurry considerably increased bacterial count on cultivated plants. Pure stands were contaminated to greater extent than plants cultivated in mixtures. More contamination increased together with subsequent applications of slurry. The highest number of species, including pathogenic bacteria, was isolated from plants of the third cut.

Microbial analysis of air-dried plants is summarised in Table 5. Plant material was significantly less contaminated with bacteria when compared with fresh samples. The number of colonies obtained from the latter varied from 4.05×10^6 to 4.56×10^8 colonies, as compared with the numbers of 3.82×10^3 to 4.39×10^4 colonies in control samples, whereas after drying of the plants only ranges of 3.39×10^3 - 3.99×10^4, and 2.51×10^2 - 2.75×10^2 colonies were determined, respectively. Dried material from the third cut of plants contained more barteria as compared with respective samples from first cut. This was directly connected with higher contamination of fresh plants.

Table 4. The bacteria identified from leaaves and stems from plants fertilised with cattle slurry and mineral fertilisers

Cattle slurry	Mineral fertilisers
Achromobacter spp., Acinetobacter calcoaceticus var. anitratus, A.calcoaceticus var. lwoffi, Citrobacter diversus, C.eundii, Edwardsiella tarda, Enterobacter aerogenes, E.cloacae, E.hafniae, E.sakazakii, E.sergoviae, Escherichia coli, F.lavobacterium multivorum, Klebsiella oxytoca, K.ozaenae, K.pneumoniae, Micrococcus spp., Neisseria spp., Pasteurella multocida, Proteus mirabilis, P.vulgaris Providencia mirabilis, P.rettgeri, P.stuartii, Pseudomonas cepacia, P.maltophilia, P.stutzerii, Salmonella arizonae, S.spp., S.typhi, Serratia liquefaciens, S.marcescens, S.odorifera, S.plymuthica, Shigella spp., Staphylococcus saprophiticus, S.xylosus, Streptococcus agalactiae, S.bovis, S.faecium, S.lactis, S.uberis, Yersinia enterocolitica, Y.intermedia	*Enterobacter agglomerans, E.cloacae, E.sakazakii, Escherichia coli, Micrococcus spp., Serratia plymuthica*
Colony count in 1 g	
$4{,}05 \times 10^6 - 5.15 \times 10^8$	$3{,}82 \times 10^3 - 4{,}39 \times 10^4$

Table 5. The bacteria identified from dried forage from plants fertilised with cattle slurry and mineral fertilisers

Cattle slurry	Mineral fertilisers
Achromobacter spp., Alcaligenes spp., Arizona hinshawii, Citrobacter diversus, C.freundii, Dicrococcus spp., Enterobacter aerogenes, E.agglomerans, E.cloacae, E.sakazakii, E.sergoviae, Escherichia coli, Flavo-bacterium multivorum, Klebsiella oxytoca, K.ozaenae, K.pneumoniae, K.spp., Micrococcus spp., Neisseria spp., Pasteurella aerogenes, Proteus mirabilis, P.spp., P.vulgaris, Providencia mirabilis, P.rettgeri, Pseudomonas cepacia, P.maltophilia, Salmonella arizonae, S.typhi, S.spp., Serratia liquefaciens, S.odorifera, S.marcescens, S.odorifera, S.phlymuthica, S.rubidaea, Staphylococcus aureus, S.saprophiticus, Streptococcus agalactiae, S.bo-vis, S.dysgalactiae, S.faecium, S.lactis, S.uberis, Yersinia enterocolitica, Y.intermedia, Y.pseudotuberculosis	*Enterobacter agglomerans, E.cloacae, E.sakazakii, Escherichia coli, Citrobacter diversus, C.freundii, Serratia odorifera, S.plymuthica*
Colony count in 1 g	
$3{,}39 \times 10^3 - 3{,}99 \times 10^4$	$2{,}51 \times 10^2 - 3{,}37 \times 10^2$

DISCUSSION

Microbiological evaluation of soil and plants fertilised with slurry and minerals disclosed large qualitative and quantitative differentiation of bacterial microflora. This indicates a significant effect of slurry on the microbiological balance of microflora. Because of epizootical and epidemiological reasons it is extremely important that microorganisms present in slurry are identified. Crude cattle slurry used in the experiments conained numerous bacteria pathogenic to humans and animals including among others, bacteria from the genera *Enterobacter, Escherichia, Klebsiella, Pseudomonas, Salmonella, Serratia, Shigella, Streptomyces*, and *Gersinia*. Other authors (Bartkowiak 1984, Jones 1986, Larsen and Munck 1983, Tamasi 1983) have also reported on the danger of pollution of the environment and impairment of soil microflora by application of slurry or sewages. The magnitude of bacterial pollution depended on degree of contamination of a slurry used, the season and plant species. Grasses and persian clover plants cultivated in mixtures fertilised with slurry were less contaminated with bacteria than plants from pure stands.

Plants dried in a natural way contained smaller amount of microorganisms. Some bacteria were killed during the drying prosess. But even the reduced number of bacteria remaining can be dangerous to animals, since pathogenic species, such as, *Klebsiella pneumonia*, *Pseudomonas malthophilia*, *Salmonella typhi*, *Shigella flexnerii*, *Streptomyces dysgalactiae*, *Gensiana enterocolitica* were identified.

Plants collected from the experimental plots fertilised with slurry did not meet existing international requirements, which exclude fodders containing bacteria from the family Enterobacteriaceae and/or the genus Pseudomonas (Strauch and de Bartoldi 1986).

Considerable presence of pathogenic microflora both in soil and plants collected from fertilised plots fully justify an urgent investigation on the effect of slurry on the environment and plants in particular as well as experiments dealing with the application of slurry to soil. There is need to develope treatments which would eliminate pathogenic microflora from collected plants. Agrotechnology offers some possibilities of partial removal. According to our earlier studies (Kluczek 1986, Kluczek and Skinder 1988) such factors as method of feeding and breeding animals, and atmospheric conditions have an influence on the composition of bacterial species.

Until the time when some clear solutions to the problem are available, advice is to use ionizing irradiation, alternate high and low temperatures and disinfectants (Larsen et al. 1988, Strauch 1988). In our studies we have concentrated predominantly on the problem of microbiological contamination of soil and cultivated plants, on which we

found numerous pathogenic bacteria, which in turn create an epidemiological threat to animals.

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Table 4. The bacteria identified from leaaves and stems from plants fertilised with cattle slurry and mineral fertilisers

Occurrence of foliar fungal diseases of grasses in the UK

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ABSTRACT

The occurrence of foliar fungal diseases of grasses was recorded in May and/or September from 1983 to 1987 on experimental plots established on areas of old grassland at 16 sites in England and Wales. The plots were cut at 4 or 8-week intervals and were given five rates of nitrogen fertiliser (0-900 kg N/ha per annum). Diseases were recorded most frequently on *Lolium perenne* and *Dactylis glomerata*, and the causal fungi recorded were Puccinia spp., *Erysiphe graminis*, Drechslera spp., *Septogloeum oxysporum*, Rhynchosporium spp., *Mastigosporium rubricosum* and *Laetisaria fuciformis*. Diseases were more common in central and southern England than in northern England and Wales, in September than in May, and on plots cut at 8-week intervals than on plots cut at 4-week intervals. Infection by *E. graminis* and Rhynchosporium spp. tended to be increased by higher rates of N fertiliser, whereas that of Puccinia spp., Drechslera spp. and *Laetisaria fuciformis* tended to be decreased.

Keywords: *Dactylis glomerata*, Drechslera spp.,*Erysiphe graminis*, foliar fungal diseases, grasses, *Lolium perenne*, *Mastigosporium rubricosum*, Puccinia spp.,Rhynchosporium spp., United Kingdom

INTRODUCTION

Descriptions of diseases of grasses occurring in the UK and Ireland have been published (e.g. O'Rourke 1976; Priestley et al 1988; Sampson & Western 1954), but there is little information on factors affecting disease incidence. The establishment of a multi-site experiment in England and Wales in 1983 (Hopkins et al 1990) provided an opportunity to monitor the occurrence of foliar fungal diseases in grass swards given similar management at a range of sites.

METHODS

Plots (size approx. 1.5 m x 6 m) were marked-out in 1983 on swards of permanent grassland at 16 sites in England and Wales (Figure 1) as part of an experiment to assess

the response to nitrogen fertiliser (Hopkins et al 1990). The swards were of mixed species composition and most were at least 20 years old. Annual rainfall at each site varied from 545 mm to 1700 mm (Figure 1). Treatments comprised five rates of nitrogen fertiliser: 0, 150, 300, 450, and 900 kg N/ha per annum, and two frequencies of cutting: 4 and 8 weeks. Not all combinations of treatments were included at some sites. Foliar fungal diseases, and their host grasses, were recorded in May and/or September from 1983 to 1987; only those diseases affecting a number of plants were recorded.

Figure 1. Location of sites in England and Wales (and annual rainfall in mm): 1. Mungrisdale (1487); 2. Barnard Castle (870); 3. Winterburn (1132); 4. Cawthorne (668); 5. St Asaph (838); 6. Pant-y-Dwr (1206); 7. Ponterwyd (1795); 8. Tredegar (1700); 9. Highclere (814); 10. Selborne (943); 11. Oxford (545); 12. Great Alne (690); 13. Exminster (762); 14. North Wyke (1035); 15. Chagford (1524); 16. Bridgwater (746)

RESULTS

The diseases recorded and their host grasses are listed in Tables 1 and 2, for September only. In May, diseases were less frequent and in May 1984 none were recorded. In May 1987 *Mastigosporium rubricosum* on *Dactylis glomerata* and *Septogloeum oxysporum* on an unknown grass were recorded at Ponterwyd, and *S. oxysporum* on *Alopecurus pratensis* at Mungrisdale. In May 1986, *M. rubricosum* was recorded on *D. glomerata* at Barnard Castle, Ponterwyd, Great Alne and Chagford, Rhynchosporium spp. on *L. perenne* at North Wyke, and Drechslera spp. on *A. pratensis* at Bridgwater.

Diseases were more frequent at sites in central and southern England than at sites in northern England and Wales (Tables 1 & 2). Most frequently recorded were Drechslera spp., Rhynchosporium spp., and Puccinia spp. on *L. perenne*, and *E. graminis*, Puccinia spp. and *M. rubricosum* on *D. glomerata*. More infection was observed on plots cut less frequently and infection by *E. graminis* and Rhynchosporium spp. tended to be increased by higher rates of N fertiliser, whereas that by Puccinia spp., Drechslera spp. and *Laetisaria fuciformis* tended to be decreased.

DISCUSSION

Although sward management was similar at all sites, the sward composition varied greatly thus confounding comparisons of disease incidence between sites. However, certain trends in disease incidence were apparent and these were in broad agreement with Priestley et al (1988). *M. rubricosum* and Rhynchosporium spp., which are spread by rain-splash were more common at wetter sites, whereas *E. graminis* and Puccinia spp., which require dry conditions for spore dispersal were more common at the drier sites. Drechslera spp., although spread by rain-splash, occurred at a wide range of sites and are the most common cause of disease in *L. perenne* (Lewis 1992). Quantitative data on the incidence and severity of Drechslera spp. and other diseases were obtained for swards reseeded to *L. perenne* at each site (Lewis 1992).

Disease incidence was lower in May than September probably because grass growth is at a maximum in May and with a rapid turnover of leaf tissue, diseases have little opportunity to spread. Similarly, the more frequent cutting regime would have curtailed disease development and it was not surprising that disease incidence was lower than with the less frequent cutting. The effects of N fertiliser on disease incidence were

in agreement with other reports (O'Rourke 1976; Priestley et al 1988). Thus the present work showed that the incidence of a particular disease depended on the abundance of the host grasses, the time of year, the climate, and the management of the sward.

Table 1. Occurrence of fungal foliar diseases of grasses in September at sites in northern England and Wales 1983-87

		Grass species infected[b]				
Site no.[a]	Disease	1983	1984	1985	1986	1987
1	Drechslera spp.		Lp	Lp		
	Rhynchosporium spp.			Lp		
	Septogloeum oxysporum					Ap
2	Drechslera spp.	Lp	Lp			
	Erysiphe graminis		Dg			
	Mastigosporium rubricosum			Dg		
3	None recorded					
4	*Erysiphe graminis*		Dg	Dg	Dg	
5	*Erysiphe graminis*			Lp		
6	*Mastigosporium rubricosum*			Dg		
	Erysiphe graminis			Fr		
7	Drechslera spp.	Lp	Lp	Lp		
	Rhynchosporium spp.	Lp			Lp	
	Mastigosporium rubricosum			Dg	Dg	
	Septogloeum oxysporum					
	Laetisaria fuciformis	Lp				
8	Drechslera spp.		Lp			
	Septogloeum oxysporum			Ap	Ag	

[a] see Figure 1 for site locations

[b] Lp=*Lolium perenne*, Dg=*Dactylis glomerata*, Ap=*Alopecurus pratensis*, Fr=*Festuca rubra*

Table 2. Occurrence of fungal foliar diseases of grasses in September at sites in central and southern England 1983-87

		Grass species infected[b]				
Site no.[a]	Disease	1983	1984	1985	1986	1987
9	Drechslera spp.	Lp	Lp			
	Rhynchosporium spp.			Lp		
	Puccinia spp.	Lp/Hl	Lp	Hl		Lp/Hl
10	Drechslera spp.		Fp		Fp	
	Septogloeum oxysporum			Ag	Ag	
	Puccinia spp.		Lp			Lp
11	*Erysiphe graminis*		Dg		Dg/Fr	
	Puccinia spp.		Dg	Dg/Fr	Fr	
12	Drechslera spp.	Lp	Lp			
	Mastigosporium rubricosum					
	Erysiphe graminis		Dg		Dg	
	Puccinia spp.	Lp/Dg				
13	Drechslera spp.		Lp			
	Puccinia spp.	Lp/Hl/Ao	Lp/Dg/Hl			
14	Drechslera spp.	Lp	Lp		Lp	Lp
	Rhynchosporium spp.			Lp	Lp	
	Puccinia spp.	Lp	Lp			
15	Drechslera spp.		Lp			
	Mastigosporium rubricosum			Dg	Dg	
	Erysiphe graminis		Dg			
16	Drechslera spp.		Lp			
	Erysiphe graminis		Dg/Hl/Ag	Dg	Dg	Dg/Hl
	Puccinia spp.		Lp/Dg/Ag/Hl		Fp/Hl	

[a] see Figure 1 for site locations

[b] Lp=*Lolium perenne*, Dg=*Dactylis glomerata*, Ap=*Alopecurus pratensis*, Fp=*Festuca pratensis*, Hl=Holcus spp., Ag=Agrostis spp.,Ao=*Anthoxanthum doratum*

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Incidence of infection of grasses by endophytic fungi in the UK, and effects of infection on animal health, pest and disease damage, and plant growth

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ABSTRACT

In the UK, 32% of grass swards examined contained *Lolium perenne* infected with the endophytic fungus *Acremonium lolii*; up to 72% of plants were infected and infection was almost entirely restricted to old swards. In SW England 0-100% of *Festuca rubra* plants were infected with *A. typhinum* at 13 sites, and 96% of *F. arundinacea* and *F. pratensis* were infected with *A. coenophialum* and *A. uncinatum* respectively, each at two sites. Epichloë spp. were detected in *Dactylis glomerata*, *Holcus lanatus* and Agrostis spp.

Concentrations of Lolitrem B, the alkaloid causing 'ryegrass staggers', a disorder of grazing animals, reached a maximum of 5.5 mg/kg in *A. lolii*-infected *L. perenne* from UK swards.

Locusts (*Locusta migratoria*) in captivity ate 68-90% of endophyte-free tillers of *L. perenne*, *H. lanatus*, *A. stolonifera*, and *D. glomerata* but only 3-47% of endophyte-infected tillers. *A. lolii* did not affect infestation of *L. perenne* by frit-fly larvae (*Oscinella frit*) or parasitic nematodes.

Infection of *L. perenne* by *A. lolii* increased herbage yield and plant survival and reduced the effect of virus infection. There was no consistent effect on herbage yield of *Trifolium repens* grown in mixture with *L. perenne*, but extracts from leaves of *A. lolii*-infected *L. perenne* reduced seedling length of *T. repens* by 36%.

Keywords: Acremonium spp., endophytic fungi, forage grasses, grass viruses, herbage yield, *Locusta migratoria*, Lolitrem B, surveys,*Trifolium repens*, United Kingdom

INTRODUCTION

Possible effects of infection of grasses with endophytic fungi in the UK on grazing animals and plant growth were first mentioned many years ago (Sampson 1933 & 1935; Bradshaw 1958). However, these effects were not established until researchers in the USA and New Zealand, from 1977 onwards, began to discover the varied effects of

agricultural importance attributable to endophyte-infected grasses (Bacon 1993). These findings were the stimulus for the initiation of a programme of research at IGER in 1983. The aims of this programme were to assess the incidence of endophyte infection in the UK and its impact on agriculture, with a view to exploiting the benefits. The results of the experimental work are reviewed in this paper.

METHODS

Surveys of endophyte infection of grasses in the UK

Lolium perenne Plants were collected from 72 swards in England and Wales between 1984 to 1992 and examined for *Acremonium lolii* infection (see Lewis & Clements 1986).

Other grasses In July 1992 plants of *Festuca rubra* were collected from 13 sites (see White et al in press), and plants of *F. arundinacea* and *F. pratensis* from two sites in south-west England. The plants were examined for infection with Acremonium endophytes. Also, areas of grassland around the North Wyke Research Station in mid-Devon were examined for stromata of Epichloë spp. on grass stems.

Effect of endophyte infection on herbage yield of *L. perenne*

Field experiment Plants of nine genotypes of *L. perenne* with and without *A. lolii* infection were established in a spaced-plant experiment at three sites (see Lewis & Clements 1990). Herbage dry matter yield was assessed for each genotype/endophyte combination on up to seven occasions, and the number of dead plants on one occasion.

Glasshouse experiment Plants of *L. perenne* with and without *A. lolii* infection and with and without infection with ryegrass mosaic and barley yellow dwarf viruses were grown from single tillers in a glasshouse and harvested at intervals (see Lewis & Day 1993).

Analysis of levels of Lolitrem B in herbage of *L. perenne*

Herbage from *A. lolii*-infected plants of *L. perenne* was analysed for Lolitrem B concentration, using an HPLC method. All of the plants were collected from fields in England and Wales; some had been grown on in a glasshouse (see Lewis & Clements 1986) and others had been planted-out in the field (see Lewis & Clements 1990).

Effect of endophyte infection on pests of grasses

Locusts Tillers of *Holcus lanatus*, *Agrostis stolonifera*, and *Dactylis glomerata* with and without stromata of Epichloë spp., and *L. perenne* plants with and without infection by *A. lolii* were placed in a cage containing adult locusts (*Locusta migratoria*) and left overnight. The amount of each tiller eaten was recorded (see Lewis et al 1993).

Other pests The effect of *A. lolii*-infection on infestation of *L. perenne* by frit-fly larvae (*Oscinella frit*) and by plant-parasitic nematodes was assessed in field experiments (see Lewis & Clements 1986; Cook et al 1991).

Effect of endophyte infection in *L. perenne* on growth of *Trifolium repens*

Field experiment The effect of *A. lolii* infection on seedling establishment and herbage yield of *L. perenne* and *T. repens* was assessed in plots sown with a mixture of the two species (see Lewis 1992).

Bioassay Tillers of *L. perenne* with and without infection with *A. lolii* were chopped, soaked in water and strained. The liquid extract was used to prepare agar plates. Different soaking times, temperatures, extract concentrations and sterilizing techniques were used (see Table 1). Seeds of *T. repens* were placed on the plates and after incubation at 20° C for 5 days, total seedling lengths (radicle+hypocotyl) were measured.

Table 1. Effect of extracts from tillers of *L. perenne* with and without infection with *A. lolii* on total length (hypocotyl+radicle) of seedlings of *T. repens*.

Treatment				Seedling length (mm)	
Soaking time (h)	Soaking temp. (°C)	Extract sterilization	Extract concn. in agar (%)	- *A. lolii*	+ *A. lolii*
24	5	autoclave	50	43.0	41.7
4	5	autoclave	50	45.4	44.1
24	20	autoclave	50	32.0	33.0
24	5	autoclave	75	40.7	36.0*
24	5	filter	50	49.7	32.0***

*, *** Significantly different from -*A. lolii* at $P<0.05$, 0.001 respectively

RESULTS AND DISCUSSION

Surveys of endophyte infection of grasses in the UK

L. perenne Infection by *A. lolii* was detected in 23 swards, of which 21 were at least 15 years old; the maximum incidence of infection recorded was 72%. Therefore *A. lolii*-infected *L. perenne* appeared to be widespread in old grassland in the UK. More recent sowings with European cultivars seemed to have little or no infection, which is in agreement with reports by Latch et al (1987) and Dapprich et al (1992).

Other grasses Infection of *F. rubra* by *A. typhinum* was detected at 10 of the 13 sites and ranged from 0% to 100%. Infection of *F. arundinacea* by *A. coenophialum* and of *F. pratensis* by *A. uncinatum* was detected at both sites examined for each species, and was 96-100%. Stromata of *E. typhina* were detected on *D. glomerata*, of *E. baconii* on *Agrostis capillaris*, and of *E. clarkii* on *H. lanatus* (White 1993).

Effect of endophyte infection on herbage yield of *L. perenne*

Field experiment Interactions between site, genotype and *A. lolii* were significant but *A. lolii* alone had no significant effect. Only two of the nine genotypes showed a consistent response to *A. lolii* infection; one genotype yielded 26% less when infected, the other 268% more. However, the yield of the latter genotype was poor even when *A. lolii*-infected and subsequently the plants were found to be infected with both ryegrass mosaic and barley yellow dwarf viruses. At one site, plant survival of this genotype was 50% greater when *A. lolii*-infected. The effect of *A. lolii* infection on the growth of *L. perenne* is not yet clear because inconsistent results have been obtained (Lewis & Clements 1990).

Glasshouse experiment The cumulative yield of virus-free plants exceeded that of virus-infected plants by 65% at the final harvest. *A. lolii*-infected plants had a higher cumulative yield at all harvests and the difference was more marked with virus-infected plants. The apparent amelioration of the effects of virus infection could be an important finding because grass viruses are common throughout the UK and cause significant reductions in plant growth.

Analysis of levels of Lolitrem B in herbage of *L. perenne*

The concentration of Lolitrem B in *A. lolii*-infected plants from UK swards ranged from 0.08-5.5 mg/kg and therefore were sometimes above the threshold of 2 mg/kg proposed for the induction of staggers (di Menna et al 1992). However, this threshold value only applies if the animal's intake is predominantly herbage from infected plants,

and in old grassland there is often likely to be a large dilution effect from endophyte-free species. Two swards examined in the surveys reported in this paper had been associated with cases of staggers and these swards had a high content of *A. lolii*-infected *L. perenne*. The recent introduction of the New Zealand cultivar 'Yatsyn' to the list of recommended varieties of *L. perenne* issued by the National Institute of Agricultural Botany may lead to an increase in swards with a high level of infection with *A. lolii*.

Effect of endophyte infection on feeding by insect pests

Locusts Locusts ate 68-90% of endophyte-free tillers, but only 3-47% of endophyte-infected tillers.

Other pests Infestation of tillers of the two cultivars of *L. perenne* by frit-fly larvae was unaffected by infection with *A. lolii*, which was predictable because adult female frit-flies do not feed on the plant before egg-laying and are not deterred. It is this deterrence of egg-laying females that is the basis of the protection against damage by larvae of Argentine stem-weevil in New Zealand. Populations of plant-parasitic nematodes in plants of nine genotypes of *L. perenne* were not reduced in the presence of infection with *A. lolii*, perhaps because they were not deterred by the presence of alkaloids, or the alkaloids are not translocated to roots.

At present there is no evidence that *A. lolii*-infected *L. perenne* has resistance to damage by pests in the UK. The implications of the deterrence of locust feeding are difficult to assess because there are no major foliar-feeding pests in UK grassland.

Effect of endophyte infection in *L. perenne* on growth of *T. repens*

Field experiment Seedling establishment of *L. perenne* and *T. repens* was unaffected by infection with *A. lolii*. Infection significantly affected herbage yield at three harvests but both increases and decreases in yield of the two component species were recorded.

Bioassay The only strong indication of an allelopathic effect of *A. lolii*-infected *L. perenne* on growth of *T. repens* seedlings was with the filter-sterilized extract, which reduced seedling length by 36% (Table 1). This suggests that the chemical involved is heat-labile.

Although the results provided no strong evidence of risk to *T. repens* from *A. lolii*-infected *L. perenne*, further work would seem justifiable in view of the increasing importance of *T. repens* in UK grassland.

CONCLUSION

Endophyte-infected grasses appear to be widespread in old grassland in the UK, and although their impact on agriculture is not yet fully known, some important effects of infection have been indicated.

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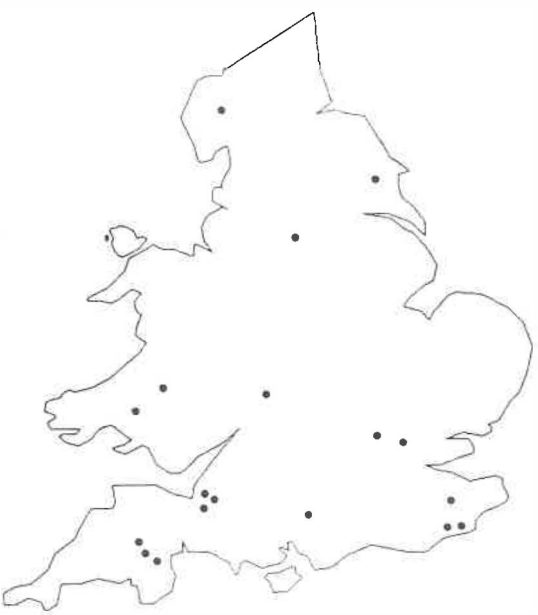

Figure 1. Location of sites in England and Wales where *Acremonium lolii*-infected *Lolium perenne* was detected (at some sites infected plants were detected in more than one field)

Effects of the sample matrix on immunological assays for mycotoxins

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ABSTRACT

Recent advances in analytical procedures have seen the rapid development of immunoassay protocols for the identification and assay of mycotoxins. These immunoassays, predominantly ELISA-based systems, have the advantage of simplicity, speed and sensitivity over classical physico-chemical techniques such as HPLC and GC. Generally ELISA assays permit the analysis of crude extracts directly, and avoid the pre-requisite clean-up procedures associated with the physico-chemical methods.

However, in a number of cases the complex sample matrix contains components which can interfere with the analysis, and which must be removed from the sample prior to assay. These interfering components generally reduce the apparent binding of antibody to sample, and may lead to false positive results in competition ELISA.

We here describe a simple extraction and clean-up procedure which minimises these matrix effects and permits the ELISA analysis of the trichothecene mycotoxin 3-acetyl-deoxynivalenol (3-AcDON) in pasture herbage samples.

KEYWORDS: 3-Acetyldeoxynivalenol, Assay, ELISA, Herbage, Sample matrix, Trichothecene.

INTRODUCTION

Many researchers worldwide have advised of the deleterious effect of contamination of processed feed by a number of *Fusarium* species, and the subsequent ingestion by livestock of toxins produced by these adventitious saprophytic fungi (*eg* Trenholm *et al.* 1989)

In New Zealand, sheep, beef, and dairy farming practices rely heavily on the grazing of improved pasture, and supplementation with processed feed is usually of little significance. However, in the warm humid autumn period, some farmers report a lack of expected weight gain and general 'ill thrift' indicative of possible trichothecene ingestion. *Fusarium* species are known to be widespread pathogens not only of grain, but also of pasture grasses, and indeed, another *Fusarium* toxin, zearalenone, has been found as a common pasture contaminant over this period. A study was initiated to investigate

whether trichothecene toxins are present in the pasture environment. The present study highlights the development of one of a series of immuno-analytical procedures required to undertake a widespread survey of possible trichothecene contamination of pasture.

A major advantage of immunological assays over conventional procedures (for example, HPLC, GC) is their ability to accurately determine and quantify a particular compound or group of compounds within a crude sample extract, without the necessity for extensive clean-up of the sample. In many cases a simple solvent extract may be assayed directly and immediately by the ELISA assay. Although ELISA assays can often be performed on such crude extracts, it is also commonly necessary to include a limited purification step in the assay preparation.

The three main factors which dictate the level of sample purification required are:

1) The properties of the antibody.
2) The ease of extraction of the toxin.
3) The complexity of the sample matrix.

The properties of the toxin-specific antibody reagents employed in the ELISA vary from preparation to preparation. Some antibodies may tolerate high solvent concentrations and wide pH ranges, while others will perform only under narrowly defined conditions.

Each toxin or toxin group exhibits a characteristic solubility, defined in part by its polarity, and hence requires the development of a suitable extraction procedure. Each sample matrix also differs in its chemical make-up and its analysis may demand anything from a simple dilution of the solvent extract to a rigorous clean-up procedure, designed to eliminate any interfering co-extracting compounds.

The extent of sample clean-up required for immunoassay thus depends firstly on the compatibility of the extraction solvent with the immunological reagents (antibodies), and secondly upon the effect that co-extracting compounds exert on the assay.

Pasture herbage is a particularly difficult matrix to work with; it contains many interfering compounds which, under a range of simple solvent extraction protocols, often co-extract with the toxin of interest. These compounds often preclude simple analysis by immunoassay, and necessitate adoption of sample clean-up and purification procedures approaching those required for analysis by conventional HPLC procedures.
The aim of this study was to develop a simple extraction / clean-up procedure for the analysis of the trichothecene 3-AcDON from pasture samples.

METHODS

Antibodies specific for 3-AcDON were raised in sheep using thyroglobulin-3AcDON-15-HG conjugates (Garthwaite *et al.* 1992). These antibodies were used in competitive indirect ELISA (cELISA) assays employing a BSA-3-AcDON plate coating conjugate. Detection of immunological binding was carried out via an enzyme labelled anti-sheep second antibody. A selection of oven dried and milled grass herbage, chosen from sites with low *Fusarium* cell counts, was subjected to a variety of extraction procedures, as below, and the extracts analysed by ELISA.

The extraction methods were compared for their ability to give a zero 3-AcDON value (*ie*, no false positives caused by interference from other compounds in the sample), and for reproducibility of results when analysed at different sample dilutions. Herbage (1g) was extracted with 10 volumes extractant for 1hr, and the extract clarified by centrifugation. Supernate was added directly to the assay, or pre-diluted to 10% in the case of organic extractants.

Extractants investigated were distilled water, phosphate buffered saline (PBS), PBS-Tween 20 (0.05%), 1-5% trichloroacetic acid (TCA), acetone, acetonitrile-methanol-water (85:5:15), and a range of aqueous methanol percentages.

Known amounts of 3-AcDON were then added to the herbage samples to enable analysis of recovery, and the assessment of a variety of clean-up or purification procedures.

RESULTS AND DISCUSSION

The dilution of a 3-AcDON standard in herbage extract and comparison with a standard curve performed in 10% aqueous methanol proved to be the most practical method for the rapid assessment of extraction procedures.

Aqueous based solvents (water, PBS, PBS-Tween 20, 10xPBS and 1-5% TCA) all depressed the maximum absorbance, which relates binding in the absence of 3-AcDON competition (B^0 value), generating various degrees of false positives (Fig. 1). Extraction with acetone gave variable recoveries and again resulted in false positives. Extraction in acetonitrile-methanol-water [85:5:15] (Lauren and Agnew 1991) decreased the assay range and Bo value. Extraction with 50-70% aqueous methanol was most promising, with only a slight decrease in the assay range and Bo value. It became clear that extraction alone did not yield a sufficiently 'clean' sample for assay, and therefore a range of common clean-up procedures were investigated.

Neither 3-AcDON nor the interfering material bound to polyvinyl pyrrolidine (PVP), a compound shown previously to be useful for the removal of interfering plant material prior to chromatographic analysis (Glenn *et al.* 1972). The alumina-carbon, clean-up procedure reported by Lauren and Agnew (1991) also failed to separate the analyte from interference. We then turned our attentions to the removal of the interference rather than the purification of the chosen analyte.

The interfering material was shown to be of low molecular weight by centrifugal dialysis (10kDa membrane cutoff); however, this property was of little value as 3-AcDON is also of low Mr. Although C-18 Sep-Pak cartridges appeared to remove the interfering mataerial, subsequent elution of the 3-AcDON from the cartridge was not consistent and the method proved unworkable. The answer was finally reached with the discovery of a procedure that appeared to separate the interfering component from the aqueous phase. By performing a solvent partition of the aqueous methanol extract against hexane, the interfering component was shown to be non-polar in nature, co-migrating with coloured material into the hexane fraction. Subsequently, 3-AcDON was confirmed to remain in the aqueous phase. Extraction of the 3-AcDON spike was most efficient at high methanol concentrations, in contrast partitioning was most efficient as the methanol percentage was reduced (Table 1).

Treatment ---------- Extractant	Sample	[3-AcDON] calculated pre-partition	[3-AcDON] from the aqueous partition phase	% final recovery
10% Methanol	Zero Control	n.d.	n.d.	---
	10 ppm	5.05	9.69	97
50% Methanol (diluted to 15% prior to partitioning)	Zero Control	n.d.	n.d.	---
	10 ppm	6.95	12.19	122
70% Methanol	Zero Control	n.d.	n.d.	---
	10 ppm	5.71	9.53	95

Table 1 Results obtained with aqueous methanol extraction, followed by solvent partitioning against hexane. Apparent 3-Acetyldeoxynivalenol concentration, [3-AcDON], increases two fold following removal of interfering compounds into the hexane phase.

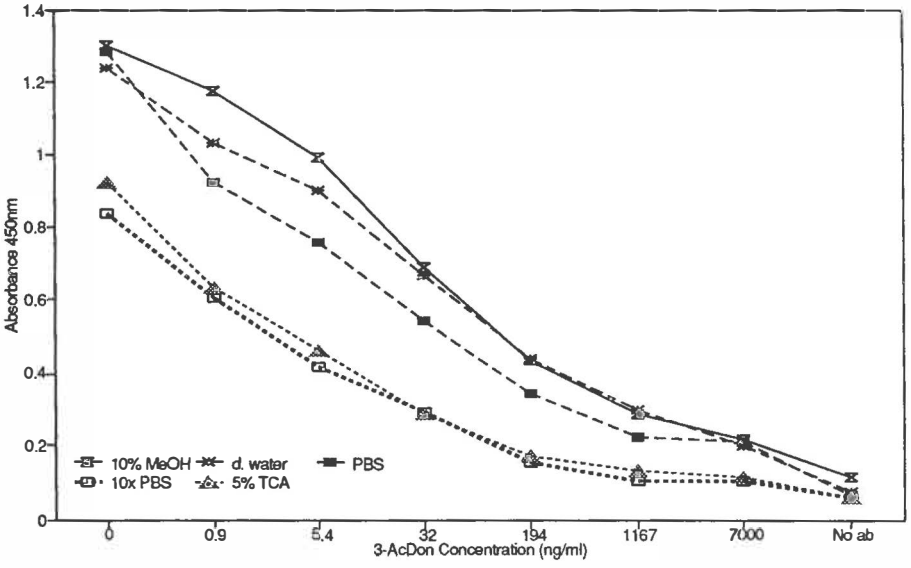

Figure 1. Effect of grass matrix on standard curve, with aqueous extraction

The extraction procedure chosen was a compromise between these two factors. The method, which reproducibly gave a 120% recovery of spike, along with the absence of false positives was performed as follows:
1g grass was extracted with 10ml 50% aqueous methanol and shaken for 1hr. 14ml distilled water was added to reduce the methanol concentration to 15% and mixed. The extract was clarified by centrifugation, 200g for 5min, and 4ml of the supernate material was partitioned against 4ml hexane with shaking for 45min and a sample taken from the aqueous phase for analysis.

With an ELISA assay detection limit of 5ng/ml, and assuming that the 3-AcDON partitioned solely into the aqueous phase (as appears to be the case), the theoretical detection limit of the extraction/assay procedure is 120 ng 3-AcDON per g dry herbage, 0.12ppm.

CONCLUSION

Ideally one would hope to develop an extraction and assay protocol that allows the routine detection and quantitation of a toxin in a particular matrix with the minimum amount of work, and at a minimum possible cost. The development of robust, inexpensive, easy to use immunoassays, and the adaption of these assays to a given

sample matrix, offers the best solution currently available. With the development of an extraction and clean-up protocol for the extraction of toxin from the pasture herbage matrix, we are now able to report such a simple, robust analytical method for the assay of the trichothecene 3-acetyl deoxynivalenol in pasture feed.

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An Immunological Approach to the Study of the Ryegrass Staggers Syndrome

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ABSTRACT

Ryegrass staggers (RGS) is a neuromuscular condition brought on by ingestion of tremorgenic mycotoxins. RGS itself is non-fatal but causes stock loss due to misadventure, for example by drowning; moving of affected stock also becomes difficult. The indole diterpenoid toxins responsible for RGS are produced by *Acremonium* endophytes present in perennial ryegrass (*Lolium perenne*) pastures. The major toxin responsible for RGS has been previously identified as lolitrem B. Antibodies have been raised against lolitrem B and its likely precursors lolitriol and paxilline. These antibodies have been successfully employed in ELISA assays, TLC-ELISAgram and HPLC/EIA procedures for the detection and quantitation of the tremorgens, and also for the affinity cleanup of crude plant extracts. These techniques have enabled the detection of novel (structurally related) compounds. Their use along with the use of antibodies to other mycotoxins produced by the endophyte/plant association is discussed.
KEYWORDS: *Acremonium lolii*, ELISA, Lolitrem B, Paxilline, Ryegrass Staggers.

INTRODUCTION

Endophytic fungi of the genus *Acremonium* live in symbiosis with many of the common pasture grasses. These fungi complete most, if not all, of their life cycle within the plant and inhabit the intracellular spaces of the leaf sheath and blade. The fungi provide their host plants with a variety of benefits such as resistance to drought and insect predation.

There is intense interest in the interaction between plant and fungus within endophyte-infected plants, with a major focus on the novel secondary metabolites produced. Two plant-endophyte combinations which in particular merit such intense study are perennial ryegrass (*Lolium perenne*)/*A. lolii* and tall fescue (*Festuca arundinacea*)/*A. coenophialum*. The former is of concern in temperate climates such as those of New Zealand, South America and parts of Europe, whilst the tall fescue system is of importance in warmer climates such as those found in the eastern United States. In the majority of cases, the endophyte/perennial ryegrass combination produces relatively high levels of peramine, lolitrem B and associated indole diterpenes including likely precursors

lolitriol and paxilline (the simplest known indole diterpene tremorgen), while the endophyte/tall fescue association is high in ergot alkaloids and loline alkaloids.

The vast majority of sown pasture in New Zealand is infected with endophyte, as original grass selections were made to promote resistance to insect pests. Peramine, which is present in plant tissues throughout the year, is the chief protective compound against Argentine stem weevil (*Listronotus bonariensis*) the major predator on perennial ryegrass in NZ (Rowan *et al.* 1986). In the sub-tropical grasslands of the eastern US, the loline alkaloids of endophyte-infected *Festuca* spp. confer upon the plant resistance to the aphid *Rhopalosiphum padi* (Siegel *et al.* 1991).

When conditions favour the survival and breeding of the insect predator, the contrast between endophyte-infected and endophyte-free pasture is considerable, with only the endophyte-infected pasture surviving predation. The presence of endophyte thus gives the farmer increased herbage production and pasture dry matter yield for feeding to his livestock.

Unfortunately, the same plant/endophyte association which produces the beneficial metabolites also leads to the production of metabolites which can cause livestock mycotoxicosis. Two distinct endophyte-related forage grass toxicities have been reported - RGS and fescue toxicosis. In the perennial ryegrass association, the neurotoxin lolitrem B is produced. This toxin is responsible for RGS, a reversible neuromuscular condition (Gallagher *et al.* 1984). The tall fescue association produces ergot alkaloids which can give rise to three sets of symptoms: Fescue foot; fat necrosis; and fescue toxicosis or 'summer slump' characterised by reduced animal performance, reduced tolerance to high temperature, lethargy and excessive salivation (Stuedmann and Thompson 1993).

Although the manifestation and severity of symptoms vary between different host/endophyte combinations, in all cases the ideal solution is to maximise benefits of endophytic pasture whilst minimising the associated livestock toxicity. In New Zealand our efforts have been concentrated on the perennial ryegrass/*A. lolii* interaction and toward the production of a combination which confers pest resistance without causing RGS. Colleagues in the US are using a similar approach on the tall fescue association.

Such a programme requires a reliable assay system for the toxin(s) which can be used for the rapid analysis of large numbers of samples for a variety of metabolites. Current analytical techniques (*eg* HPLC) generally require a high level of extract purification prior to analysis, are laborious, and often difficult to interpret. This is especially true for herbage extracts which contain other UV-absorbing materials.

We report the development of simple, highly sensitive immuno-analytical procedures for the analysis and determination of paxilline and lolitrem families of toxins.

METHODS

Antibody Production

Toxin-protein conjugates were used to raise murine and ovine antibodies against paxilline and the lolitrems as described by Garthwaite *et al.* (1993). Mice with high antiserum titre were selected as splenocyte donors for hybridoma production and screened for the secretion of antibodies specifically binding the free toxin. Ovine sera were monitored for antibody titre and also selected for ability to bind free toxin in a competitive ELISA.

Sample Extraction

Dried and milled herbage material was extracted in 2:1 dichloromethane-methanol. The extract was clarified by centrifugation and an aliquot dried for storage at -20°C until analysis. Filtered or freeze dried mycelial material was treated as above, while culture broth was simply diluted 1:1 with phosphate buffered saline (PBS) before analysis. For assay, the extract was dissolved in methanol and diluted with PBS to a 10% methanol solution.

ELISA Assay

ELISAs were developed for each toxin family using the competitive antibody capture technique. The toxin-protein conjugate was coated onto the surface of a microtitre plate. Further binding sites were blocked by the addition of 1% milk proteins in PBS. A sample or standard in 10% methanol was added, followed by antibody solution, and plates incubated for 2 hrs. Following a brief wash, plates were incubated with horseradish peroxidase (HRP) linked-anti-species IgG. The immunological reaction was disclosed by incubation with the chromogen TMB for 15 min, and read spectrophotometrically on an EIA reader (Garthwaite *et al.* 1993). The optimum antibody dilutions were determined empirically.

HPLC-EIA detection

Samples were chromatographed by HPLC (Gallagher *et al*, 1985). Fractions were collected via narrow bore tubing, and a portion of each dried down and stored at -20°C overnight before dissolution in 10% methanol for ELISA assay.

TLC ELISAgram

The method of Pestka (1991) was followed; sample was spotted onto TLC plates, and run in 10:1 chloroform-acetone for 15 min, and the plates air dried. The plate was then lightly wetted with a spray of 10% methanol, and blotted onto nitrocellulose which had been pre-coated with antibody and blocking agent. The nitrocellulose membrane was then

incubated with HRP-labelled paxilline, washed, and incubated with chromogen which yields an insoluble coloured product on conversion by the enzyme.

RESULTS AND DISCUSSION

Antibody production and characterisation

A panel of hybridoma cell lines was raised, which each secreted monoclonal antibodies capable of binding free paxilline. Two were chosen for use: M-03/01, a broad specificity antibody binding paxilline and a number of analogues, and M-03/02, of similar affinity but with higher specificity. Ovine antiserum M-06/01 was selected for its high titre and wide dynamic range for use in ELISA. This antiserum shows 2-3 times more avidity for lolitriol than lolitrem B.

Sample extraction

A number of extraction procedures were investigated for extraction efficiency and for potential matrix effects. Dichloromethane-methanol (2:1) or chloroform-methanol (2:1) proved the most efficient extractants, with the dichloromethane mixture chosen for its higher volatility. Assays performed using this extraction procedure were not subject to matrix interferences.

HPLC-EIA detection

By taking 15-second fractions from the HPLC system, and subjecting these to ELISA assay, we have identified 'immuno-reactive' fractions. This method in combination with analysis of the corresponding fluorescence and diode-array UV peaks has led to the identification of many previously unidentified indole-diterpenoid compounds from perennial ryegrass.

TLC-ELISAgram

Conventional TLC chromatography, though simple to perform, is limited in its application by the relatively high limit of detection of standard chromogen or fluorogens. In this study, on disclosure, regions of the nitrocellulose where the paxilline-immunoreactive material had bound were seen as colourless areas against a uniform coloured background. In this way, we were able to detect much smaller quantities of toxin and structurally related compounds than by TLC with conventional detection.

Toxin production *in vitro*

Production of indole diterpenoids has been demonstrated in a range of endophytic *Acremonium* endophyte species by ELISA in both surface and liquid culture (Penn *et al.* 1993). Using the standard extraction and ELISA procedure the lower limit of detection of

paxilline in culture broth is 5ng/ml and in mycelial extracts, 10ng/g DWT; for lolitriol 3ng/g DWT, and for lolitrem B 25ng/g DWT.

Paxilline production in endophyte-infected perennial ryegrass
A number of ryegrass samples, either endophyte-free or infected with various endophyte strains were analysed for paxilline and lolitrem B by ELISA and HPLC respectively. Generally, herbage extracts required further 10- to 30-fold dilution to obtain paxilline concentrations within the working range of the assays. No paxilline or lolitrem B were detected in endophyte-free ryegrass; paxilline was observed in all endophyte-infected grasses in concentrations up to 14mg/kg DWT.

CONCLUSION

Since the discovery of the beneficial effects that the *A. lolii* endophyte confers on its ryegrass host by way of insect resistance, attempts to manipulate the endophyte/plant association to avoid RGS have focused on the selection of endophytes that produce little or no lolitrem B. However, evidence is accumulating that strategies aimed solely at eliminating lolitrem B may not be the best approach. The structural similarity between paxilline and lolitrem B suggests that the toxins have similar modes of action and therefore may show additive effects. Our findings support the selection of paxilline-free endophytes as the best way of reducing tremorgen exposure.

Employing the ELISA, we have found that the ability to synthesise paxilline appears common to virtually all wild type *Acremonium* endophytes (Penn *et al.* 1993). The potential of this technique for the rapid, aseptic screening of endophyte cultures and identification of mutants whose indole diterpenoid biosynthetic pathway is blocked prior to paxilline production has also been shown (Gurney and Mantle 1993; Itoh *et al.* 1993). The ability of immuno-analytical methods to detect low levels of these toxins with only a simple extraction procedure has made them a key technique in endophyte research.

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PRELIMINARY RESULTS ON THE DETECTION OF SOME CHARACTERISTIC ALKALOIDS OF *ACREMONIUM* SPP. IN *LOLIUM PERENNE* IN GERMANY

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Key words: loline alkaloids, *Acremonium* spp., *Lolium perenne*, Germany

Abstract

Intensive work has been carried out in New Zealand, Australia and the United States to investigate the significance of alkaloids produced by *Acremonium* endophytes living in grasses, especially in perennial ryegrass (*Lolium perenne* L.) and tall fescue (*Festuca arundinacea* Schreb.). It is known that some of these metabolites are toxic to cattle and others cause insect resistance as well as better stress resistance in their hosts. However, very little is known about *Acremonium*/grass interactions in Europe, particularly in Germany.

Our intention was to develop a simple biochemical method to detect some of the characteristic alkaloid compounds of the *Acremonium*/*Lolium* complex, and to estimate whether these substances are produced under the climatic conditions of Germany.

As characteristic compounds we chose the loline alkaloids loline, N-acetylloline and N-formylloline. Separation and detection was made on high performance thin layer chromatography-plates (HPTLC). As plant material we used the perennial ryegrass cultivars 'Ellet', 'Pennant' and 'Repell' cultivated in pots in a greenhouse and outside in the summer of 1992. Plant material of three cutting harvests was collected and examined. Plants were tested microscopically for the presence of endophytes before each cut.

Detection of the loline alkaloids with Dragendorff solution revealed a difference in alkaloid contents between the endophyte infected perennial ryegrass cultivars. The highest content with all three alkaloids was found in the cultivar 'Ellet' with N-acetylloline beeing dominant. In samples of 'Pennant' no alkaloids were detectable with this method, and in 'Repell' we found only low amounts of N-acetylloline.

Further investigations of these interactions are necessary.

Introduction

According to BUSH et al. (1982) there are characteristic substances of the group of pyrrolizidine alkaloids (PA) synthesized in endophyte infected perennial ryegrass (*L.perenne*), meadow fescue (*F.pratensis*) and tall fescue (*F.arundinacea*). They include loline and its derivatives N-formylloline and N-acetylloline. SIEGEL (1985) reports these substances to be involved in the resistance to certain pathogenic organisms induced by endophytic fungi in grasses. These resistances include that against aphids (PRESTRIDGE et al., 1982), nematodes (PEDERSEN et al., 1988) and fungal diseases (WHITE & COLE, 1985).

The PA are common secondary plant metabolites in many plant families (e.g. *Apiaceae*, *Boraginacea*, *Rubiaceae*) derived from the amino acids glutamic acid (RICHTER, 1982) and/or arginine (SMITH, 1975). In endophyte infected grasses they normally occur only in extremely low concentrations at growth temperatures below 25°C. These concentrations rapidly increase at higher temperatures (BACON et al., 1977). In endophyte free grasses PA are not detectable (BUSH et al., 1982).

Besides the favourable effects of some alkaloids, others synthesized at high temperatures, e.g. the ergot alkaloids and the lolitrem derivatives are toxic to grazing animals and cause the `ryegrass stagger syndrome' (BACON et al., 1977) or the 'eczema solare' disease (VERMEULEN, 1986).

As the latter substances can only be detected with highly sophisticated apparatus we decided to develop a simple detection method. In addition we were interested in obtaining information about the potential toxicity of endophyte infected grasses in Germany.

Loline alkaloids can be isolated specifically with organic solvents in a Soxhlett extractor (RIZK, 1991). Separation of the substances can be carried out on thin layer chromatography plates (TLC) and detection is possible with Dragendorff solution (STAHL, 1967) resulting in red spots or lines on the TLC-plates. Though Dragendorff solution does not react specifically with alkaloids but with every nitrogen (N) atom with three or four substituents this staining solution is favoured because alkaloids are characterized by the contents of at least one of such an N-atom. So due to the extraction procedure the probability of preferentially detecting alkaloids with Dragendorff is very high.

Materials and Methods

As plant material we used endophyte infected and endophyte free plants from the perennial ryegrass (*L.perenne*) cultivars 'Ellet`, 'Reppell' and 'Pennant' as well as two unknown endophyte infected cultivars from Germany designated 82/83 and 82/89, kindly provided by Dr. Oldenburg. As a reference we used an unknown cultivar of meadow fescue (*F.pratensis*) from The Netherlands, found to be infected with endophyte to a high degree and producing high levels of the alkaloids loline, N-formylloline and N-acetylloline (confirmed by combined gas chromatography and mass spectroscopy).

Plants were grown in 10 cm plastic pots in a greenhouse and outside in the summer of 1992. In the greenhouse plants were cultured under semi-controlled conditions with temperatures allowing to rise up to 50°C in sunlight. Plants were harvested whenever temperatures rose above 25°C for at least 5 hours a day for more than a week. From these plants the basal 1 - 5 cm were cut and freeze-dried. The dried material was ground in a hammer mill (screen diameter 1 mm). 10 g of the ground material was extracted in a Soxhlett extractor with methanol over night. The organic solution was evaporated at 50°C to dryness and the residue was resuspended in 100 ml 1N hydrochloric acid. After washing three times with 50 ml ethyl acetate and once with 50 ml chloroform the pH of the aquous solution was adjusted to 10 with 37 ml of a 25% ammonium hydroxide solution. Subsequently the basic solution was extracted three times with 50 ml chloroform and the organic phases were combined and evaporated at 40°C to dryness. The residue was resuspended in 2 ml chloroform.

Separation of the samples was made on 10 x 10 cm high performance thin layer chromatography plates (HPTLC, G 60, Merck, Art.No. 5631). Separation was performed in a solution of chloroform / methanol / ammonium hydroxide (25%) (74 : 24 : 2).

Samples were applied in spots 1.5 cm above the base of the plates. The separation occurred in a separation chamber of 11 x 6 x 18 cm sealed with a glas cover. The solution was added in such a quantity that it did not reach the samples. Separation was ended when the separation solution reached up to 2-3 mm beneath the top of the HPTLC-plates.

Detection of the alkaloids in the samples was performed by dipping for a few seconds in Dragendorff solution according to Munier (1953).

Results and Discussion

Alkaloid compositions of plants grown inside the greenhouse or outside were the same, but the concentrations were different. Plants in the greenhouse produced higher concentrations of alkaloids in all cases, and only these results are considered further.

Alkaloid detection on the HPTLC-plates (Fig. 1) shows several Dragendorff positive spots in every sample. Due to the extraction procedure and the reaction of the stain only with molecules containing one or more three or four binding N-atoms the probability that these spots are alkaloids is very high.

The reference sample of *F.pratensis* is shown in lines 8 (plate 1) and 5 (plate 2). The loline alkaloids are (from bottom to top) loline, an unknown alkaloid, N-formylloline and N-acetylloline.

Plate 1 shows all four plant extracts with lines 1+2 for 'Ellet', 3+4 for 'Pennant', 5+6 for 'Repell' and 7+8 for *F.pratensis*. The lines with the even numbers contain the samples of endophyte infected plants the others are samples from endophyte free plants.

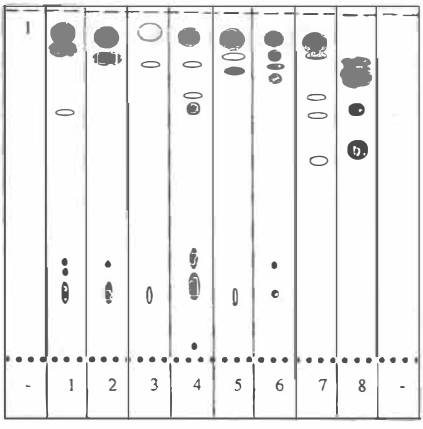
In the sample of the cultivar 'Ellet' all three loline alkaloids were detected with N-acetylloline being dominant (line 2). In the cultivars 'Pennant' (line 4) and 'Repell' (line 6) only N-acetylloline could be detected by this method.

Plate 2 shows besides endophyte infected 'Ellet' (line 1+2), 'Pennant' (lines 3+4) and *F.pratensis* (line 5) the spot of ergotamine tartrate (line 6), an ergot alkaloid, in a concentration of 7.5 μg. Compared with line 8 (sample 82/89) it can be assumed that in this line an ergot alkaloid is present.

The alkaloids present in almost every line in the lower half of the plates are grass specific alkaloids because of their presence in the non infected plant samples.

The results presented indicate that endophyte infected perennial ryegrass and meadow fescue are indeed able to produce loline alkaloids and possibly even ergot alkaloids under German climate conditions. This stresses the importance of further investigations to determine to the exact kind of alkaloids synthesized in endophyte infected grasses in Germany. It is also important to determine whether they are toxic for grazing animals, or whether they are able to induce resistances against insect pests or fungal diseases in their hosts.

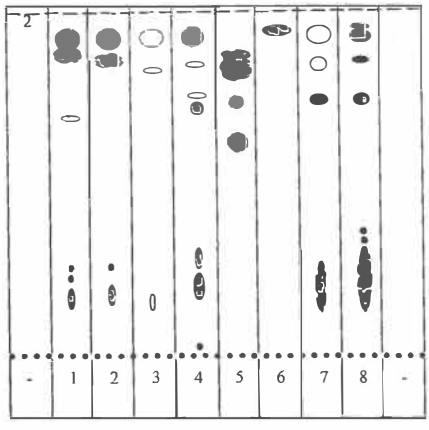
Figure 1 HPTLC-Plates No. 1 and 2, crude alkaloid extracts of *L.perenne* cultivars



Samples and extract volumes

Line	Sample	vol. (μl)
1	Ellet (-)	30
2	Ellet (+) >25	30
3	Pennant (-)	30
4	Pennant (+) >25	30
5	Repell USA (-)	30
6	Repell USA (+) >25	30
7	Barpresto* (-)	5
8	Barpresto (+)	5

(-) = endophyte free
(+) = endophyte containing
>25 =growth temperature more than 25°C for at least 5 days
**Festuca pratensis* cultivar
– – – top of the mobile phase
• • • baseline



Samples and extract volumes

Line	Sample	vol. (μl)
1	Ellet (-)	30
2	Ellet (+) >25	30
3	Pennant (-)	30
4	Pennant (+) >25	30
5	Barpresto* (+)	5
6	Ergotamine tartrate (standard 1mg/ml)	7,5
7	82/83 (+)	30
8	82/89 (+)	30

(-) = endophyte free
(+) = endophyte containing
>25 =growth temperature more than 25°C for at least 5 days
**Festuca pratensis* cultivar
– – – top of the mobile phase
• • • baseline

Dragendorff positive spots on the original HPTLC-plates are red on a yellowish background. The dithering of the spots resembles the intensity of the Dragendorff positive spots on the original plates.

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Results of one trial examining the influence of *Acremonium uncinatum* and leaf-spot diseases on different chemical components and nutritive value of *Festuca pratensis*

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ABSTRACT

Meadow fescue cv. Predix - endophyte free and infected with *A. uncinatum* - was grown in a glasshouse and inoculated with six leaf-spot pathogens by spraying mycelial suspensions on the leaves. The pathogenic fungi were : *Drechslera dictyoïdes*, *D. andersenii*, *D. siccans* (two different isolates), *D. sorokiniana* and *Microdochium nivale*. Ten days after inoculation, the plants were freeze-dryed for chemical analyses of components related to nutritive value of the grass. The digestibility of the organic matter (OMD) was calculated using a formula elaborated by J. SCEHOVIC. Multifactorial analyses of variance based on two replicates of the trial gave the following significant results :

Presence of **endophyte increased** total nitrogen, carotens and OMD by **decreasing** the total fibre content (NDF), lignocelluloses (ADF), true celluloses, silica, and certain phenolic compounds. The alkaloid content (total bases) was also significantly decreased (in contradiction to the formation of loline alkaloids) by the endophyte.

All **leaf-spot diseases decreased** OMD and **increased** the phenolic compounds (single, polymerised and phenolic acids). NDF and ADF were increased by *D. siccans* and *D. andersenii*.

Analyses of meadow fescue samples from other origins gave partly confirmatory and partly contradictory results to this trial.

Keywords : *Acremonium uncinatum*, *Drechslera* spp., endophyte, Festuca pratensis, leaf-spot diseases, nutritive value

INTRODUCTION

Meadow fescue (*Festuca pratensis* L.) is frequently infected by *Acremonium uncinatum* (Gams *et al.* 1990), a fungal endophyte which lives in symbiosis with the plant

and is transmitted by the seed to its descendants. The first objective of this experiment was to determine if the symbioses influenced the main chemical components of nutritive value of the fodder.

A. uncinatum shows a very high antifungal effect *in vitro* against *Dreschlera* spp. and other leaf-spot pathogens of grasses (Schmidt 1991). The second objective was to determine if infection of meadow fescue with *A. uncinatum* reduced leaf-spot development caused by specific pathogens.

Leaf-spot diseases mainly affect fodder quality, palatability and intake. The third objective was to determine to what extent this was the case with these specific pathogens. A multifactorial trial using one meadow fescue cultivar, endophyte free and infected with *A. uncinatum*, artificially inoculated with six different leaf-spot pathogens was designed to answer the above questions.

MATERIAL AND METHODS

Fifty endophyte-free or infected seed of meadow fescue cultivar Predix were sown in pots of 15 cm diameter. The experiment took place during summer in a glasshouse cooled down to 5°C below outside temperature of the air. At the age of two months, the leaves were sprayed until run-off with a mycelial suspension produced in a liquid medium of either of five leaf-spot pathogens. Five pots with endopyte infected and five with endophyte-free plants were inoculated with each fungal isolate. After inoculation, the pots were kept in a moist chamber for 48 h, avoiding leaf contact between them. Species and isolate designations of the five leaf-spot pathogens are listed in Table 1.

Ten days after inoculation, the leaves were scored for disease symptoms using a scale fromä 1 (all leaves healthy) to 9 (whole leaf surface necrotic). Then the plants were cut at 1 cm above soil level and immediately fixed in liquid nitrogen for freeze-drying. The same procedure was repeated on the regrowth one month after the first cut. This enabled statistical analyses based on two replicates.

Chemical analyses mentioned in Table 1 were done after the usual methods described by Scehovic (1979, 1990 and 1991) and the organic matter digestibility (OMD) calculated according to the formula elaborated by Scehovic (1979) : OMD % = 113,707 - 1,222 acid detergent fiber % + 0,462 true celluloses % - 10,849 phenolic acids %.

RESULTS

he pathogens causing the most severe necroses, covering between 20-40% of the whole leaf surface, were *Drechslera siccans* (isolate 742, clear gray), *D. andersenii* and *D. sorokiniana*, followed by *D. siccans* (isolate 1/79, red tinge). *D. dictyoides* and a

thermophile strain of *Microdochium nivale* isolated from *F. pratensis* caused only light leaf damage. Leaf symptoms were rather similar on endophyte infected and free plants. In the second infection cycle, the scores were slightly, but significantly, lower for endophyte infected compared to endophyte-free plants.

The results of the chemical analyses are presented in Table 1. Organic matter digestibility was most affected by the pathogens causing the most severe leaf-spots. Neutral and acid detergent fibers (NDF and ADF) were significantly increased by *D. siccans* and *D. andersenii*, while true celluloses and total nonstructural carbohydrates were decreased. Attack by all pathogens increased the methanol soluble phenolic compounds and the phenolic acids (esterified).

Presence of endophyte had a beneficial effect on fodder quality by increasing OMD, total nitrogen and carotens and decreasing the fibers, true celluloses, silica, the soluble phenolic compounds, and total alkaloids. The decrease in lignin was statistically not significant.

DISCUSSION

The slight but significant reduction of disease scores in presence of endophyte should not be considered important. All scores are only subjective estimations, to which application of statistical analyses might be unsuitable. In other experiments, the size of leaf-spots after a standardized, punctual inoculation did not show any relation to presence or absence of endophyte (Schmidt 1993).

The expected degradation of fodder quality by leaf-spot pathogens could be demonstrated by this experiment, as well as its correlation to the degree of necrotisation. This degradation was however rather low, with differences in digestibility of about 2%, which would probably have very little influence on the performance of cattle. Fiber content was increased by leaf necroses with the exception of black leaf-spots caused by *D. sorokiniana*. This explains why digestibility was less tending to decrease by *D. sorokiniana* attack then by *D. siccans* and *D. andersenii* attack.

The increase of total nitrogen by the endophyte and some pathogens is probably due to an enrichment with fungal proteins. The increase of the caroten faction of the terpenic compounds could be favorable for fodder quality. The decrease of silica content in the presence of endophyte has sometimes been found in previous analyses. It cannot be explained so far.

The increased OMD by presence of endophytes is related to a decreased content of fibers and phenolic acids. OMD is an estimation based on approved correlations with certain compounds. It does not consider at all eventual interactions with the microflore of the rumen, which could occur with endophyte infected fodder. Hannah *et al.* (1990) found

a decreased digestibility *in vivo* by *A. coenophialum* with sheep fed a seed-based diet of *F. arundinacea*, while Goetsch *et al.* (1987) did not find any such effect with steers fed endophyte infected and free tall fescue hay. Many authors agree that fodder intake is affected by presence of endophyte in tall fescue (Schmidt and Osborn 1993) as the animal develop toxicoses symptoms. In such a situation, small differences in digestibility may not be relevant to fodder quality because the animal health becomes the most significant factor. However, no animal experiments have been undertaken with meadow fescue, and it only contains loline alkaloids, while tall fescue contains also ergovaline and peramin, as presented by Bush and Schmidt at this same conference. If it should be confirmed that N-acetyl- and N-formyl-loline do not affect the animal performance and rumen digestibility, our predicted improvement of fodder quality by endophyte presence in meadow fescue would be very interesting. The cummulative effect of all positive influences results in a calculated net energy available for milk production of 6,67 MJ/kgDM in the endophyte infected control plants versus 6,09 MJ/kgDM in the endophyte free ones, a very large difference.

The most amazing phenomenon is the highly significant decrease in total alkaloids (total bases) by the endophyte. Similar observations were evident in previous analyses, also of *F. arundinacea* / *A. coenophialum* and *Dactylis glomerata* / *A. tyhphina* symbiota. This is in contradiction to the accumulation of certain alkaloids in endophyte infected grasses, e.g. of pyrrolizidine alkaloids in meadow fescue/*A. uncinatum* associations, which can exceed levels of 1%. The increase of these total alkaloids was very much enhanced by leaf-spot pathogens in endophyte-free meadow fescue, as well by those causing little leaf damage (e.g. *M. nivale*). We cannot explain which compounds account for these differences, possibly a pollution with non alkaloid basic compouds in plant extract.

These results illustrate the complexity of the biochemical reaction of a plant to a symbiotic and/or pathogenic influence.

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Table 1. Results of the chemical analyses of samples of meadow fescue infected with *Acremonium uncinatum* (+) or endophyte-free (-) attacked by leaf-spots, (percentage except for carotens and total alkaloids expressed in optical density)

Chemical analyses	Endo. level	leaf-spot pathogens						Control	Total means	Influence by*	
		D. dictyoides H2/87	*D. andersenii* H3/73	*D. siccans* 742	*D. siccans* H1/79	*D. sorokiniana* FpChg.	*M. nivale* Fp86			endo	path.
Total nitrogen	+	26.150	22.150	23.850	21.250	23.050	26.150	24.600	23.886	↑↑	↑
	-	20.400	18.800	18.650	18.150	21.550	19.950	17.400	19.271		
Tot. fibers (NDF)	+	41.000	43.550	43.550	43.050	42.600	40.800	41.800	42.336	↓↓	↓
	-	44.100	46.250	45.800	44.400	43.800	44.350	45.200	44.843		
Acid deterg. fiber	+	25.100	26.000	26.400	26.200	25.800	25.400	25.050	25.707	↓↓	↑↑
	-	27.300	28.350	28.350	27.800	26.300	27.250	28.400	27.679		
Lignin	+	3.345	3.475	3.070	3.390	3.410	3.205	3.055	3.279		
	-	3.515	3.190	3.735	3.470	3.265	3.370	3.365	3.416		
True celluloses	+	20.750	21.300	21.950	21.500	21.050	21.100	21.000	21.236	↓↓	↓
	-	22.450	23.550	23.150	23.000	21.700	22.700	23.650	22.886		
Silica	+	0.975	1.250	1.365	1.325	1.345	1.110	1.045	1.202	↓	
	-	1.305	1.625	1.500	1.370	1.305	1.190	1.390	1.384		
Total solub. sugar	+	10.365	11.135	9.850	12.265	11.620	11.290	12.450	11.282		
	-	11.520	10.255	10.445	11.215	11.645	12.215	12.045	11.334		
Solub. phenolics	+	1.172	1.299	1.219	1.337	1.304	1.182	1.088	1.229	↓↓	↑↑
	-	1.284	1.371	1.389	1.379	1.370	1.306	1.212	1.330		
Phenolic acids	+	1.143	1.215	1.201	1.134	1.227	1.139	1.100	1.166		↑↑
	-	1.130	1.233	1.215	1.164	1.224	1.130	1.101	1.171		
Carotens	+	4.110	3.696	3.913	3.712	3.461	4.018	3.816	3.818	↑↑	
	-	3.345	3.085	3.140	3.498	3.351	3.340	3.323	3.297		
Total alkaloids	+	15.087	12.577	8.961	11.037	11.692	12.927	12.173	12.065	↓↓	
	-	18.432	14.667	16.159	13.723	23.777	19.616	12.558	16.990		
OM digestibility	+	80.250	78.600	78.550	79.300	78.550	80.050	80.850	79.450	↑↑	↓↓
	-	78.450	76.550	76.600	77.700	78.300	78.650	78.000	77.750		

* ↑ (↑↑) increased, ↓ (↓↓) decreased at p = 0,05 (0,01)

Biological Control of Phytopathogens and Insect Pests

Scoring of grass diseases for the evaluation of varieties

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KEYWORDS: Rusts, Drechslera, Xanthomonas, Grasses, Breeding, Resistance, Scoring.

ABSTRACT

In the course of the evolution of integrated or extensive cultivation methods hitherto unimportant grasses and their diseases will become important. In variety testing disease resistance is estimated by assesments based on scales used by of the official testing institutions. Because of deficiencies in using such scales for grasses, we developed special methods for some grass diseases: *Puccinia* sp., *Drechslera* sp., *Xanthomonas* sp. We considered the specific characeristics of the diseases (obligate/facultative parasites, local/systemic diseases), and formulated instructions for the use of the scales.

INTRODUCTION

For many years integrated and extensive cultivation methods have been constantly developed. As part of this process, hitherto less important genera of crop plants and their diseases will become more important. Some genera of grasses belong to this category and thus numerous grass diseases gain importance.

Disease resistance is a part of the value for culture and use of grasses. In most cases, assessing of grass diseases is carried out using scales which are in accordance with disease assessment regulations of official institutions. These scales have different ranges in disease descriptions in words and/or percentages (Boeker et al. 1975, BSA 1988, INRA 1985, NIAB 1986/87, RIVRO 1987). The scales try to combine the diseases or scoring characteristics in one system and thus illustrated scoring scales are not available. Standard diagramms are most useful tools when the estimator has to assess disease levels in percentage terms. Such scales exist for cereals (James 1971, Ministry of Agricultures, Fisheries and Food 1974), but these are not sufficiently differentiated in the lower and more frequent disease classes found in grass disease work. For cereal rusts there are extensive illustrated scoring scales (Melchers & Parker 1920, Petersen et al. 1948), which are limited in use because of very different illustrations of rust symptoms. We have therefore developed special scoring scales for some grass diseases. In the following paper I will present scoring scales for three disease groups.

RUSTS

To infect leaves rusts require moisture for a certain time. Under field conditions free leaf moisture develops when dew settles on the leaves during the night and younger

leaves can be infected if spores are present. In controlled conditions this must be simulated by inoculating successively and maintaining a leaf wetness period after inoculation.

Grasses have a marked tillering capacity, and a rusted plant consists of many shoots and leaves of different age, so the rust attack varies considerably within the plant. Therefore giving one score per plant is difficult. If the disease attack is relative low, scoring is much harder and differentiation of varieties is much more complicated. After a successful first inoculation it is possible to obtain a sufficient differentiation of varieties by scoring single plants, on the basis of % leaf area infected and illustrated scoring scales are useful.

Crown rust, *Puccinia cornata* var. *coronata,* causes irregulary distributed, powdery pustules with a yellow-orange colour. A scoring system for this disease was developed in stages. First we scored all leaves of a plant, i.e. three differently positioned leaf blades on all tillers of the plant. Then we estimated only the most severely diseased leaves or leaf segments. We assumed that enough spores had been deposited on the leaves by the inoculations thus showing the possible susceptibility best. In the last step we scored the two most severely attacked leaves per plant. The correlation between all scales was highly significant. Reduction to one severely diseased leaf per plant thus appears possible, if operators have sufficient training in disease assessment (Birckenstaedt 1990) (Fig. 1).

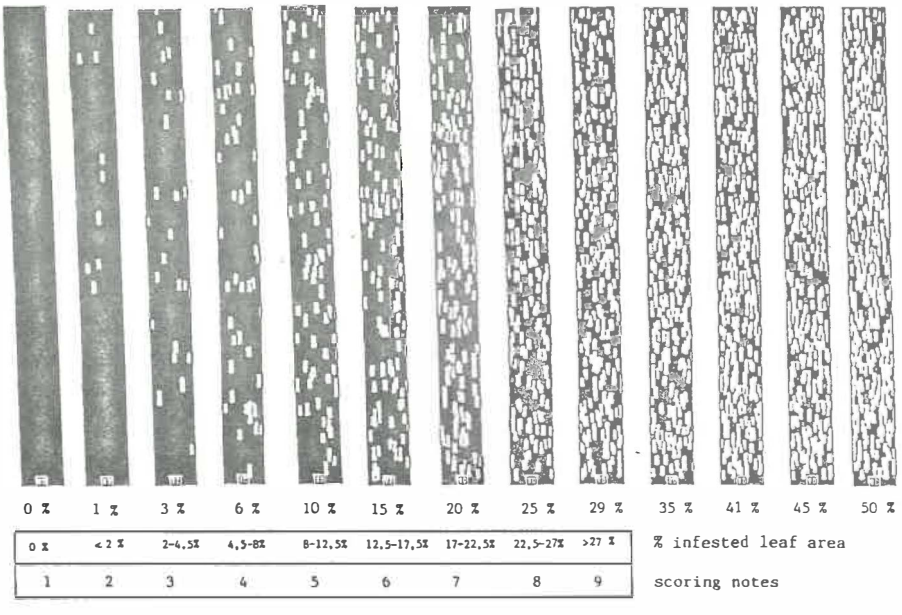

Fig. 1: Scoring scale for *Puccinia coronata* var. *coronata* produced with help of a software by Dölz & Dölz (1988) (Birckenstaedt 1990)

Puccinia brachypodii var. *poae-nemoralis* is a rust on Kentucky bluegrass. The symptoms are relatively small, regular separated littel pustules with a matt-brown colour. The scoring system for *P. brachypodii* var. *poae-nemoralis* is the same as for crown rust (Fig. 2).

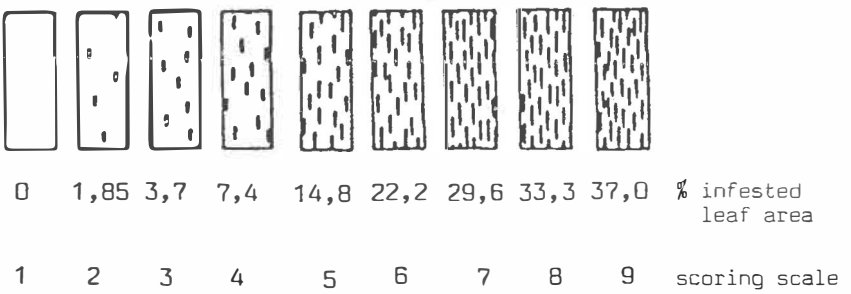

Fig. 2: Scoring scale for *Puccinia brachypodii* var. *poae-nemoralis* (Petersen et al.1948 ,varied)

Puccinia striiformis var. *poae* is also a rust on Kentucky bluegrass. The symptoms are irregular, discrete pustules, yellow in colour, arranged more or less in stripes. The scoring system for yellow rust is the same as for crown rust (Fig. 3). The yellow rust attacks the plant very severely, and high per cent values are often found.

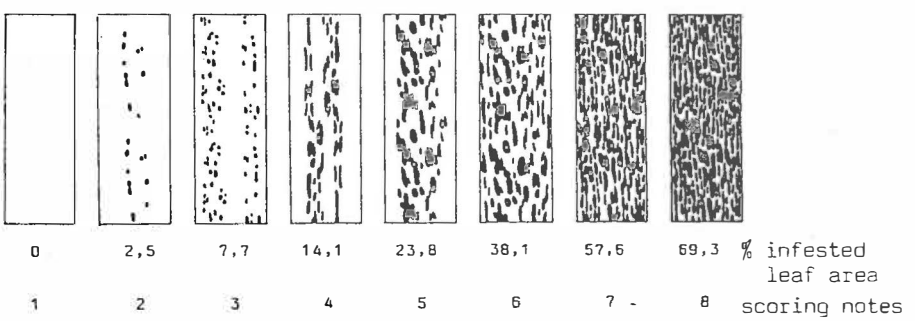

Fig. 3: Scoring scale for *Puccinia striiformis* var. *poae*

DRECHSLERA

Leaf spots on grasses are caused by *Drechslera* - species, commonly *Drechslera andersenii, D. dictyoides, D. poae* and *D. siccans.* Irregularly formed and irregularly

dispersed spots with a range of brown colours appear, *D. poae* and *D. siccans* can make saimilar spots and *D. andersenii* and *D. dictyoides* netting spots. The grass plant can be killed if the infection appeares in the lower shoot area. The available scoring scales (Burhenne 1992, Lam 1983) refer to the average leaf attack. The scoring of a *Drechslera* attack is difficult because of the irregular spots. The following components of symptoms vary: The number of spots per leaf, the size of the spots and the number of diseased leaves per plant. The degree of the attack depends on the age of leaves, old leaves are more susceptible than the younger ones. It is also important to know how long the infection persists. Diseased leaves, especially at the base of the plant, are a good nutrient medium for saprophytic fungi, which cause the final death of the leaves. It is not correct to include this in the scoring of *Drechslera*. Ten days old infections with *Drechslera* show a good differentiation between plants. Because there was a correlation between the degree of attack and the frequency of attack within a plant, it is convenient to estimate the average attack per plant. The disease assessment can be made in per cent diseased leaf area or by giving scores, as shown in Fig. 4, 5 and 6.

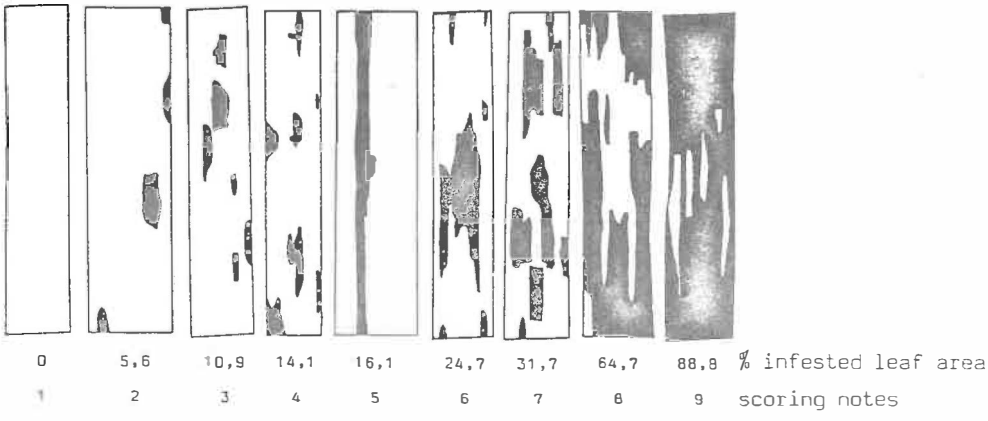

Fig. 4: Scoring scale for *Drechslera poae*

Scoring notes	Symptoms
1	no attack
2	single spots
3	scattered spots
4	frequent spots
5	many spots

Fig. 5: Quantitative scoring scale for *Drechslera poae*

Scoring notes	Symptoms
1	no attack
2	black dots
3	with central necrosis dots
4	small spots
5	medium spots
6	large spots

Fig. 6: Qualitative scoring scale for *Drechslera poae*

BACTERIAL WILT

Bacterial wilt is caused by *Xanthomonas campestris* pv. *graminis* and *X. c.* pv. *arrhenatheri* and is found in many grasses. It causes striped chlorosis and necrosis along the midrib of the leaf-blade, curling or drying of severely attacked leaves, withering and drying of tillers and death of the plant. Because the bacterium spreads in a systemic way the withering symptoms can only be estimated gradually, so scoring is done with a semi-descriptive scale from one to nine (Fig. 7).

Scoring scale	Degree of symptoms
1	no visible attack
2	at least one leaf is dried but still green, or chlorotically striped, plant groth depressed
3	first wilting symptoms on tillers
4	< 50 % of the tillers are diseased
5	50 % of the tillers are diseased
6	> 50 % of the tillers are diseased
7	plant heavily diseased, but still able to develop thin and small tillers
8	plant completely diseased, no regrowth is observable
9	plant entirely killed

Fig. 7: Scoring scale for *Xanthomonas campestris* p.v. *graminis* and *X.c.* p.v. *arrhenatheri* (Paul & Birckenstaedt 1989)

Most grasses can be scored easily with this system, only species with small, rough leaves like *Festuca rubra* show untypical leaf symptoms. In this case the degree of the leaf sheath becoming woody or the accumulation of dead leaf sheaths can be estimated (Fig. 8).

Scoring scale	Symptoms
1	no visible attack
2	
3	Degree of leaf sheath becoming woody
4	
5	The leaf colour of growing leaves become paler
6	
7	Deterioration of quality in new growth
8	
9	plant entirely killed

Fig. 8: Scoring scale for *Xanthomonas campestris* p.v. *graminis* for grass species with small leaves

CONCLUSIONS

The scoring method and time to score the disease - especially in controlled climate experiments - is dependent on the ability of the pathogen to colonize the host and on the development of symptoms. For diseases which spread systemically (*Xanthomonas c.* p.v. *graminis*) disease assessment using scales with descriptions of symptoms is very useful because assessing percentages is difficult. The optimum time to score must be determined by observation of disease development and experience is necessary for this task. For diseases which affect a host only locally withperhaps special conditions for inoculation (leaf wetness for the rust inoculation) determination of the time of assessment relatively easy and occurs with the pustules erupt. But many diseases lie between these extremes. In our investigation *Drechslera* species and yellow rust are examples of such diseases, growing in the leaf in a partially systemic manner. In this case the estimation of the optimum scoring time needs considerable experience.

Diseases which cause clear and regular leaf symptoms can be easily scored as per cent of attacked leaf area. The reduction to one or two leaves per plant for scoring is an important simplification. Like Analytis & Kranz (1972) we found a good correlation with scoring all leaves per plant. In case of more irregular symptoms it is better to use descriptive scales. The reliability of a scoring system can be influenced strongly by the choice of the codes used in the scale. With these methods we have obtained good differentiation between levels of disease in variety tests with rusts and bacterial wilt. The differentiation for *Drechslera* was not successful. This was attributed to the characteristics of the pathogen which is a facultative parasite with saprophytic properties.

We hope that the scoring systems described here are a further advance in the disease assessment of grasses.

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Disease Resistance in Grass Variety Testing Systems A Review of Results from the UK

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ABSTRACT

Disease resistance in grass varieties for agricultural use is evaluated as part of statutory and advisory grass testing systems in the UK. Data have been collected since 1978. There were marked differences in geographical distribution of disease, but five pathogens, *Puccinia coronata*, *Erysiphe graminis*, *Drechslera* spp, *Rhynchosporium orthosporum*, and ryegrass mosaic virus (RMV) appeared to be common and severe. The effects of fungal diseases on grass yield and quality were investigated in a series of fungicide trials and, though average effects were small, some large and significant effects did occur at certain sites in certain years. Though most varieties had at least moderate resistance to most diseases, a significant number of the more popular varieties were susceptible to one or more diseases. The role of disease resistance information within the testing systems and in advising farmers is discussed.

Keywords: Disease resistance, ryegrass, variety testing systems

INTRODUCTION

Grass and herbage legume varieties for agricultural use in the UK are evaluated at the National Institute of Agricultural Botany. Evaluation of disease resistance forms a part of both statutory and advisory testing systems, ie the National and Recommended Lists.

Resistance to a number of diseases in perennial and Italian ryegrass has been evaluated since 1978. In an attempt to establish the effects of disease on grass yields and quality, a series of fungicide experiments was carried out during 1983-86. Since that time, a commercial recommendation for fungicide use on grass has been approved, and trials associated with this have given further evidence of the effects that disease can have on grass performance. This paper will review the results obtained from resistance evaluation over the last 10 years, summarise the extent of disease effects seen in fungicide trials, and discuss the ways in which disease resistance information is used by the testing authorities when decisions on varieties are made, and how the information is used for advising farmers.

METHODS

National and Recommended List trials were established and managed according to standard protocols. These included conservation (a five cut system) and grazing (a nine cut system) for perennial ryegrass, and a combined system for Italian ryegrass. National List trials were carried out at 11 sites (latterly eight) and Recommended List trials, including "on-farm" plots, managed according to local commercial practice, at around 14 sites each year. All trials were examined for disease incidence usually about four times each year in the early spring (February-March), the late spring (early May), mid-season (June and early July) and late season (September-October). Foliar fungal diseases were assessed using an incidence and severity key (Thomas, 1985) and ryegrass mosaic virus was assessed using a disease index (DI) where symptoms were weighted according to severity (adapted from Doherty and Doodson, 1985). Trials were only assessed when infection exceeded 5% (or a DI of 5) on one or more varieties in the trial. Percentage or DI data were analysed directly using a fitted constants technique to adjust for years and trials when varieties were not included. The adjusted means from this analysis were scaled when necessary against control varieties which had well established levels of susceptibility. This procedure was used when particularly high or low levels of disease occurred in a season. Finally, scaled means were converted to a 1-9 resistance rating, where 9 = 0% disease, using a curvilinear relationship, the curved section of the scale being at the lower end from point 4.0 (equivalent to 10% disease) to point 1.0 (equivalent to 40% or more disease cover).

Fungicide trials with six varieties were established at eight sites during 1983-6, and managed using a five cut system. Plots were sprayed with propiconazole at 0.5 l/ha two weeks after each cut, and at approximately monthly intervals during the remainder of the year. Later trials were carried out with single varieties at a total of three sites using one spray of propiconazole four weeks before the first silage cut in early May.

RESULTS

There was a marked geographical distribution of diseases, with *P. caronata* occurring in the south, *Drechslera* spp. and *E. graminis* being widespread, though with a tendency for the former to be more severe in the west, and the latter in the east, *R. orthosporum* being confined to the west, and RMV common and severe everywhere except the extreme west. The mean and maximum infection levels for foliar fungal diseases corresponding to a range of resistance ratings are shown in Table 1. LSDs generally indicated that differences between two rating points were statistically significant, though there were exceptions in some years for some diseases. (1993 LSD range only included in Table 1). It was also apparent that, under high infection pressure, even varieties with a rating of 6 could become severely infected, especially in the case of *P. coronata*.

Table 1. Mean and maximum % infection values for foliar fungal diseases of ryegrasses corresponding to 1-9 resistance ratings.

Resistance rating	2	4	6	8
Mean % (common to all diseases)	20	10	6	2
Maximum %				
Rust	70	50	30	5
Mildew	30	28	12	2
Drechslera	*	22	13	2
Rhynchosporium	*	17	8	*

LSD range (P=0.05) 2.67 - 12.21 (1993 data only, from all diseases)
* = no data available.

Over a 10 year period, there appeared to be relatively little change in the distribution of resistance ratings for those varieties considered for inclusion on the UK Recommended List (Tables 2 and 3). Most varieties had at least ratings of 6 or 7 to most diseases, but there were significant numbers of more susceptible varieties occurring over the whole period for all diseases.

Table 2. Numbers of varieties in four ratings groups from three sample years during the period 1983-1993 for perennial ryegrass diseases.

		Resistance rating group											
		1-3			4-5			6-7			8-9		
		83	87	93	83	87	93	83	87	93	83	87	93
a) Crown rust													
	E*	3	3	1	3	4	4	3	6	11	11	8	4
	I	2	0	3	0	0	2	6	1	12	6	11	10
	L	1	0	0	1	0	3	9	9	13	14	11	9
b) Mildew													
	E	4	3	2	4	3	5	11	12	10	1	3	3
	I	4	0	0	4	2	0	4	5	15	2	5	12
	L	3	1	0	2	1	2	15	16	15	5	2	8
c) Drechslera													
	E	1	0	0	7	5	5	9	16	10	3	0	5
	I	0	0	0	3	1	1	9	11	12	2	0	14
	L	1	0	0	6	2	3	12	18	15	6	0	7

* Maturity groups : E early heading, I intermediate heading, L late heading
Within perennial ryegrass, there was a tendency for the early heading group to contain a higher proportion of susceptibles than the intermediate and late heading groups. For Italian ryegrass, a rather high proportion of varieties was susceptible to RMV.

Table 3. Numbers of varieties in four ratings groups from three sample years during the period 1983-1993 for Italian ryegrass diseases.

	Resistance rating group											
	1-3			4-5			6-7			8-9		
	83	87	93	83	87	93	83	87	93	83	87	93
a) Rhynchosporium	1	0	1	7	14	6	5	9	20	0	0	0
b) Mildew	1	0	0	5	5	5	3	11	19	4	6	3
c) RMV	3	3	5	8	3	9	1	6	10	0	3	3

Table 4. Effects of management systems on the severity of disease on susceptible varieties of perennial and Italian ryegrass.

a) Severity of crown rust on Barlano under different defoliation and nitrogen regimes at a single site (Cambridge, autumn 1986).

	9 cuts 340 kg/ha N	5 cuts 350 kg/ha N	Sheep grazing 200 kg/ha N
% rust	0	10	24

b) Severity of mildew and Rhynchosporium on Barcolte under different defoliation regimes (mean of five years data for each management).

	7 cuts (with early April cut)	5 cuts (without early April cut)
% mildew	6	12
% Rhynchosporium	5	14

Disease levels were invariably lower on grazing type managements than conservation (Tables 4a and 4b for examples with perennial and Italian ryegrass respectively) though other factors such as the level of nitrogen fertilisation were clearly important in the case of crown rust on perennial ryegrass (Table 4a).

Fungicide trials from 1983-86 produced a large range of positive and negative responses to treatment for both yield and quality. Only a few cases gave results which indicated a relation between the incidence of disease on varieties with differing resistance and size of response. Examples for Drechslera spp. and crown rust are shown in Table 5. Single spray and single variety trials gave positive and statistically significant responses in yield and quality parameters in association with the control of high levels of mildew, (Table 6).

Table 5. Effects of fungicide on yield and quality of ryegrass varieties with different levels of resistance to *Drechslera* spp. and crown rust.

a) Drechslera

Variety	Yield Response t/ha (Treated-Untreated)	Drechslera %
Lidura	+1.24*	18
S24	+0.76*	9
Basta	+0.14	10

* significant response at P=0.05

b) Crown rust

Variety	Increase in WSC% (Treated-Untreated)	Crown rust %
S24	+3.9*	15
Gremie	+2.5*	9
Basta	+1.2	3

* = significant response at P=0.05
WSC = water soluble carbohydrate

Table 6. Effect of a single fungicide spray on the yield and quantity at the first cut of perennial and hybrid ryegrass varieties infected with mildew.

Variety	Barcolte			Barkate		
	Yield (t/ha)	D value	Mildew %	Yield t/ha	D value	Mildew %
Treated*	7.78	70.3	1.8	6.69	67.0	0.4
Untreated	7.42	69.0	31.0	6.37	65.5	15.8
LSD (P=0.05)	0.250	0.70		0.270	0.90	

* Treated = sprayed with propiconazole 0.5l/ha four weeks before cut 1.

DISCUSSION

The pathogens identified as being most frequent and severe in trials are likely to be an accurate reflection of the major disease problems occurring in commercial temporary grassland given the distribution of trials and the range of managements applied to them. However, other diseases may be severe from time to time, and it is accepted that

pathogens such as *R. orthosporum* and *P. coronata*, which are only assessed on Italian and perennial ryegrass respectively at present, can cause problems on both species.

The level of statistical significance between resistance rating points (normally a two rating point difference is significant) has resulted in a division of the 1-9 scale where ratings of 1-3 are regarded as very susceptible, 4-5 as susceptible, 6-7 as moderately resistant, and 8-9 as resistant. Though most varieties reaching the UK National and Recommended Lists are moderately resistant to most diseases, there is caused for concern when certain varieties with particularly desirable agronomic characteristics also have low resistance ratings. Recent examples include Merlinda and Condesa, both rather susceptible to crown rust, but comprising 28% and 20% of total seed production in their respective maturity groups. The level of seed production is usually reflected in sales, and these varieties are popular because of their high yields and good quality. In addition, even though many varieties have moderate resistance, they do not remain free from disease especially under high inoculum pressure. A greater number of varieties with ratings of 8 or 9 is desirable, since these do not appear to become significantly infected even under conditions very favourable to diseases.

Table 7. Influence of disease susceptibility on decisions for National and Recommended List status in the UK.

	Conservation yield	Grazing yield	D value	Crown rust rating	Potential* decision
Variety A	Low	Low	Low	3	Not NL
Variety B	High	Moderate	Low	3	Not RL
Variety C	High	Moderate	High	3	RL with warning
Variety D	Low	High	High	3	RL with warning

* NL = National List, RL = Recommended List

Though evidence of the effects of grass disease is very limited compared to that available for most arable crops, results from fungicide trials indicate that a number of diseases cause yield and quality losses of a magnitude which is considered relevant in terms of grass variety performance and animal productivity. These losses have exerted increasing influence on the decision processes by which varieties reach National and Recommended List status in the UK. This can be illustrated by considering the four hypothetical varieties described in Table 7. All are equally susceptible to crown rust. Variety A has a poor agronomic performance overall, and may not reach the National List. Variety B has a high conservation yield, with some grazing value, but its D value (digestibility) is poor. Poor D value does not allow any compensation for the poor disease resistance, and the variety is not likely to be recommended. Variety C is identical, but with a better D value. This could compensate for disease susceptibility, and the variety could be recommended, but a warning would be issued to growers that they should avoid

using high proportions of the variety in mixtures under managements which are likely to encourage crown rust or in areas where the disease is prevalent. Variety D is primarily a grazing variety, and is not likely to be used in situations where crown rust is a problem, and so could be recommended, but again a warning would be issued against its use in high risk situations.

Disease resistance thus has an important role in variety evaluation systems in the UK, but it is necessary to consider resistance together with the potential use and management of a variety, and the areas in which it is likely to be grown. In this way, it should be possible to make maximum use of a variety's characteristics without incurring extensive losses due to disease.

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Breeding for bacterial wilt tolerance of *Lolium multiflorum* using artificial conditions.

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Keywords: Italian ryegrass, bacteral wilt, selection for resistance

Bacterial wilt in grasses is caused by *Xanthomonas campestris* pv. *graminis* which was described first by Egli and al. in 1975.

It leads to extensive damages in forage crops, mainly italian ryegrass, tall fescue and their hybrids *Festulolium.* Both yield and persistency are seriously affected.

First observed in breeding stations, bacterial wilt is now spreading in any forage production areas causing more and more damage. A survey of italian ryegrass fields in 1989 showed that *Xanthomonas campestris* pv. *graminis* was widely spread in Western France (Fig. 1). The rate of infested fields found in Normandy, in the basin of Rennes and in Poitou was 16 %, 75 % and 44 % respectively. No *Xanthomonas* was collected in Anjou because the survey was probably carried out too too early in the spring.

Using less susceptible varieties seems to be the only way for overcoming the disease. A breeding programme is in progress at the Forage Plant Breeding Station of Lusignan for improving tolerance to bacterial wilt.

Materials and method

88 families of tetraploïd italian ryegrass (*Lolium multiflorum*) were inoculated. As the average family susceptibility was high, 39 families were discarded. Then, the 49 remaining families were passed through 3 cycles of mass selection within family. At each cycle, 80 plants per family, divided into 4 replicates, were inoculated and between 6 to 24 of the most tolerant individuals were selected within each family. "Lipo", Turilo" were usually used as resistant and susceptible controls respectively. Artificial inoculation was carried out in a greenhouse in July on one-month old seedlings by cutting plants 3-4 cm high with scissors previously dipped into a 10^8 bacterial/ml inoculum. The mortality rate was scored on successive dates. Finally, tolerant individuals within a family were selected and transferred at field conditions for seed multiplication the following summer.

Results

Symptom expression of bacterial wilt was highly dependant on environmental conditions, mainly temperature. 25°C seemed to be an optimum temperature for screening susceptible families. By comparing the mortality rate within a family over generations, it was shown that selection is effective for improving tolerance. 19 families in the second cyle of selection reached a tolerance level similar to or even higher than Lipo (Fig. 2). Moreover, a large variability between families was maintained over generations of selection suggesting that further improvement is possible.

Prospects

- long term progress and selection limits in diploïd and tetraploïd italian ryegrass
- genetic control of bacterial wilt tolerance
- pathogenicity among strains of *Xanthomonas campestris* pv. *graminis*

However, as most of the species within the *Festuca* complex are susceptible to bacterial wilt, the procedure using artificial inoculation is currently applied in breeding of ryegrass x fescue hybrids.

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Figure 1 : *Xanthomonas campestris* **pv.** *graminis* **in Western France**

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• Field without Xanthomonas

▪ Field with Xanthomonas

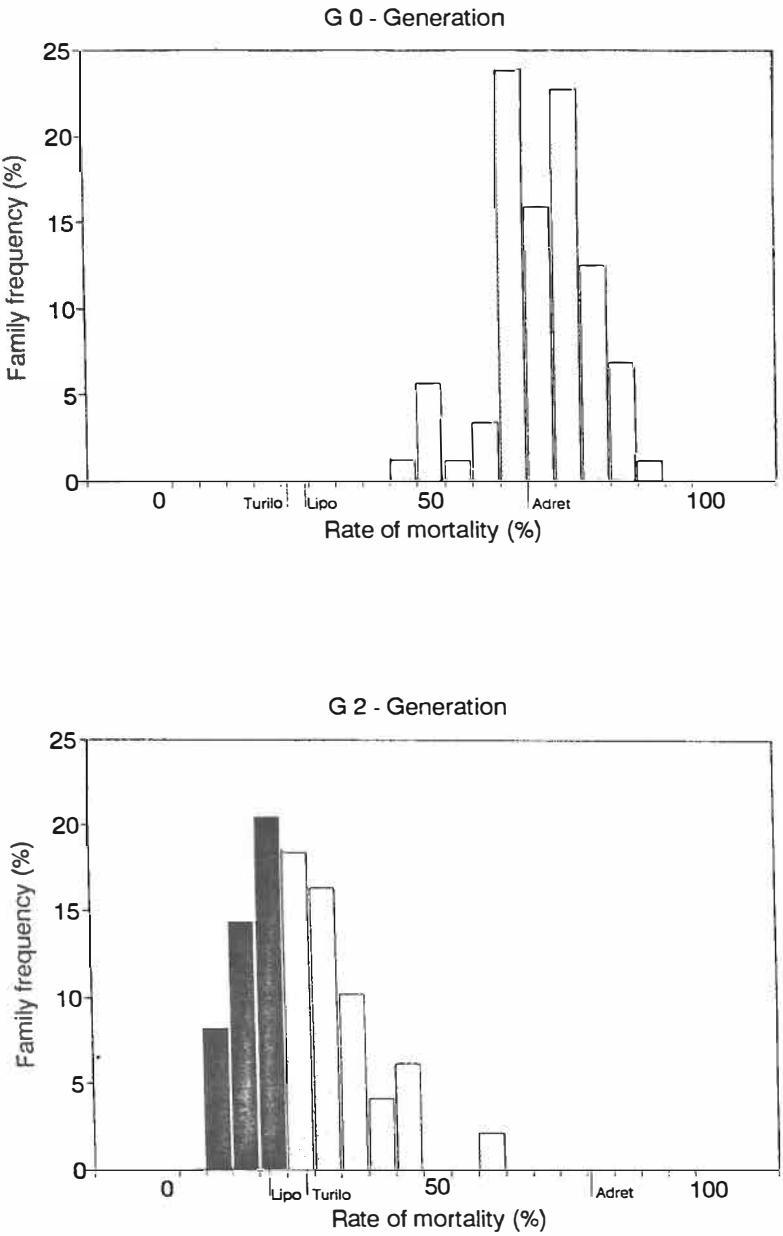

Figure 2 : Family distribution of the rate of mortality caused by X. campestris

BEHAVIOUR OF PERENNIAL RYEGRASS CULTIVARS TOWARDS *PUCCINIA CORONATA* UNDER FIELD CONDITIONS IN CENTRAL ITALY(1)

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KEWORDS: Crown Rust, resistance, inoculated field and grass xy tests

ABSTRACT

The results of a two-year investigation on the resistance of 18 cultivars of perennial ryegrass (*Lolium perenne* L.) to crown rust, carried out in Umbria (Central Italy) are reported.

The cultivars were tested in field experiments using a four-replicate randomized block design. Plants of the susceptible cultivar Vejo, located along two edges of the experimental plots, were inoculated in the first week of July of each year with a single-pustule isolate of *Puccinia coronata* Corda. The inoculum of the fungus produced on 'Vejo' leaves spread naturally to the 18 cultivars.

During the summer each plot was assessed weekly for incidence of infection using an arbitrary scale 1-5 where 1 = no visible symptoms or traces of symptoms; 2-5 = increasing percentage (up to 80-90%) of plot surface affected by rust pustules.

The most severe attacks of *P. coronata* were observed during the first year (1991), probably due to favourable infection conditions, i.e. frequent rainfalls, recorded during the second half of August.

The cultivars Artal and Alpi were significantly more resistant to crown rust (average score < 2) than the remaining cultivars during the two-year investigation period. 'Artal', in particular, confirmed the results obtained with artificial inoculations carried out with the same fungal isolate in greenhouse trials. The cultivar Score, resistant in greenhouse tests, was susceptible in the field.

INTRODUCTION

In Italy the diseases of perennial ryegrass (*Lolium perenne* L.) have not yet been extensively studied. To date *P. graminis* Pers. and *P. coronata* Corda have been recorded in experimental plots in Italy (Govi *et al.*, 1974; Cappelli *et al.*, 1993), while *Drechslera siccans* (Drech.) Shoemaker and *D. andersenii* Lam. have been isolated from turfgrasses and seed samples (Cappelli, 1991).

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In the period 1989-93 we have often observed rust attacks on ryegrass in Umbria, both on weeds and turfgrasses and the pathogens were identified as *P. coronata* and *P. graminis* (the former being more frequently detected) according to the criteria proposed by Cummins (1971). An isolate of *P. coronata*, obtained from a single-pustule (uredosoric) inoculum was then repeatedly inoculated on seven different cultivars, two of which showed a certain degree of resistance to the pathogen under greenhouse conditions (Cappelli *et al.*,1993).

Here we present the results of a two-year field investigation designed to study the behaviour of some ryegrass cultivars in addition to the seven tested in greenhouse towards *P. coronata* under natural conditions.

MATERIALS AND METHODS

A single-pustule isolate of *P. coronata*, the same as used in our previous greenhouse experiments (Cappelli *et al.*, 1993), was employed in these trials. The pathogen was cultured on plants of the susceptible cv Vejo, which were routinely inoculated by dusting inoculum uredospores diluted with talc (1:13 w/w) on the previously wetted upper leaf surface (6 mg of inoculum/plant). Inoculated plants were incubated in a moist chamber for 36-48 h and then returned to the natural humidity conditions of the greenhouse at 18-22 °C. Two weeks later uredospores were collected and stored in glass vials at 5 °C.

Field trials

Seeds of 18 ryegrass cultivars were sown in sterile peat disks on15/12/90 and about two months later on 28/2/91 four replications of 20 plants of each cultivar were transplanted in the field, a replication consisting of a single row 1 m long, with a 30 cm space between rows.

Two hundred plants of the cv Vejo, serving as inoculum sources, were planted in two rows, 5 m each, along opposite borders of the field, so that any plant in trial would not be more than 3 m from these rows.

All the 'Vejo' plants composing the borders were inoculated at the end of June, following the same method described for the greenhouse. The uredospores successively formed on the surface of the inoculated leaves were naturally spread towards healthy plants.

The degree of infection was scored for each plot during the months of August and September according to an arbitrary scale 1-5 (1 = traces of disease; 5 = 80-90% of leaf surface attacked by the pathogen). After the last survey the grass was cut and subjected to current cultural practices.

The following year the infection and the observations on disease development were repeated.

Statistical significance of the results of each year was assayed by the variance analysis and Duncan's Multiple Range test.

RESULTS

In both years uredospores appeared on artificially infected 'Vejo' plants on the two borders of the field ten days after inoculation (first week of July) and the inoculum was naturally spread towards the eighteen cultivars of perennial ryegrass. During the months of August and September the attacks of *P. coronata* became heavy and the fungus was detectable, especially on the most susceptible varieties.

The maximum severity of the disease was observed during the first week of September in 1991 and the third in 1992 (Tab. 1). The intensity of infections decreased afterwards and only mild symptoms developed on new foliage, in consequence of weather conditions, unfavourable to the fungus.

None of the 18 cultivars tested were completely resistant to *P. coronata* during the two years considered. Only 'Artal' and 'Alpi' showed a certain degree of resistance with less severe attacks of the fungus, while 'Vejo' was the most susceptible. The remaining cultivars showed an intermediate reaction.

On the whole, the degree of the infection registered in the first year was higher than in the second year.

Summer weather in 1991 was characterized by moderate rains in the second half of August, while in 1992 rain occurred later, in the first half of September.

CONCLUSIONS

The present investigation shows that in field conditions, the ryegrass cultivars tested react in different ways to *P. coronata*, confirming in some cases the results obtained in previous experiments carried out with young plants in the greenhouse (Cappelli *et al.*, 1993). In fact, a comparison between the two series of data shows that six cultivars among the seven tested in the greenhouse ('Artal', 'Pleno', 'Patora', 'Sisu', 'Elka' and 'Vejo') behaved almost identically in field conditions, while the last one ('Score') appeared much more susceptible in the field. This different behaviour of the cv Score in the two investigations may be explained considering that resistance of young plants is not always related to the adult reaction to a pathogen. This phenomenon has also been observed in the stem rust of perennial ryegrass (*P. graminis*) (Rose-Fricker *et al.*, 1986; Welty *et al.*, 1992). On the whole, the higher intensity of attacks registered in 1991 compared to 1992 could be a consequence of rainfalls during the second half of August 1991.

Problems caused by crown rust in ryegrass have been observed in several geographic areas (Kopec *et al.*, 1983; Lowe *et al.*, 1983; Cagas, 1986; Potter *et al.*, 1990). Some findings have shown an excellent behaviour of 'Artal' (Potter *et al.*, 1990). These have also been confirmed in our experimental conditions, while 'Elka', very

susceptible to our isolate, had previously given good results in other environments (Meyer, 1982; Kopec *et al.*, 1983)

On the basis of our data, cultivars more or less susceptible to *P. coronata* could be identified early in greenhouse conditions by quite simple methods, in limited space and a relatively short time. Developing new research programmes aimed at identifying the physiological races of the pathogen would be very important for genetic improvement and selection of resistant varieties to the crown rust fungus.

Tab. 1 - Severity of P. coronata attacks on 18 ryegrass cultivars in a two years experiment (1991/92).

Cultivar	1991	1992
	Sept.4	Sept.18
Aber	3.5 bc	2.2 bc
Alpi	1.5 a	2.0 ab
Artal	1.5 a	1.2 a
Barlenna	4.0 bc	3.0 bd
Chantal	3.7 bc	2.2 bc
Citadel	3.2 b	2.0 ab
Condensa	3.7 bc	3.7 df
Danny	3.7 bc	2.5 bc
Elka	3.0 b	2.7 bc
Meltra	3.5 bc	4.2 f
Mirvan	1.7 a	2.2 bc
Pamir	3.5 bc	2.2 bc
Patora	3.2 b	2.0 ab
Pippin	3.0 b	2.2 bc
Pleno	4.2 bc	3.0 bd
Score	4.0 bc	3.2 ce
Sisu	3.2 b	2.5 bc
Vejo	4.7 c	4.0 ef
average	3.3	2.6

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Histological investigations on resistance of perennial ryegrass to crown rust

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ABSTRACT

The infection process of an Italian isolate of *Puccinia coronata* Corda on leaves of *Lolium perenne* L. plants resistant (cv. Artal) or susceptible (cv. Vejo) to the pathogen isolate was studied by light microscopy. Pathogen growth was approximately the same in all plants during the first 2-3 days after inoculation with the sole exception of a reduced rate of stomatal penetration by fungal appressoria in 'Artal'. Thereafter fungal development stopped in 'Artal' and haustoria remained small and round-shaped whereas they became bigger and lobate in 'Vejo'. Intercellular hyphae frequently collapsed and hypersensitive necrotic signs, mainly affecting haustorium containing cells, appeared in resistant plants at 3-4 days. In 'Vejo' leaves abundant uredospore production began on days 6-8 whereas the pathogen formed no uredospores at all in 'Artal'. The results indicate a very effective hypersensitivity-associated resistance to *P. coronata* isolate in the 'Artal' plants examined.

Keywords: Crown rust, Histology, Hypersensitive reaction, Infection resistance, *Lolium perenne*, Perennial ryegrass, *Puccinia coronata*

INTRODUCTION

Variable levels of resistance to the crown rust fungus *Puccinia coronata* Corda are found in perennial ryegrass (*Lolium perenne* L.) cultivars or varieties (Cruickshank 1957; Wilkins 1975; Potter et al. 1990). Resistance is under polygene or major gene control (references in Potter et al. 1990). Perennial ryegrass cv. Artal plants which were highly resistant to an Italian isolate of *P. coronata*, i.e. showed no visible macroscopic symptoms when inoculated with the pathogen, were selected from a wider plant population of the same cultivar used in previous experiments in our laboratory (Cappelli, Marte and Paul in press).

Histological observations on incompatible plant-pathogen interactions could contribute in characterising the plant resistance involved and give indications for further studies on its genetical and physiological bases. The penetration and development of the Italian isolate of *P. coronata* in leaf tissues of the highly resistant *L. perenne* cv. Artal

plants and susceptible plants of the cv. Vejo were therefore investigated, along with host responses to the pathogen, by light microscopy. The results obtained are described in the present paper.

MATERIALS AND METHODS

Inoculation procedure

Eight of the greenhouse *L. perenne* plants susceptible to the *P. coronata* isolate (cv. Vejo) and eight resistant (cv. Artal), which had been selected in previous experiments (Cappelli, Marte and Paul in press), were used. Four re-growth leaves for each plant were inoculated with the pathogen isolate (a single-pustule one) by brushing a uredospore suspension (about 300 000 spores/ml of distilled water) on the adaxial (upper) leaf surface. Inoculated plants were incubated in a moist chamber for two days and then returned to the natural humidity conditions of the greenhouse at 18-22°C with natural illumination supplemented by artificial light for a total photoperiod of 14 h.

Light microscopy

Two inoculated leaves per plant from two plants per cultivar were collected at 12 and 24 h then at 2, 3, 4, 5, 6 and 8 days after inoculation. Four to six pieces, 4-5 x 8-12 mm, were cut from each sampled leaf, decolourised and stained in alcoholic lactophenol cotton blue, cleared in chloral hydrate and mounted in glycerine, according to the Shipton and Brown (1962) method.

Other leaf pieces were fixed in formalin-acetic acid-ethanol (FAA, Johansen 1940), embedded in Paraplast Plus (Sherwood Medical) and 10-12 mm thick sections were cut by a microtome. Transverse sections were either stained with cotton blue or double stained with safranin and aniline blue (Darrow 1944) for bright field microscopy. Unstained sections were observed under an interference contrast microscope. Some pieces were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.2, postfixed in osmium tetroxide in the same buffer, dehydrated in ethanol and embedded in a mixture of Epon and Araldite resins. Semi-thin sections from resin embedded material were stained with toluidine blue.

At least 800 randomly chosen uredospores inoculation procedure per leaf were examined in cleared preparations at 12 and 24 h after inoculation and the percentage of spores with a germ tube longer than the spore diameter (= germinated spores) was recorded. The proportion of vesicle-forming appressoria, average number of hyphae produced per vesicle and hyphal growth were determined by selecting 180-200 stomata, covered by appressoria, per leaf in cleared preparations at the subsequent collection times. Statistical significance of data was assayed by variance analysis and Duncan's Multiple Range test.

RESULTS

Uredospore germination reached the maximum level in all plants 24 h after inoculation when values higher than 80% were usually recorded. The mean germination percentage was slightly higher on 'Artal' leaves at 24 h but the difference between the two cultivars was not significant (Table 1). At 12 and 24 h a number of stomata were covered by one to six fungal appressoria and each appressorium produced a cone- or sausage-shaped substomatal vesicle which in turn usually gave rise to one or two thin infection hyphae (Figs. 1, 2).

TABLE 1. Development of an Italian isolate of *Puccinia coronata* in leaves of *Lolium perenne* plants susceptible (cv. Vejo) or highly resistant (cv. Artal) to the fungal isolate, determined at 12 and 24 h after inoculation[a].

Host cultivar	% spore germination[b]	% substomatal vesicle-forming appressoria		Number of infection hyphae/vesicle	
		12 h	24 h	12 h	24 h
Vejo	81.6 A	59.4 A	89.8 A	0.14 A	1.34 A
Artal	86.5 A	52.6 A	61.7 B	0.16 A	1.79 A

[a]. Each figure is the mean of 10-12 replicates per cultivar. Numbers in each column followed by a different letter are significantly different at $p \leq 0.01$ (Duncan's Multiple Range test).
[b]. At 24 h after inoculation.

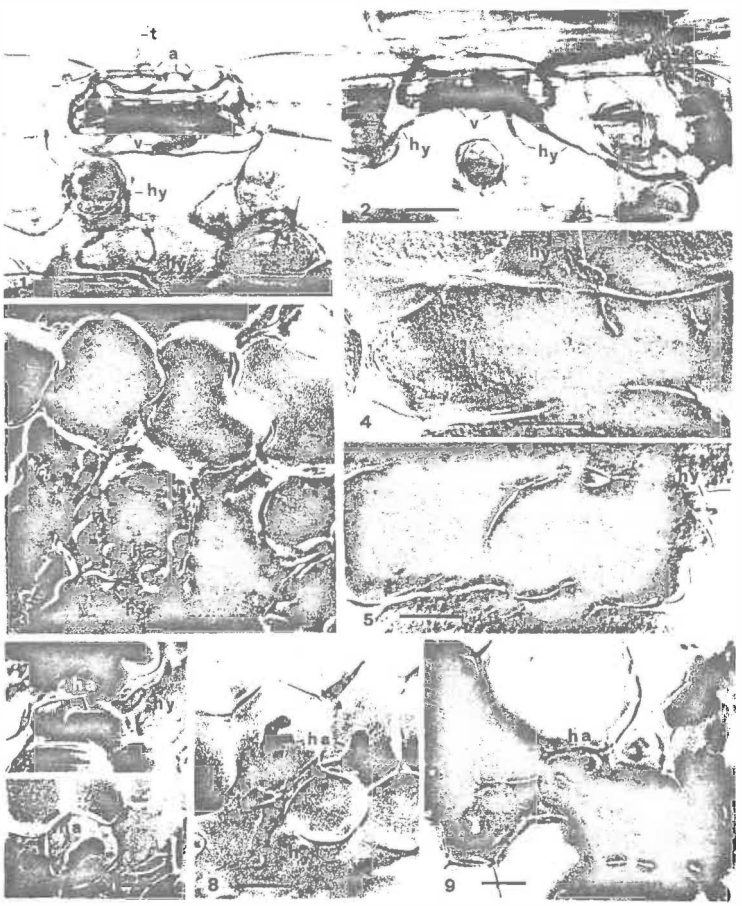
Table 1 shows that the proportion of vesicle-forming appressoria (rate of stomatal penetration) at 24 h was significantly lower in resistant plants (about 62% compared to 90% in susceptible plants) but no other appreciable effects of plant resistance were detected in fungal infection at this stage of development.
Infection hyphae were elongated and branched to the same extent in both cultivars during the subsequent 1-2 days. Their development became more and more difficult to observe in cleared preparations due to the morphological features of *L. perenne* leaves, especially the markedly undulated upper surface and abundance of vein tissues. However, small round haustoria (3-4 mm in diameter), with a very thin neck, were observed in mesophyll cells of all infected plants 2-3 days after inoculation. Haustorium invaded cells, showing distinct signs of degeneration, i.e. plasmolysis, cytoplasm coagulation, cell collapse and increase in stainability of cell content were seen in both cleared preparations and sections from infected 'Artal' leaves at 3-4 days (Figs. 3-5). Occasionally one cell or rarely two, adjacent to those containing haustorium also became necrotic (Fig. 5). No further fungal

development was observed in the same cultivar at this time and most of the previously formed infection hyphae appeared empty, frequently collapsed. On the contrary, pathogen growth continued vigorously in infected leaf tissues of 'Vejo' plants where many haustoria became larger, often elongated, irregular in shape or lobate at 3-4 days or later (Figs. 6-9). The number of collapsed necrotic cells tended to increase slightly in each infection site of resistant plants during the subsequent days reaching a maximum of 3-5 cells per site. Subepidermal stromata producing abundant uredospores began to differentiate in the infected leaves of the susceptible cultivar at 6-8 days and erumpent uredosori were detected at 8 days. Uredospore production was never observed in inoculated leaf tissues of the 'Artal' plants.

DISCUSSION

Resistance to *P. coronata*, associated with hypersensitive reaction (HR) of host tissues, was reported and histologically investigated in *Avena sativa* L. (Rothman 1960; Naito et al. 1970; Tani et al. 1975). Although in the cleared preparations we examined some details were not easily observed, it can be concluded that the resistance we investigated is also associated with HR. The high efficiency level of this resistance was confirmed by the arrest in growth of the pathogen which failed to differentiate even microscopically detectable amounts of uredospores in 'Artal' leaves during the observation period. Under our experimental conditions, the number of host cells involved in HR remained very low in each infection site. The hypersensitive necrosis mainly affected haustorium invaded cells and appeared as a reaction to haustorium formation. Histological responses elicited by the first haustorial development (posthaustorial reactions) are usually observed in the resistance of host cultivars to rusts and often include plant cell death, i.e. HR (Heath 1981). This kind of resistance to rusts is normally controlled by major genes (references in Niks and Dekens 1991).

A prehaustorial response, i.e. the decreased rate of stomatal penetration by the fungal appressoria at 24 h after inoculation, though apparently of limited effectiveness, was also observed in the 'Artal' plants examined. No further resistance signs, e.g. failure of some hyphal tip to produce haustoria, were documented. Histological expressions of resistance preceding HR (and haustorial formation) were detected in oat-*P. coronata* interactions (Rothman 1960; Naito et al. 1970; Tani et al. 1975) and, at least in some cases, are thought to be induced independently of host cell necrosis (Tani et al. 1975). The occurrence of more than one histological response in a single plant-rust fungus interaction may indicate that multiple resistance mechanisms are activated by the plant against the invader organism (Heath 1981).



Figs. 1-9. Light microscopy of leaf tissues from susceptible (cv: Vejo) and resistant (cv. Artal) *Lolium perenne* L. plants infected by an Italian isolate of *Puccinia coronata* Corda. **Figs. 1** and **2**. Stomatal penetration, formation of substomatal vesicles and infection hyphae in 'Artal' (Fig. 1) and 'Vejo' (Fig. 2) leaves 24 h after inoculation. A germ tube and three appressoria are visible in Fig. 1. Cleared whole mounts stained with cotton blue; bright field optics. Magnification bars = 20 mm. **Fig. 3**. Mesophyll cell containing a small, round haustorium of the fungus in a resistant 'Artal' plant 3 days after inoculation. The invaded cell shows incipient plasmolysis and coagulation of its content. Unstained section of formalin-acetic acid-ethanol (FAA) fixed material; interference microscopy. Bar = 10 mm. **Figs 4** and **5**. Two examples of haustorium containing cells showing signs of degeneration, i.e. plasmolysis, incipient cell collapse and increased affinity for the dye (especially Fig. 5) in leaves of resistant 'Artal' plants 3 days after

inoculation. Note the small size and round shape of the haustoria. Cleared whole mounts stained with cotton blue; interference contrast microscopy. Bars = 10 mm. **Figs. 6, 7, 8** and **9**. *P. coronata* haustoria in mesophyll cells of susceptible 'Vejo' plants 6-8 days after inoculation. Haustorial shape ranges from elongated (Fig. 6, 6 days; Fig. 8, haustorium on right, 8 days) to irregular (Fig. 7, 8 days) or lobate (Fig 8, haustorium on left; Fig. 9, 8 days). Sections from FAA fixed material, stained with cotton blue. Figs. 6-8, interference microscopy. Fig. 9, bright field optics. Bars = 10 mm.

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In vitro selection for snow mould resistance in perennial ryegrass.

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INTRODUCTION

Winter survival of perennial ryegrass (*Lolium perenne*) depends mainly on the resistance to low temperature and to snow mould fungi. Among them, *Microdochium nivale* is the most important pathogen causing snow mould. Like many Fusarium species, M. nivale is able to produce toxins like Deoxynivalenol (DON). The aim of our work was to investigate whether improvement in toxin resistance is associated with an improvement in pathogen resistance, and if the resistance is transmitted to sexual progeny of the host.

Keywords: snow mold, ryegrass, resistance, selection, toxins

MATERIAL & METHODS

In vitro selection

From 2 populations of *L. perenne* (cv. Loretta=WD02 and an experinental Synthetic= WD51) callus cultures were established from mature embryos (Schmidt & Posselt 1991). Callus was induced from 1000 explants in each variant: a control without DON (D0) and a selection variant with 200 µg DON (D200). For the latter many calli did not survive, and the number of calli were reduced before subculture to 200 in each of the 3 variants: 1. control with no DON during subculture (D0/D0), 2. no DON followed by DON in subculture (D0/D400), and 3. DON during induction and subculture (D200/D400). Induction lasted 5 weeks and subculture 4 weeks, respectively. After transfer to toxin free medium, Ro-plants could be regenerated from all variants. Sexual progenies were produced by intercrossing the Ro-plants of each variant separately, resulting in HS-families from which R1-plants were raised.

Snow mould test

Inoculum was produced from a toxigenic isolate of *M. nivale* following a procedure outlined by Miedaner et al (1993). In brief: mycelium growing on malt extract solid medium was used to induce mass propagation in a liquid shaken culture. Medium saturated vermiculite was inocu-lated with the shake culture. Within 2 days incubation the vermiculite was uniformly colonized by the fungus and 35 ml was spread evenly on the surface of a 7 x 7 cm pot containing one plant. The plants were kept for 3 weeks in the

dark at +1 °C maintaining high humidity. All plants except the noninoculated controls had been colonized by the pathogen. After one week under favourable conditions plants were scored on a 1 - 9 scale (9 = dead plant). Each genotype was represented by 4 clonal replicates.

Toxin test

Mature embryos from the HS-families were plated on artificial medium (MS, 30 g/l maltose, 0.2 mg/l kinetin) containing D1400 (1400 µg DON). After 14 days, germination percentage was counted and seedling growth scored.

RESULTS

Snow mould tolerance of Ro-plants

As can be seen from Table 1 a seed control was included in the snow mould test. However, no significant differences between seed derived plants and the in vitro regenerants (D0/D0) could be found. The same holds true for plants where toxin selection took place only during subculture (D0/D400). However, in both populations the two step selection variant (D200/D400) showed a significant improvement in its snow mould tolerance. Though the forage type WD51 was already better in its resistance than the turf type WD02, the improvement by means of in vitro selection by 1.8 units was even more pronounced. The very best genotype from WD51 was scored 3.25. Each selection variant in Table 1 was represented by 4 genotypes, each tracing back to an individual explant. Plants originating from the same callus showed a similar level of resistance.

Table 1 Snow mould tolerance after in vitro selection

Variant	**WD02**	**WD51**
seed control	6.75	5.75
D0 / D0	6.13	5.19
D0 / D400	6.25	5.25
D200 / D400	5.44	3.88
LSD 5	0.89	0.93

Toxin test (R1-plants)

From each population and selection variant 3 HS-families (40 explants each) were tested for DON-tolerance. Between D0/D0 and D0/D400 no significant differences in

germination percentage and seedling growth could be found. In contrast, D200/D400 had a 55% higher germination rate and 72% faster seedling growth than the control. Among the HS-families significant differences could be observed.

Snow mould test (R1-plants)

The same HS-families used in the toxin test were subjected to a snow mould test (Table 2), but only D0/D0 and D200/D400 were compared. On average of the two populations and within WD02 no significant differences between the 2 variants could be found. However, in WD51 a significant difference (0.6) could be observed. No significant differences among HS-families could be detected.

Table 2 Snow mould tolerance of sexual progenies (R1-plants)

Variant	average	WD02	WD51
D0/D0	5.60	5.63	5.57
D200/D400	5.39	5.80	4.98
LSD 5	0.26	0.40	0.33

DISCUSSION & CONCLUSION

Toxin tolerance is a heritable character. However, because DON is a non host specific toxin the relationship between toxin and pathogen resistance is not very close. According to the data presented, in vitro selection with DON cannot be recommended as an alternative to direct selection for snow mould resistance. However, in crops where toxin contamination is a severe problem either in vitro selection or the toxin test could be valuable tools in population improvement.

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GENETIC ASPECTS OF CROWN RUST RESISTANCE IN THE RYEGRASSES

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ABSTRACT

Several populations of italian and perennial ryegrass were analysed for their resistance against crown rust by means of quantitative genetic experiments. Additive genetic variance is predominant in most populations. However, in some cases specific combining ability plays an important role indicating the presence of major gene resistance.

KEYWORDS: crown rust resistance, quantitative inheritance, ryegrasses

INTRODUCTION

Crown rust (*Puccinia coronata* Corda) is a cosmopolitan pathogen that causes one of the most important foliar diseases in grasses. Crown rust reduces both yield and quality (POTTER, 1987; CHOSSON & SINGLA, 1989). Because chemical control is not desirable in forage crops, disease resistance breeding is one of the major goals in most breeding programs. Different levels of crown rust resistance among cultivars of italian (MANSAT & BETIN, 1979; BIRCKENSTAEDT, 1990) and perennial ryegrass (BIRCKENSTAEDT, 1990; KOPEC et al, 1983) were demonstrated. Selection experiments were carried out in both ryegrasses (MANSAT & BETIN, 1979; HIDES & WILKINS, 1978; CAGAS, 1990). Examples of quantitative genetic analysis of crown rust resistance are very limited (HAYWARD, 1977; WILKINS, 1975; SCHMIDT, 1980). The present report concerns the nature of inheritance of crown rust resistance in the ryegrasses and its implications in breeding practice.

MATERIAL & METHODS

Plant material

Crossing experiments were carried out with plants from italian (cv. Lema, Lemtal, Limulta) and perennial (cv. Cropper) ryegrass. Both the genetic material and the type of mating design (diallel or NC II) are specified in each table. Crosses were made in the glasshouse by bagging heads of two different genotypes. From most of the parents selfed seed could be obtained as well. Selection experiments were carried out with cv.'s Lemtal, Limulta, Aurora and variety A, B, C.

Inoculum

Rust spores were first collected in the field on as many plots and genotypes as possible, seperately for both ryegrasses. Multiplication was performed on either cv. Limulta or cv. Aurora, respectively. For short term storage, the spores were kept in a refrigerator.

Resistance tests

Plants were raised from seed and transplanted to pots (7 x 7 cm) filled with a peat-soil compost. At the time of inoculation plants had 5 to 6 tillers and were about 10 weeks old. The resistance tests were carried out in a glasshouse as complete block designs with six replicates and five plants per replicate (i.e. 30 plants/entry). If still available, ramets from the parents were included in the tests. The uredospores (20 mg/100 plants) were diluted in 10 times their own volume of talc and dusted evenly on the leaves by means of a cyclone duster. Surface water for spore germination and penetration was maintained for 48 hours by means of humidifiers. 16 days after inoculation plants were scored for susceptibility. On three leaves per plant lesion coverage in % was estimated (BIRCKENSTAEDT, 1990). These data can be converted into 1 to 9 scores according to the following table:

score	1	2	3	4	5	6	7	8	9
%	0	0-2	2.1-4.5	4.6-8.0	8.1-12.5	12.6-17.5	17.6-22.5	22.6-27.0	> 27

Biometrical analysis

All computations were done with PLABSTAT (UTZ, 1990). Diallels were calculated according to Griffing's method 4, model 1 (GRIFFING, 1956). Factorials (NC II) were analysed according to COMSTOCK & ROBINSON (1952).

RESULTS

Italian ryegrass

The results of a 6-parent-diallel (cv. Lema) are given in Table 1. The parental values range from 0 to 7.3 % leaf area infected with an average of 4.6. The mean of the F_1-progeny is 4.3. While regarding gca-values one has to bear in mind, that due to scoring the disease, negative gca is positive from the breeders point of view i.e. reduction of the disease. Thus, parent 1 is the best and parent 5 is the worst in terms of rust resistance inheritance. Analysis of variance (AOV) shows significant variation among crosses. If partitioned into gca and sca components it can be seen from Table 1, that the largest part of genetic variation is due to gca effects. Specific combining ability is of minor importance and not significant.

Table 1 : Six parent diallel (cv. Lema) (parental data on the diagonal)

	P1	P2	P3	P4	P5	P6	GCA
P1	*0*	2.5	3.0	2.8	4.2	2.4	-2.1
P2		*3.3*	3.3	5.1	6.2	5.9	-0.1
P3			*3.1*	6.1	5.6	6.0	0.2
P4				*6.0*	6.7	4.4	0.4
P5					*7.3*	5.9	1.3
P6						*6.2*	0.3

A N O V A :

	d.f.	F-test	V.C.
reps	5	1.4^{ns}	
crosses	14	2.3^{**}	
gca	5	5.2^{**}	1.1^{**}
sca	9	0.7^{ns}	0.2^{ns}
error	70	0.6	

AOV of the factorial crosses of cv. Limulta and cv. Lemtal is presented in Table 2. The 6 parents which had been crossed 3 females (F) x 3 males (M) resulted in 9 F_1's per set. The two Limulta sets were not different from each other, while a significant difference occurred in Lemtal. Within the Limulta progenies the F : S and M : S were of the same order. The variance components were translated into the additiv (δ^2_A) and dominant ((δ^2_D) components of variation. In Lemtal the male parents of both sets did not posess a significant amount of variation. While dominance was of no importance in Limulta, the opposite was true for Lemtal.

Table 2: Factorial crosses (NC II) (six parents in two sets)

A N O V A cv. LIMULTA

	F	V.C.	gen. Var.
Sets	0.4^{ns}		
F : S	2.8^{*}	0.09^{*}	$\delta^2A = 0.36$
M : S	3.3^{*}	0.11^{*}	$\delta^2A = 0.44$
F x M : S	1.1^{ns}	0.02^{ns}	$\delta^2D = 0.08$
Error	1.7		

A N O V A cv. LEMTAL

	F	V.C.	gen. Var.
Sets	11.8**		
F : S	2.9*	0.224*	$\sigma^2A = 0.90$
M : S	1.0ns	0.002ns	
F x M : S	2.1*	0.393*	$\sigma^2D = 1.57$
Error	4.2		

Perennial ryegrass

The results of one set of 6 x 6 factorial crosses within cv. Cropper are given in Table 3. The parents could be tested together with their progenies. The parents ranged from 0 (F4) to 8.5 % (M 10) diseased leave area. The marginal means are the average value across all progenies of each of the 12 parents. If one compares these means with the grand mean (2.7. %), all smaller numbers are indicative for good general combining ability of the respective parent. According to AOV, the females posess a seven fold higher genetic variation than the male parents. The male x female interaction (i.e. sca variance) was not significant and eight times smaller than the total gca variance.

Table 3: 6 x 6 factorial crosses (cv. ropper)

	M	7	8	9	10	11	12	
	3.4	6.5	3.3	8.5	2.1	2.0		
F								
1	3.1	5.2	5.0	6.3	7.6	1.3	3.7	4.9
2	1.5	5.5	4.3	2.3	0.9	1.5	3.8	3.1
3	4.5	5.3	3.3	3.4	5.4	2.7	1.2	3.6
4	0	0.1	0.3	0.4	0.6	0.3	0.5	0.3
5	1.0	1.3	2.7	1.5	2.0	0.3	1.9	1.6
6	1.0	2.2	3.6	2.5	3.6	2.4	2.1	2.7
Mean		3.2	3.2	2.7	3.3	1.4	2.2	2.7

A N O V A	M.S.	V.C.	F-test
Female	29.57	2.18	**
Male	6.97	0.30	*
M x F	3.32	0.28	ns
Error	2.74		

Selfed progenies

Two susceptible plants from cv. Lema and two resistant ones from cv. Cropper yielded enough selfed seeds for testing. Because of the heterozygous state of the parents, the offsporing can be considered to equal a segregating F_2-generation. According to Table 4, at least for the Lema progenies, continuous variation is obvious, ranging from 0 to 20 % diseased leaf area. Because of the high level of rust resistance in both, the parents and the progenies, scale limitations occur.

Table 4: Variability among selfed progenies

	LEMA		CROPPER	
no.of plants	71	72	34	53
% leaf area				
0	2	2	25	20
1	2	1	8	24
3	2	1	1	6
6	21	18		3
10	3	42		
15	0	7		
20		1		
25	-			
x prog.	9.7	9.8	0.3	1.2
x parent	7.3	6.2	0.1	1.5

Selection experiments

Phenotypic selection was practised in both ryegrasses (see Table 5). From 200 plants of each population, the 30 most resistant were selected and recombined. The improved population (G1) was compared with the original material (Go). In all cases a significant improvement in rust resistance could be demonstrated.

Table 5: Selection experiments

	G o	G 1
Limulta	9.8	7.8
Lemtal	7.2	2.5
	G o	G 1
Aurora	13.5	-
A	4.8	2.9
B	4.2	1.6
C	4.9	1.5

DISCUSSION

The occurrence of resistance of the ryegrasses to crown rust has been well established (HAYWARD, 1977; WILKINS, 1975). Due to the continuous variation of the disease scores in populations (i.e. cultivars) rust resistance is considered to be a quantitative character (HAYWARD, 1977). Frequency distributions are indicative of the mode of inheritance. A bimodal type of distribution suggests presence of major genes (KOPEC et al, 1983). A similar interpretation was given for skewed distributions towards high resistance (HIDES & WILKINS, 1978). Among the selfed progenies in Table 4 there is only one case of a skewed distribution. However, due to scale limitations it is not conclusive whether major gene resistance is present or not. Besides the data presented, many more S1's have been analysed and there was no single case of clear cut segregation as one would expect if resistance was be of qualitative nature.

Quantitative genetic analysis is the only way to get an insight into the nature of quantitative inheritance. In the Lema diallel, and also in the Limulta and Cropper factorial crosses, gca was predominant as compared to sca. However, in Lemtal, sca (i.e. dominance) was more important than gca. WILKINS (1975), who applied the same design in this cultivar did not find great importance of sca. However, in two of the progenies typical dominant expression occurred. The very rapid improvement of rust resistance in Lemtal as compared to Limulta (see Table 5) is certainly due to dominant resistance genes. The pro and cons of the utility of of either dominant or recessive resistance genes have been discussed by HAYWARD (1977). Breeders have to be aware that ambidirectional dominance may be present and thus make selection response unpredictable. As was shown by MANSAT & BETIN (1979) in italian ryegrass, in most cases population improvement for crown rust resistance followed a linear pattern which is indicative for additivity. This additive nature of inheritance and the relative high heritability of the character under consideration suggests that phenotypic selection is an effective means of raising the resistance of a population. According to our unpublished data, a close relationship between field data and those from artificial inoculation experiments exists. However, this is only true, if a broad based rust population is applied. There is some evidence that different virulence genes occurs in rust populations from different geographic origin. Further research in this respect is needed. Breeders can avoid false selection by choosing the correct testing sites.

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Experiences with artificial *Puccinia coronata* infections in ryegrass breeding

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Keywords: crown rust, artificial injection, resistance breeding, rygrass

1. Objectives

Testing the effectiveness of artificial infections (AI) with *Puccinia coronata* in ryegrass breeding.

1.1. Is the artificial infection reliable and repeatable ?

1.2. Are plants, selected after an artificial infection, less susceptible to a natural epidemic?

2. Materials and methods

During the winter 1992-1993 we inoculated 20 000 ryegrass plants with spores of a mixture of European *Puccinia coronata* isolates, obtained from the Labor für Biotechnologie und ökologische Phytomedizin, Soest, Germany. The plants were two to three months old at the time of inoculation. They belonged to genetically diverse families and varieties of perennial and Italian ryegrass, all represented by 90 plants.

The spores were dispersed over the plants by a Micron Ulva Controlled Droplet Applicator (CDA) mounted on a motorized device, moving slowly (6 cm/s) on a track. The Micron Ulva CDA applied droplets of about 100 μm at a flow rate of 16 ml/minute. The plants were kept at a 100% relative humidity and a temperature between 20 an 25°C for 24 hours.

The infection became visible after about a week. All the plants were assessed individually two to three weeks after the inoculation. We used a scale from 1 to 9 (1 : not diseased ; 9 : leaves fully covered with acervuli). In practice most plants fell in the range 1-5. We selected and planted the best plants in the field. On average the selection intensities fluctuated around 35%.

2.1. To study the repeatability of the AI, we inoculated 50 families of *Lolium perenne* twice with an interval of six weeks. The first inoculation occurred when the plants were ten weeks old. After both inoculations, all the plants were assessed individually as described above.

2.2. We inoculated 13 varieties of *Lolium multiflorum* and selected the best plants. These selected strains were planted in the field as well as their corresponding original varieties. During a *Puccinia coronata* epidemic in August 1993 we compared the reaction of the original varieties and the selected strains. All the plants were assessed individually in a scale from 1 to 5 (1 is healthy ; 5 is most diseased).

3. Results

3.1. Figure 1 shows the percentages of healthy plants (scored as 1) in each family after the 2 infections (each dot represents 1 family). The graph shows an overall good consistency. A linear regression $y=21.78+0.90x$, $R^2=0.68$ was calculated. Y = % healthy plants after AI2 ; X = % healthy plants after AI1.

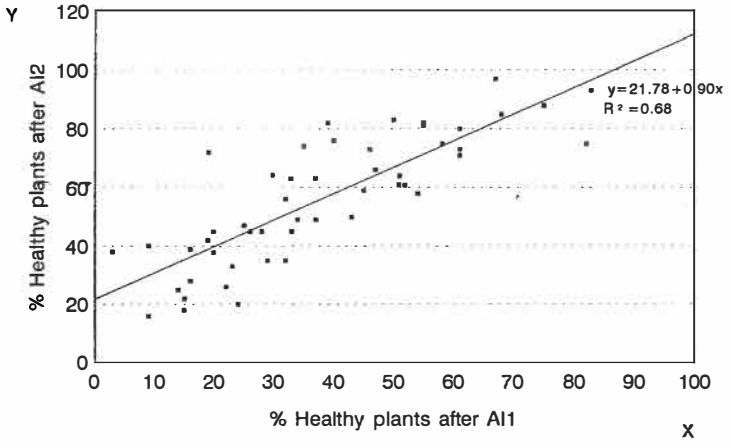

Figure 1. Percentage healthy plants (scored as 1) in 50 families of Lolium perenne after 2 artificial infections with Puccinia coronata.
Score range 1-9 : 1=healthy ; 9=totally diseased

We looked at the paired scores (AI1 versus AI2) of 700 plants out of the 8 most diseased families. Figure 2 shows how these paired scores matched : 27% of the plants were scored identical and 39% of the plants one unit lower or higher. This means that 2/3 of the plants reacted with an acceptable stability.

3.2. Figure 3 shows the differences in reaction of the original varieties and the selected strains to a natural *Puccinia coronata* epidemic. The selection after the AI had a negative effect in one variety and no, or only a slight positive effect in three varieties. A substantial improvement occured in the nine remaining varieties.

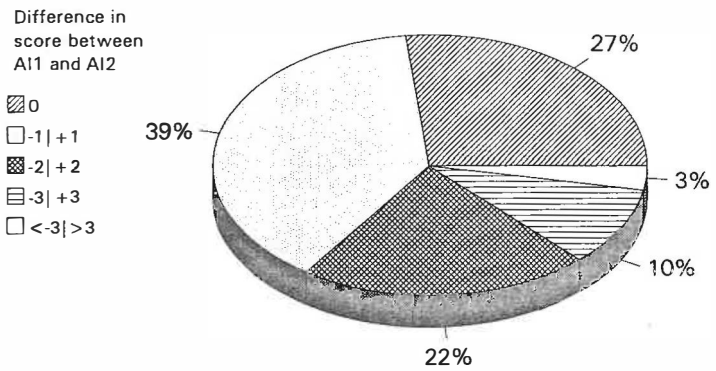

Figure 2. Correspondence of the individual scores of 700 plants after 2 artificial infections with Puccinia coronata
Score range 1-9 : 1 = healthy ; 9 = totally diseased

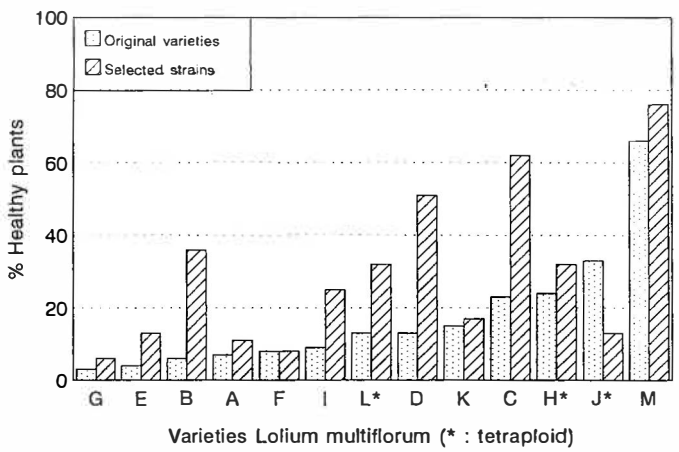

Figure 3. Percentage healthy plants scored as 1 in a scale of 1-5 (5 is most diseased) in 13 varieties of Lolium multiflorum and the selected strains during a natural epidemic of Puccinia coronata

4. Conclusion

We conclude that the variability of the described technique is acceptable in the screening for less rust susceptible plants. In most cases the selected plants are less infected during a natural epidemic.

Management of stem rust (Puccinia graminis subsp.graminicola) in perennial ryegrass (Lolium perenne L.) grown for seed.

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ABSTRACT

In 1991 through 1993 stem rust epidemics in perennial ryegrass grown for seed in the Willamette Valley in Oregon began on different dates each year. When epidemics started early, disease severity was greater by the time of seed harvest. A "critical month" model, developed by K.S. Chester in 1946 for the increase in leaf rust in wheat, was examined for use with stem rust in perennial ryegrass. Preliminary results indicate this model may be useful in predicting development of stem rust in perennial ryegrass. In addition, the model may be helpful in timing fungicide applications to obtain maximum control of stem rust, or reduce production costs by reducing the number of fungicide applications.

KEYWORDS: Stem rust, perennial ryegrass, Puccinia graminis subsp. graminicola, Lolium perenne, seed production, disease management, fungicides, critical month.

INTRODUCTION

Stem rust, caused by Puccinia graminis subsp. graminicola, is a serious disease of perennial ryegrass (Lolium perenne L.) grown for seed in the Willamette Valley of Oregon. In most years, grass seed producers control stem rust by one to three applications of a fungicide. In years when disease severity is high, a 3- to 10-fold increase in seed yield was observed among cultivars treated with propiconazole (126 g a.i./ha) compared to nontreated controls (R.E. Welty and M.D.Azevedo, 1993, unpublished data). In 1991, seed of perennial ryegrass, produced on 45 100 ha, had an estimated farm value of $54.6 million (Miles 1991). One application of propiconazole (190 g a.i./ha + cost of application) to treat all fields of perennial ryegrass in the Willamette Valley in 1991 was estimated to be $2.1 million (M.A. Mellbye, personal communication). Control of stem rust through disease resistance or through judicious applications of fungicides would expose the environment to fewer fungicides and seed producers would reduce production costs by $2 to 6 million, depending on the number of fungicide sprays eliminated.

In our laboratory, progress is being made (Welty and Barker 1992) to develop stem rust resistant perennial ryegrass germplasm, an "environmentally friendly" strategy to control rust. Another friendly strategy would be to use weather conditions to predict

when stem rust will occur to reduce the number or frequency of fungicide applications and still provide stem rust control in perennial ryegrass.

Epidemiology and modeling of rust diseases in cereal grains within and between years has been reviewed (Roelfs 1985; Teng and Bowen 1985). Many reports related rust development to one or more environmental factors, including temperature, precipitation, relative humidity, degree days, hours of free moisture on the plant surfaces, initial inoculum, etc. While most factors were poor predictors of within-year increase of stem rust, a predictive model that may be useful for perennial ryegrass in the Willamette Valley is Chester's "critical month" method. (Chester 1946). Chester proposed that 30 consecutive days (a "critical month") with daily temperature means at or above 10 C were needed for sufficient inoculum to be produced to start an epidemic of leaf rust in white winter wheat. When the critical month occurred, Chester found disease increase was unrelated to subsequent weather conditions. He used this strategy to identify March as the critical month in Oklahoma and used the model to predict occurrence of leaf rust. He extended his model for leaf rust in white wheat to establish April as the critical month for Illinois and Iowa, and April 15 to May 15 as the critical month for New York (Chester 1946). In the Willamette Valley, epidemics of stem rust usually begin between 1 to 15 May and appear as a sigmoid, or S shape, progression when disease is graphed against time (Welty and Barker 1992). Fungicides must be applied before the appearance of stem rust lesions to obtain optimum plant protection. If applications are delayed, fungicides do not adequately control the disease.

When seeds reach 40-45% moisture, plants are cut and windrowed, usually in early July. Seeds are dried in the field and combined 7 to 10 days later when moisture content reaches about 10%. After seed removal, plants resume growth and stem rust is maintained on summer and fall regrowth, and survives winter in infected plants. In most winters, weather conditions allow slow growth of the host, but prevent stem rust from increasing (i.e. low temperatures limit growth and sporulation; urediospores that form are often washed from air by rain; or low temperatures are unfavorable for germination, infection and incubation.) In late winter and early spring, epidemics of stem rust occur in perennial ryegrass.

The purpose of this study was to establish when stem rust epidemics begin in perennial ryegrass in the Willamette Valley, compare monthly temperature means in late-winter and spring, and determine if the critical month method would apply to stem rust development in perennial ryegrass.

METHODS

Seedlings of perennial ryegrass (cv Delray) were grown in single plastic cones (3.8 x 21 cm) in plastic racks and watered daily and fertilized weekly (20-20-20; N-P-K; 473 ppm N; liquid Peters) from below. Care was taken to avoid wetting foliage during plant growth and handling. Seedlings were grown 6-wks in an isolated greenhouse free of stem rust; sowing dates were staggered to provide plants of a uniform age when set out in fields.

Five seedlings were placed in each of five locations (each corner and center) in a field of perennial ryegrass (cv. Delray) at Hyslop Field Laboratory (a University farm) near Corvallis, Oregon. The experiment was done for 12 wks in each of three years beginning on 24 April 1991, 4 April 1992, and 16 April 1993. Twenty-five seedlings (5 x 5) were exposed to natural inoculum and environmental conditions for 7-days in 1991 and for either 3- or 4-day intervals in 1992 and 1993. Exposed seedling were removed from fields and replaced with another set of greenhouse-grown seedlings. Seedlings from the field were returned to a second greenhouse at 20 C +/- 5 C for further growth. Fourteen-days after initial placement in the field, each plant was examined for stem rust and percent infection determined. In 1991, percent infection was based on 25 plants; in 1992 and 1993, data for the 3- and 4-day exposures were combined and percent infection was based on 50 plants.

Daily maximum and minimum temperatures were measured 1 m above soil and used to calculate daily temperature means. Monthly temperature means were calculated from daily means for February to June for 1991 to 1993. Weather data were provided by G.H. Taylor, Oregon Climate Service, Oregon State University, Corvallis 97331-2209.

RESULTS AND DISCUSSION

The first stem rust infections of 6-wk-old seedlings (trap plants) of perennial ryegrass occurred on 19 June 1991, 13 April 1992, and 25 May 1993. The difference between the earliest (13 April) and latest (19 June) initiation of the epidemic was 67 days. However, once the epidemic started, stem rust development followed a sigmoidal progression (Figure 1).

Mean monthly temperature during February through May in 1991 to 1993 and the date stem rust first appeared in trap plants are shown in Table 1. In 1991, when stem rust was first trapped on 19 June, monthly temperature means were 14.1 C for June, 11.3 C for May, and 9.4 C for April. In 1992, when stem rust was found in trap plants on 17 April, monthly temperature means were 12.1 C in April, 10.3 C in March, and 8.6 C in February. In 1993, when urediospores were trapped first on 25 May, monthly temperature means were 14.9 C for May, 10.2 for April, and 9.6 for March.

Temperature means for the month in which urediospores were first trapped were 14.1 C in 1991, 12.1 C in 1992, and 14.9 C in 1993. These means were available too late to be of much benefit for predicting stem rust, especially when urediospores were trapped early in the month, as occurred for May, 1993. These means ranged from 12.1 to 14.9 C which indicate these temperatures may be too high to provide sufficient time to predict stem rust development.

Monthly temperature means for the month preceding infection of trap plants were 11.3 C in 1991, 10.3 C in 1992, and 10.2 C for 1993 (Table 1). These data indicate May 1991, March 1992, or April 1993 may represent a "critical month", based on a 10-11 C "critical temperature." Field observations on the severity of stem rust in those three years support these results (Table 1).

Monthly temperature means at or below 9 C (April 1991, February 1992, and March 1993), generally occurred two months before arrival of urediospores. Temperature means in winter and early spring around or below 9 C, as seen for February, March, and April, 1991, did not allow stem rust development.

Results reported here are based on data for only three years, an inadequate time to evaluate variation in occurrence of stem rust in perennial ryegrass. However, serendipity happened in this study and provided three consecutive years with variable weather conditions. Refinement of the critical month concept will continue to determine if a running temperature mean at or about 10 C for 30 days is the critical month to predict stem rust development. In addition, fungicide application schedules will be tested using the critical month model to determine if timing and number of fungicide applications can be reduced and still control stem rust. Related studies are in progress that include stem rust development in tall fescue and include both grass species growing at another field location.

Results from this study indicate stem rust epidemics in perennial ryegrass are initiated on widely different calendar dates in different years. Furthermore, these yearly date differences contribute to the difficulty for determining optimum time to begin fungicide applications for stem rust control. The critical month method provides an opportunity to test a model to supplement visual field observations for predicting initiation of yearly epidemics of stem rust. Growers that regularly scout their fields for initial small pockets of stem rust might find a critical month temperature useful for beginning searches for stem rust in their fields.

If a monthly temperature mean is to be a useful predictor for development of stem rust, a threshold temperature selected for a critical month should provide sufficient lead time to plan fungicide applications and adjust schedules for minor changes in weather. Selection of the temperature for a critical month must not be too early so fungicides are applied needlessly, nor too late so fungicides applied do not provide stem rust control.

Eventually, a predictive model will be developed to establish a time to begin fungicide application to obtain maximum stem rust control with the fewest number of applications.

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Table 1. Mean monthly temperatures during winter and spring in the Willamette Valley in Oregon and appearance of stem rust in trap plants exposed 3-7 days in a field of perennial ryegrass.

YEAR	MONTHLY TEMPERATURE MEANS (C)					FIRST APPEARANCE STEM OF STEM RUST IN TRAP PLANTS	STEM RUST SEVERITY
	Feb	Mar	Apr	May	June	Day/month	Field scores
1991	9	7	9.4	**11.3***	14.1	19 June	light
1992	8.6	**10.3***	12.1	15.8	18.6	17 April	severe
1993	3.8	9.6	**10.2***	14.9	15.7	6 May	moderate

Bold* = Proposed critical month for predicting development of stem rust in perennial ryegrass in the Willamette Valley in Oregon.

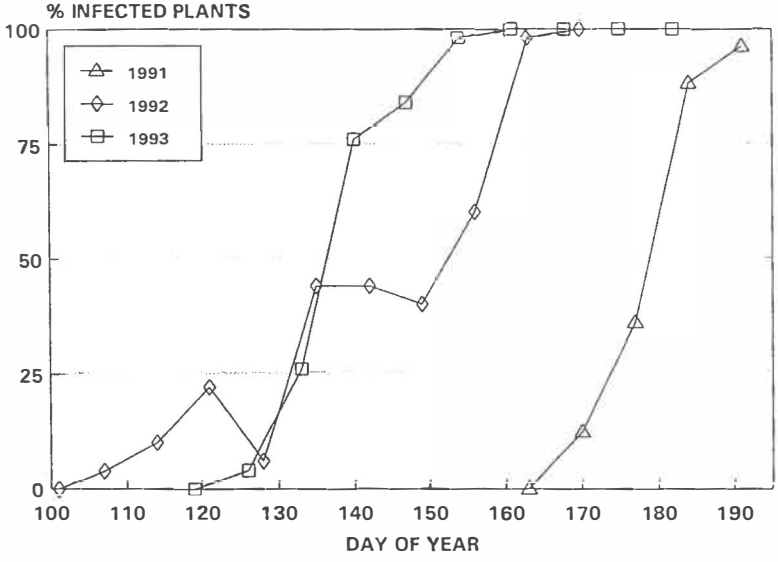

Figure 1. Percent stem rust-infected plants used to trap urediospores in a field of perennial ryegrass. Each experiment was done 12 weeks in each year, but only one initial 0% value was included in the figure.

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SELECTION FOR *DRECHSLERA POAE* RESISTANCE IN KENTUCKY BLUEGRASS (*POA PRATENSIS*) FOLLOWING ARTIFICIAL INFECTION

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ABSTRACT

Persistency under a close cutting regime in the wet and cool climate of North-Western Europe depends in a large part on resistance to the melting out disease, caused by *Drechslera poae*. Two selection methods using artificially produced inoculum were compared with an earlier method in which dried, shredded *D. poae* infected leaf litter was spread across individual plants in seedling boxes. The first method involved inoculation with artificially produced conidiospores, the second method was with mycelium. Inoculation with mycelium fragments proved to be the most rapid and discriminating method for resistance screening

KEYWORDS: *Drechslera poae*, Kentucky bluegrass, *Poa pratensis*, resistance, selection

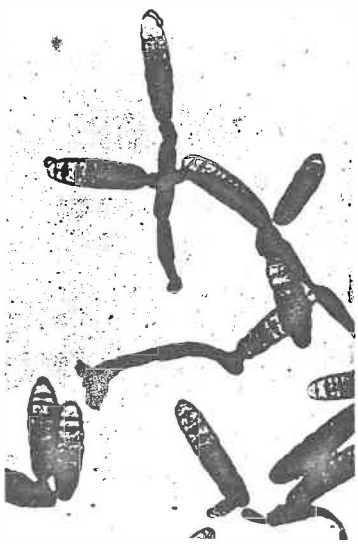
INTRODUCTION

Persistency under turf conditions is a major selection criterion in the breeding of Kentucky bluegrass (*Poa pratensis* L.). Persistency under a close cutting regime in the wet and cool climate of North-Western Europe depends for in a large part on resistance to the melting out disease, caused by *Drechslera poae* (Baudys) Schoem. In 1987 an efficient and reliable screening test was developed which screens individual plants in seedling boxes within a six-month period (Den Nijs & Winkelhorst, 1988). Seedlings were inoculated by spreading dried, shredded *D. poae* infected leaf litter across the boxes. Resistance of varieties was based on the frequency of plants which had died back after seven weeks of repeated close cutting. Limitations of this method are the difficulty to spread the shredded leaves uniformly, and the availability of infected leaves. Therefore methods for artificial inoculum production and inoculation were developed.

MATERIALS AND METHODS

Five varieties of *Poa pratensis* with different resistance levels were used. Pre-germinated seedlings were transplanted in seed boxes, allowed to establish and trimmed at least twice a week to promote turf development. In each box there were two rows of each variety. All inoculations took place 11 weeks after sowing.

D. poae-infected leaves stored in the freezer were plated on water agar to induce sporulation. Conidiospores were isolated and plated on water-agar. After six days the cultures were subcultured by transplanting pieces of hyphae. Conidiospores (Figure 1) were produced either on PDA-plates, or on surface-sterilized *P. pratensis* leaves on water-agar. Spores were washed off the plates, the concentration of the suspension was diluted to 50.000 spores/ml and sprayed on the plants till run-off. Mycelium was produced in liquid Czapek Dox medium incubated for nine days. The mycelium was filtered from the medium, washed with distilled water and blended. A suspension was prepared with 40 mg mycelium/ml, and sprayed on the plants till run-off. After inoculation the boxes were covered with plastic for 72 h to ensure a high relative humidity.



Firure 1: Conidospores of *Drechslera poae* produced *in vitro*

The two artificial inoculation methods were compared with the original shredded leaves inoculation method and a water-sprayed control. All boxes were placed under cold glass and cut back at least twice a week comparable to a field situation in the growing season.

The experiments were carried out in three replicates. Disease severity, as the frequency of plants which had died back, was assessed over a period of seven weeks.

RESULTS AND DISCUSSION

The effects of the inoculation method was already apparent after a few days for all genotypes. Figure 2 shows the severity of disease developing the incubation period on the

susceptible Apoll for each inoculation method. The differentiartion between inoculation methods appeared to be restricted to the earlier part of the incubation period, after which relative differences were relatively stable. This was also found for the other genotypes (results not shown). After the first cutting the infection pressure exerted by infected leaves will be the same for all methods. In Figure 3 the disease severity levels for each genotype seven weeks after incubation are compared with the rating from the Dutch variety list. At that time the susceptible control genotypes Arista, Delft and Apoll showed *D. poae* infection.

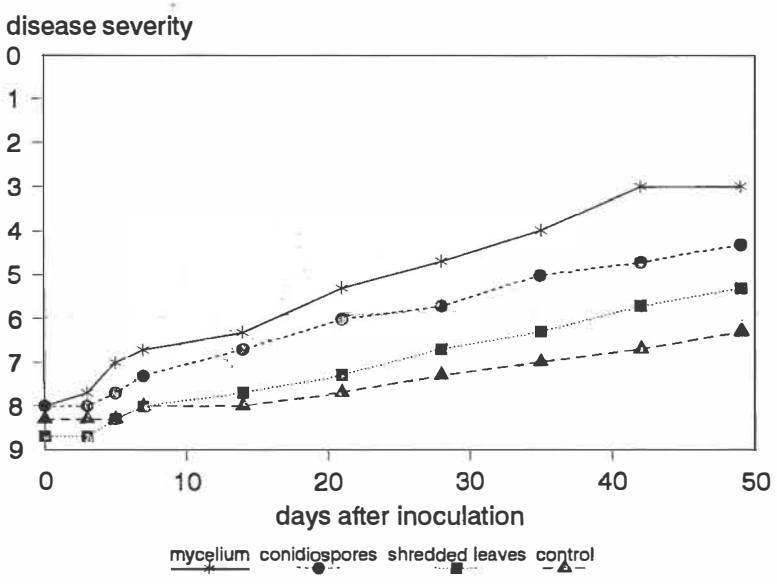


Figure 2: Development of D. poae infection on cultivar Apoll according to the scale (0-9) used in the variety list where 9 indicates absence of symptoms

Inoculation with mycelium fragments proved to be the most rapid (Figure 2) and discriminating method (Figure 3). The resistant Barblue was differentiated from the susceptible genotypes after an incubation period of only four weeks.

The inoculation method with mycelium provides considerable gains in time compared to the original screening method with *D. poae*-infected leaves which to differentiate clearly between genotypes (Den Nijs & Winkelhorst 1988). Differentiation in field trials takes two years.

Future research involves the elimination of environmental effects by developing the test for glasshouse conditions, and determining the optimal concentration of mycelium or spores.

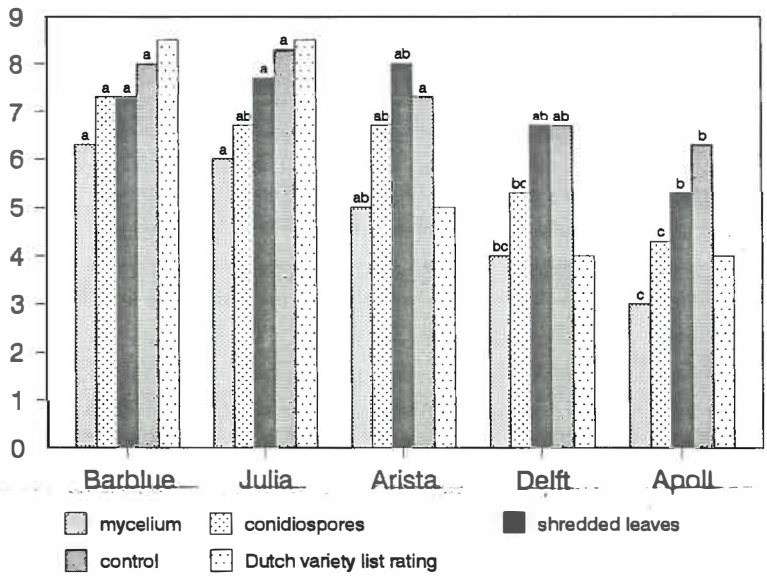


Figure 3: Ratings of Kentucky bluegrass seven weeks after inoculation according to a scale (0-9) used in the variety list where 9 indicates absence of symptoms. Varieties with similar letter do not differ significantly at P≤0.05

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Possibilities of breeding Poa pratensis L. for resistance to ergot (*Claviceps purpurea /Fr./ Tul.*)

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ABSTRACT

Field observations and artificial infection trials with a selection of Kentucky bluegrass (Poa pratensis L.) cultivars carried out during the years of 1988-1991 showed different levels of resistance to ergot. In 1992 another trial was conducted in two locations (Zubří - CZ, and Halle/Saale - GER) to screen the sensitivity of a subset of seven cultivars plus additional ones at each site four ergot isolates under conditions of artificial infection to determine physiologic specialisation and obtain the plant material with a higher level of resistance. In parallel, the level of field infection due to local ergot populations was recorded. In spite of different screening methods used in the two laboratories it was possible to conclude the following: Some cultivars had identical resistance (*cv. Lovegreen*) or susceptibility (*cvs. Moravanka, Haga*), others showed different reaction (e.g. *cv. Sheba*). It was probable that ergot isolates differed in pathogenicity (eg. the marked virulence of USA II). There were clear differences in the morphological characteristics of isolates. Trials should continue in an increased number of locations.

Keywords: ergot, Poa pratensis L., resistance, breeding

INTRODUCTION

Ergot (*Claviceps purpurea /Fr./ Tul.*) is a serious fungal disease mycosis affecting both the qualitative and quantitative parameters of grass seed yield. The economic importance of this Ascomycete fungus is variable. In one of the largesrt world seed production centres in the north-west of the USA (Oregon/Washington) it is one of the limiting factors on seed yields particularly in Kentucky bluegrass. It is also a serious pathogen in mid-European grass seed production (Germany, Czech Republic) causing damage mostly to Kentucky bluegrass, annual meadow- grass and red fescue. In other areas its occurrence and the degree of damage it causes are less important. Despite numerous problems, the ergot sclerotia content in grass seed lots can be reduced by fungicide application (Johnston et al. 1992, Cagas 1991). Non-chemical treatments, e.g. thermosanitation, are used less frequently in both the USA and Europe. There is little documentation concerning the possibilities of Kentucky bluegrass breeding for resistance

to ergot: nevertheless, numerous observations and experimental results have shown that this approach could be realistic.

METHODS

Initial data on the potential differences in Kentucky bluegrass resistance to ergot were obtained on the basis of field evaluation of natural infestations within a selection of 30 cultivars grown on the experimental plots of the Grassland Research Station at Zubrí in the years of 1988-1990. The amount of sclerotia per 1000 spikelets was recorded, but their size and weight were not estimated. A basic collection comprising 7 cultivars, namely Bluebell, Haga, Lovegreen, Nuturf, Rugby, Sheba, Tendos, was established on the basis of known differences in varietal resistances. The cultivars were then artificially infected in a glasshouse using a conidial suspension in water consisting of local ergot populations according to the procedure described previously (Cagas 1986). Sclerotia were obtained from two locations in the USA (Corvallis/Oregon, North Idaho/Washington), one in Germany (Halle/Saale) and one in the Czech Republic (Zubrí) to assess physiological specialization of ergot and pathogenicity of its various populations. Parallel trials with an identical variety set were established in two locations, Zubrí and Halle. At each site the basic set was supplemented with optional cultivars and/or new selections. Inoculation was carried out in a glasshouse and the infection level was estimated as the number of sclerotia per 100 panicles and/or per 0.5 g natural seed. Similar assessments were made of natural field infections with local ergot populations.

RESULTS

The evaluation of ergot sclerotia occurrence within the 30 Kentucky bluegrass cultivars during the years of 1988-1990 showed the following:

a) The infestation level of cvs. Lovegreen, Tendos and Sheba was very low under conditions of natural infection in the field even in 1990 which was characterized by a high selection pressure, i.e. a maximum 0.734 sclerotia per 1000 panicles (Table 1). In contrast, the cultivars Bluebell, Haga, Nuturf and Rugby were heavily infected with a maximum count of 87.93 sclerotia per 1000 panicles.

b) Artificial infection with conidial suspensions in a glasshouse correlated well with the observations in both groups of the cultivars. Cvs. Lovegreen, Tendos and Sheba showed a much lower occurrence of sclerotia (maximum 10 sclerotia per 1000 panicles) in contrarst to cvs. Rugby, Bluebell, Haga and Nuturf (up to 42.8 sclerotia per 1000 panicles). The results of the first year of the joint international trial established at Zubrí (CZ) and Halle (GER) in 1992 can be summarized as follows:

- in both laboratories the results showed identical resistances of cv. Lovegreen and susceptibility of cvs. Haga and Moravanka after inoculation with conidial suspension of 2 ergot isolates (CZ, GER);

Table 1 Ergot incidence in a collection of Kentucky bluegrass cultivars under natural infection pressure

Cultivar	Origin	Number of sclerotia per 1000 panicles			
		1988	1989	1990	Mean
Krasa	CZ	0	4.634	0.4839	1.706
Alicja	PL	0.245	9.524	0.1864	3.318
Ampellia	NL	0.877	1.991	0.1749	1.015
Bluebell	NL	0.269	20.406	0	6.892
Geronimo	NL	0	3.912	0	1.304
Cleopatra	NL	3.393	6.837	3.434	4.555
Cynthia	NL	0	3.599	0.063	1.221
Harmony	NL	1.938	3.811	1.4251	2.391
Mosa	NL	0.757	1.418	0	0.635
Saskia	NL	0.476	0.487	-	0.482
Sheba	NL	0	0.429	0	0.143
Welcome	NL	0	4.389	0	1.463
Camilla	DK	0	3.325	0	1.108
Conni DP	DK	0.651	1.600	0	0.750
Trampas	DK	3.331	0.336	0	1.222
Kyosti	SF	0	3.762	0.1058	1.289
Amason	S	0	9.009	0	3.003
Haga	S	0.383	21.928	0.1	7.470
Primo	S	0	7.673	0	2.558
Nutop	D	0	1.116	0	0.372
Nuturf	USA	7.823	20.039	0.9	9.587
Tectus	D	0.186	18.904	0.05	6.38
Tendos	D	0	0.734	0	0.245
Lovegreen	D	0	0.134	0	0.045
Glade	USA	0.538	8.526	0	3.021
Park	USA	0.347	1.735	0.0534	0.712
Rugby	USA	2.660	87.935	0	30.198
Hz - 9	CZ	-	15.697	0	7.849
Slezanka	CZ	-	13.431	-	13.431
Klon 19	CZ	-	8.678	0.1055	4.392
SL + KL 19	CZ	-	6.010	0.104	3.057
Mean		0.884	9.420	0.248	

- in both laboratories there were differences in the response of numerous cultivars (e.g. cv. Sheba) to artificial infection;
- at Zubrí results showed a high level of pathogenicity of the isolate USA II (North Idaho/Washington), much higher than other isolates;
- pathogenicity of CZ (Zubrí) and GER (Halle) isolates was about the same in both laboratories;
- the level of natural field infection by ergot was rather low at both sites.

The results of the artificial infection trials are summarized in Tables 3 and 4.

Table 2
Ergot incidence in a set of selected Kentucky bluegrass cultivars under conditions of artificial infection

Cultivar	Number of sclerotia per 1000 panicles
Lovegreen	2.1
Tendos	10.0
Sheba	8.0
Rugby	21.0
Haga	5.7
Nuturf	42.8
Bluebell	14.0

DISCUSSION

Considerable economical losses are caused by ergot in the Czech Republic irrespective of fluctuation incidence of the disease in the last decade. This prompted a screening programme to search for some prospective donors of resistance to the local ergot population causing damage to Kentucky bluegrass. The initial field screening of 30 cultivars where differences in resistance were estimated from natural infection only, lead to the concept of basing resistance breeding on a broader geographical basis. Co-operation with specialists in the USA (W.Johnston, S.Alderman) where ergot causes very severe damage to Kentucky bluegrass seed yields has been a major feature of the project. There is need to establish up a small international team of specialists working in various places using common protocols and identical plant material (a collection of previously chosen Kentucky bluegrass cultivars), but differt ergot isolates. Such a study could bring not only new theoretical knowledge on host/parasite biodiversity , but also practical results in the form of plant material carrying a higher level of resistance. The collection of the seven selected cultivars comprises two groups characterized by different levels of infestation

(Group I - Lovegreen, Sheba, Tendos, Group II - Bluebell, Rugby, Haga, Nuturf). The results of the 3-year-field observations were supported by artificial infection tests with the populations from Zubrí. This result prompted the establishment of parallel trials at Zubrí and in Halle using the basic set of cultivar, which was then supplemented with other optional cultivars at both sites. Infection trials with all the isolates in both the locations were impossible to conduct due to technical reasons. Nevertheless, it seems possible to draw some conclusions based on the results of the first joint experimental year.

Varietal resistance

Cv. Lovegreen showed the very high resistance in both laboratories. At Zubrí, with four isolates involved, it ranked together with cv. Sheba as the least infested cultivar (1.72 sclerotia per 100 panicles) as compared with the average level of infection, i.e. 5.11 sclerotia per 100 panicles. Similarly in Halle, where two isolates were used, this cultivar did not reach the average level of infection (1.23 sclerotia per 0.5 g seed). The cultivars Haga and Moravanka were found susceptible in both laboratories. The response of other cultivars differed considerably. Nevertheless, it is important to stress that we have only one year of trials, and the data obtained in 1993 have not yet been processed.

Pathogenicity of the isolates

The results from Zubrí show different pathogenicity of both the European isolates and the American population USA II (North Idaho/ Washington) in particular. All of the cultivars tested were infected most heavily by this population. The differences are shown in Table 3. Pathogenicity of another American population (USA I - Corvallis/Oregon) was very low. Both the mid-European isolates of ergot showed nearly the same level of pathogenicity at Zubrí (4.79 and/or 4.97 sclerotia per 100 panicles) as well as Halle (1.516 and/or 1.306 sclerotia per 0.5 g natural seed - see Tables 3 and 4).
Differences among populations could be also ascribed to some morphological traits and differing ability of their mycellium to produce conidia in culture on yeast agar. During inocula preparation it was found that various ergot populations differed from one another in the amount of conidia produced. The populations USA I, USA II, the Czech and German ones contained 87.5 10^6, 15.0 10^6, 17.5 10^6, and 30.0 10^6 conidia per 1 ml of water, respectively. The study on physiological specialization in ergot and at the same time the response of the selected Kentucky bluegrass cultivars in various locations have shown that there are realistic possibilities of obtaining donors of resistance which should provide promising material for future trials.. It will be important to extend the range of localities, but not to increase the number of ergot isolates and cultivars under study for at least a further 3 years.

Table 3
Number of sclerotia after inoculation with four different ergot isolates and the level of natural (field) infection caused by the local population, Zubrí Laboratory (CZ)

	Ergot populations					
	CZ	USA I	USA II	GER	Field	
	Zubrí	Corvallis	Washington	Halle	infection	Mean
Cultivar	Number of sclerotia per 100 panicles					
Lovegreen	2.71	0.0	6.8	0.81	0.0	2.064
Rugby	12.15	4.4	26.65	1.16	0.04	8.88
Nuturf	13.75	6.10	22.8	15.0	0.4	11.61
Bluebell	0.54	0.57	13.08	0.9	0.0	3.018
Tendos	3.60	2.12	30.62	14.46	0.0	10.16
Sheba	0.66	0.47	4.56	3.24	0.0	1.78
Haga	5.38	1.30	18.78	8.13	0.14	6.71
Mean	5.54	2.14	17.61	6.24	0.08	6.32
Slezanka	1.10	0.09	0.30	1.41	0.13	0.60
Moravanka	4.97	1.22	9.35	4.24	0.18	3.99
Krasa	3.12	0.37	2.70	0.38	0.19	1.35
Mean	4.79	1.66	13.56	4.97	0.11	

Table 4
Number of sclerotia after inoculation with two different ergot isolates and the level of natural (field) infection caused by the local population, Halle Laboratory (GER)

	Ergot population			
	Number of sclerotia per 0.5 g seed			
Cultivar	CZ	GER	Field infection	Mean
Haga	1.1	2.5	0.0	1.20
Rugby	0.9	1.5	0.0	0.80
Bluebell	4.2	6.0	0.0	3.40
Lovegreen	0.4	0.9	0.1	0.46
Sheba	0.5	1.6	2.8	1.63
Tendos	1.2	1.3	0.0	0.83
Nuturf	0.0	0.3	0.0	0.10
Mean	1.18	2.01	0.41	1.20
Hz - 9	1.2	2.3	0.0	1.16
Krasa	0.9	0.6	0.0	0.50
Slezanka	0.1	0.6	0.0	0.23
Roznovská	1.6	0.2	0.0	0.60
Kompakt	0.3	0.2	0.0	0.16
Broadway	8.6	4.0	0.0	4.20
Berbi	0.0	0.05	0.0	0.016
Ziera	0.4	0.9	0.0	0.43
Kutzlebener	1.4	0.3	0.0	0.56
Parade	0.5	0.7	0.0	0.40
Leuroba	1.6	1.3	0.0	0.96
Leuroba	6.7	2.1	0.0	2.93
Ziera	0.6	1.0	0.0	0.53
Leugra	0.8	0.5	0.0	0.43
PPR 11-85	2.8	2.0	0.0	1.60
PPR 15-88	0.2	0.1	0.0	0.10
PPR 18-90	0.4	0.4	0.0	0.26
Mean	1.516	1.306	0.12	

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Alkaloid content of meadow fescue and tall fescue with their natural endophytes

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ABSTRACT

The objective of this study was to determine the accumulation of alkaloids in *Festuca pratensis* (meadow fescue)/*Acremonium uncinatum* and *F. arundinacea* (tall fescue)/*A. coenophalium* associations in two diverse environments. Swards of *F. pratensis* cv. Predix and *F. arundinacea* cv. Kentucky 31 were established in 1991 in Changins, Switzerland and Lexington, Kentucky, USA. The alkaloids N-acetylloline, N-formylloline, ergovaline and peramine were determined in herbage greater than 5 cm above the soil surface. Loline alkaloids were the predominant alkaloids and were in higher concentration in meadow fescue than tall fescue at both locations. Ergovaline was found in tall fescue but not in the meadow fescue. Peramine was only found in the tall fescue/*A. coenophialum* association and only from the Lexington location.

KEYWORDS: Acremonium, alkaloids, endophyte, ergovaline, lolines, meadow fescue, tall fescue, *Festuca arundinacea*, *Festuca pratensis*

INTRODUCTION

There have been many reports of biochemical differences between endophyte infected and non-infected plants. Siegel et al. (1990) reported that only host-endophyte symbiota that included Acremonium species accumulated loline alkaloids (saturated aminopyrrolizidine alkaloids). Tall fescue/*A. coenophialum* is the most common symbiotum that produces loline alkaloids and is a prominent forage grass in north-south transition zone of eastern United States. However, Schmidt (1991) and Bush et al. (1993) reported that meadow fescue infected with *A. uncinatum* grown in Switzerland accumulated large amounts of N-acetylloline and N-formylloline. Petrini et al. (1992) suggested that high selection pressure would be exerted on fungal endophytes infesting distinct ecological niches, which would result in formation of site-specific symbiotum within a host species. This premise could explain the observed loline alkaloid accumulation in *F. pratensis*/*A. uncinatum*, but would suggest that alkaloid accumulation may be vastly different in different environments. The objective of this study was to determine location effect on alkaloid accumulation in *F. pratensis*/*A. uncinatum* and *F. arundinacea*/*A. coenophialum* symbiota grown in Switzerland and the United States.

MATERIALS AND METHODS

Small plots of *F. pratensis* cv. Predix, with and without its natural fungal endophyte *A. uncinatum*, and *F. arundinacea* cv. Kentucky 31, infected with and without its natural fungal endophyte *A. coenophialum*, were established in April 1991 in Changins, Switzerland and Lexington, Kentucky, USA. Seeds from the same seedlots were used at both locations. Infection levels were approximately 80% for meadow fescue tillers and 100% for tall fescue tillers. Acremonium endophytes were not detected in tillers from the uninfected plots. Plots at Changins were fertilized with 90 and 280 kg/ha of P and K prior to seeding. At the Kentucky location, 112 kg/ha of N fertilizer was applied each spring and, at Changins, 50 kg/ha N was added after each harvest. All green herbage material, 5 cm above the soil surface was harvested for alkaloid analysis. Initial samples were taken on 24 June 1991 at Lexington and 22 July 1991 at Changins and approximately every 4 weeks thereafter during the growing season. Initial harvests in 1992 were 24 April and 14 May for Lexington and Changins, respectively. Herbage was freeze-dried, ground to pass 40-mesh sieve, and stored at -20°C.

N-acetyl and N-formyl loline alkaloids were determined by capillary gas chromatography (Yates et al. 1990). Ergovaline was measured by a modified HPLC procedure of Rottinghaus et al. (1991). Peramine was measured by the tlc procedure described by Fannin et al. (1990).

Precipitation for the first harvest or growth period in each year was calculated from the previous 28 days. Precipitation for each growth period ranged from a low of 38 mm prior to the initial harvest at Changins in 1991 to a high of 275 mm for the second harvest at Changins in 1992 (Fig. 1). For the sampling season in 1991 the Lexington location received 415 mm and the Changins location 379 mm, which included 76 mm of irrigation. In 1992, Lexington and Changins received a total of 750 mm and 832 mm precipitation, respectively, during the sampling period.

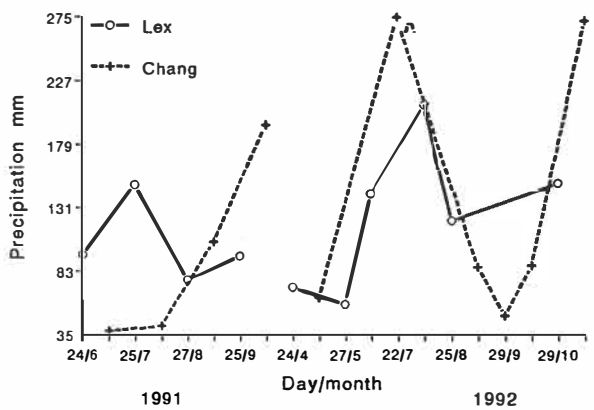

Fig. 1. Precipitation for each growth period at Lexington, Kentucky and Changins, Switzerland during 1991 and 1992.

The average daily temperature was higher in both years at Lexington compared to Changins (Fig. 2). During the sampling period in 1991, the average daily temperature at Lexington was 23.2°C and 17.8°C at Changins. In 1992, these temperatures were 18.2°C and 13.9°C, respectively.

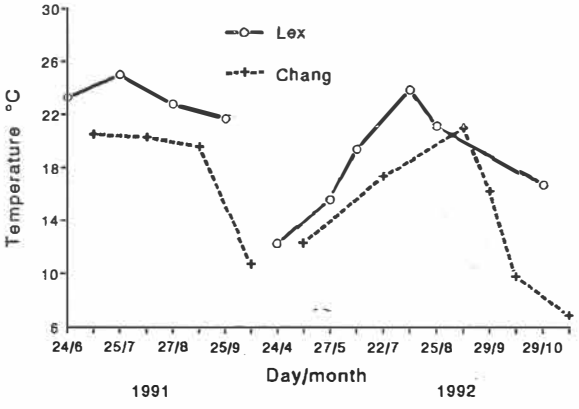

Fig. 2. Average daily temperature for each growth period at Lexington, Kentucky and Changins, Switzerland during 1991 and 1992.

RESULTS AND DISCUSSION

Loline alkaloids were found in higher concentrations in meadow fescue than in tall fescue at both locations Fig. 3 and 4). The absolute difference between the two species was greater in 1991 than 1992 at Changins, but greater in 1992 than 1991 at Lexington. At Changins the growth period with high concentration of lolines in 1991 followed two very dry growth periods and the105 mm precipitation reported for this growth period was mainly from two irrigations.

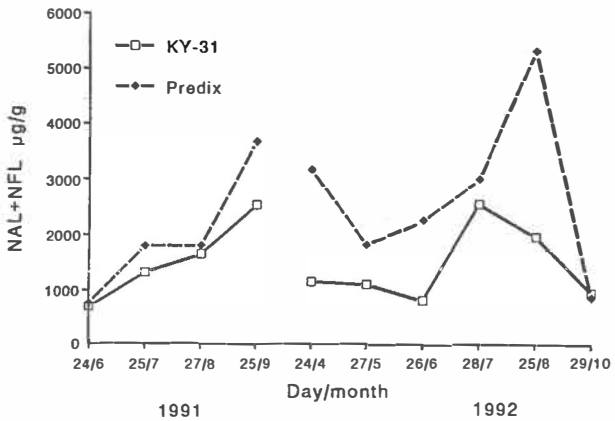

Fig. 3. N-acetyl + N-formyl loline content of Ky 31 tall fescue and Predix meadow fescue at Lexington, Kentucky during 1991 and 1992.

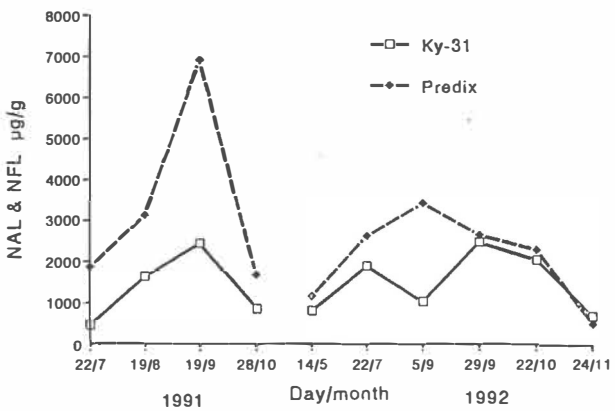

Fig. 4. N-acetyl + N-formyl loline content of Ky 31 tall fescue and Predix meadow fescue at Changins, Switzerland during 1991 and 1992.

At Lexington the maximum loline accumulation occurred during a growth period of moderate precipitation following a very high precipitation period. Maximum concentration (6900 mg/g) of loline alkaloids was measured in meadow fescue in September 1991 in Changins while the maximum concentration in Lexington was 5250 mg/g in August 1992. Loline alkaloid accumulation was similar in the two species at both locations. Mean values of all samples for loline accumulation in Kentucky 31 were 1422 and 1451 mg/g at Changins and Lexington, respectively, and 2610 and 2423 mg/g for Predix for the respective locations. There was no significant difference between the

locations in loline alkaloid accumulation. Loline alkaloid accumulation during late summer was similar to previous reports and has been related to depression in growth due to water and temperature stress more than a direct effect on alkaloid biosynthesis (Kennedy and Bush 1983; Stuedemann et al. 1985; Belesky et al. 1989).

Ergovaline was found in tall fescue at both locations but was not found in meadow fescue at either location (Fig. 5). At both locations, herbage from the first year had a higher accumulation of ergovaline than the succeeding year. Greatest accumulation of ergovaline (2.2 mg/g) occurred at Lexington. Mean ergovaline content was 0.72 and 0.88 mg/g at Lexington and Changins, respectively. Evidently, the higher temperature and lower precipitation at Lexington compared to Changins in 1992 did not affect measurably ergovaline accumulation. There was no significant difference between the two locations for ergovlaine accumulation in tall fescue.

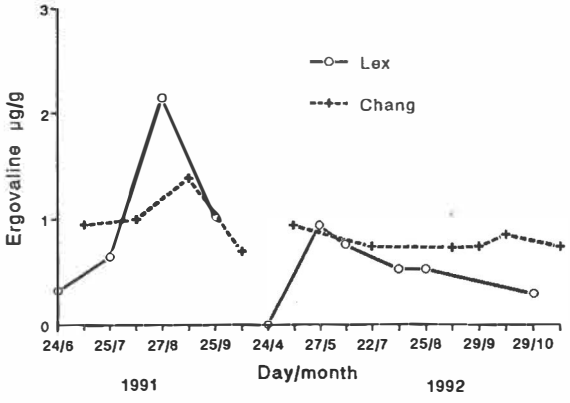

Fig. 5. Ergovaline content of Ky 31 tall fescue at Lexington, Kentucky and Changins, Switzerland during 1991 and 1992.

Peramine also was found only in tall fescue, but in contrast to ergovaline, peramine was found in tall fescue only at Lexington (Fig. 6). Peramine levels were highest in the establishment year but were always less than 1 mg/g. The lack of peramine accumulation at Changins cannot be explained from the temperature and precipitation data. The tall fescue/*A. coenophialum* symbiotum had similar accumulation of lolines and ergovaline at the two locations, which indicates that the environmental conditions were adequate for accumulation of these alkaloids, thus peramine would be expected to be present at Changins. Perhaps peramine biosynthesis is more sensitive to growth conditions or stresses not measured and thus the qualitative difference between the two locations.

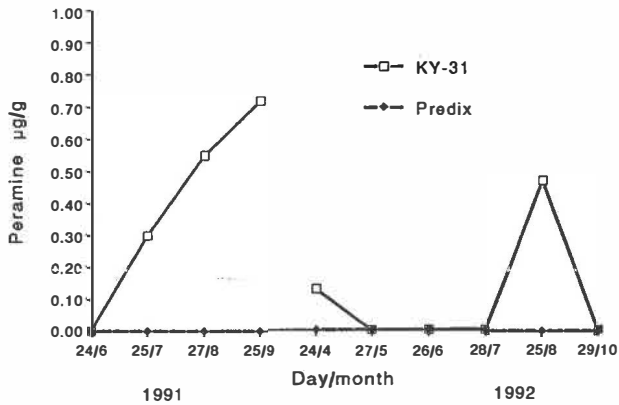

Fig. 6. Peramine content of Ky 31 tall fescue and Predix meadow fescue at Lexington, Kentucky during 1991 and 1992.

The results obtained in this study indicate that of the common alkaloids found in fescue/Acremonium symbiota only the loline group of pyrrolizidine alkaloids accumulate in meadow fescue/*A. uncinatum*, thus confirming the preliminary reports of Bush et al. (1993) and Christensen et al. (1993). This result would strongly suggest using *A. uncinatum* infected and *A. uncinatum*-free meadow fescue forage to measure animal responses to the loline alkaloids in forage grasses. The relative contribution of the *A. uncinatum*, or the meadow fescue host genotype, to the high accumulation of loline alkaloids cannot be determined from these data. Artificial inoculation of this *A. uncinatum* biotype into other meadow fescues and tall fescues will be required to ascertain the contribution of the macro- and micro- symbiont in these associations.

There was less effect of environment on accumulation of lolines and ergovaline than expected. Even within meadow fescue/*A. uncinatum* symbiotum, which accumulated much greater amounts of lolines than tall fescue/*A. coenophialum* there was measurable effect on loline accumulation only in regrowth tissue following drought. This lack of location effect was probably due to the lack of severe temperature and moisture stress in both years.

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Influence of endophytes of *Festuca pratensis* on damping-off diseases of seedlings

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ABSTRACT

Seed of meadow fescue (*Festuca pratensis*) cultivars or breeding families infected and free of endophytes were sown in soil contaminated by different pathogens suspected to cause root and shoot diseases or damping-off.

Mortality caused by *Fusarium culmorum* increased progressively with the age of the seed, even when stored at 5°C and low humidity.

Endophyte infected seed lots often had reduced emergence when inoculated with *F. culmorum* or other preemergence pathogens compared to the endophyte free seed. This might be explained by a slight delay in primary development caused by *Acremonium* infection.

On the other side, infection with *A. uncinatum* resulted in a slightly better survival to attack by *Drechslera sorokiniana,* and, in certain cases, by a thermophil strain of *Microdochium nivale* and by *Rhizoctonia cerealis*.

Yield after two months was increased by the endophyte as well in the control plots as with most inoculations, indicating a certain faculty of compensation by the surviving plants.

Keywords : *Acremonium uncinatum*, damping-off, *Drechslera sorokiniana*, endophyte, *Festuca pratensis*, *Fusarium culmorum*, *Microdochium nivale*, *Rhizoctonia cerealis*

INTRODUCTION

Meadow fescue (*Festuca pratensis* L.) is frequently infected by an *Acremonium* endphyte distinct from those found in other grasses, named *A. uncinatum* (Gams *et al.* 1990). It can be associated or not with another fungus, identical to the "*Phialophora*-like" endophyte found by Latch *et al.* (1984) in tall fescue (An *et al.* 1993), but named by error *A. typhinum* in the first publications (Gams *et al.* 1990, Schmidt 1991). Both endophytes are seed transmitted.

The symbioses with *Acremonium* endophytes may be beneficial to the plants. Ma references exist about increased vigour, insect-, nematode- and eventual disease-resistan (e.g. Siegel *et al.* 1987) in different grass species. Meadow fescue often shows a bett persistance when infected with endophytes, especially in hot dry summers (perso observations). Root and shoot diseases might be envolved in summer dye-out.

This paper reports observations of some fungal pathogens on meadow fesc seedlings, in relation to presence or absence of endophytes.

METHODS

Endophyte free and infected seed were produced on clones of naturally infecte plants freed from the endophyte by fungicide treatment. Predix is a synthetic cultiv based on nine clones, all infected with *A. uncinatum.* Endophyte-free and infected see lots of the M_1 generation had been multiplied at the same place and year.

The inoculum consisted of a culture of the respective fungi on a mixture of whe bran (10%) and humid sand (90%) autoclaved twice. The inoculum was spread in drills trays of steam treated peat. Twenty seed were put in each row and covered with anoth layer of inoculum. The control plants were sown in sterile substrat. High humidity wa maintained by covering the germination trays with plastic sheets until emergence of th seedlings. All infection trials took place in a glasshouse with minimum temperatures 20°C day and 16°C night. Each trial used twenty seeds per seedlot and treatment in thre replications.

RESULTS

Pathogenicity of the strains

In a first test using an endophyte-free commercial seed lot of Predix, all strains *Fusarium culmorum* and *F. avenaceum* were highly pathogenic prior to emergenc showing no differences between isolates from wheat or from fodder grasses. *F. oxysporu* and *F. graminearum* were slightly less aggressive, but they still reduced emergence to high extent. The same was observed with *Drechslera sorokiniana* and a thermophil stra of *Microdochium nivale*, isolated from *F. pratensis* in summer. However, these latter tw pathogens also induced post-emergence damage. Other isolates of *M. nivale* caused muc less mortality. *Rhizoctonia cerealis*, a *Hendersonia* sp., *Pseudocercosporel herpatrichoides* and *Septoria nodorum* were only weakly pathogenic.

Influence of endophytes on seedling damping-off

In a next trial using recently harvested seed of Predix (tab. 1), *F. culmorum* induced much less mortality than in the first trial; and it affected the endophyte free seed less than the endophyte infected seed. The most severe damage was caused by the thermophil strain of *M. nivale* and, again, mortality was lower in the endophyte free lot.This tendency was less striking with *D. sorokiniana* and *R. cerealis* inoculations. With these two pathogens, the number of surviving plantlets one month after sowing was favourably influenced by the presence of endophytes. Even if this difference was not significant, it indicates that *A. uncinatum* could have a beneficial influence on post-emergence damage caused by these two fungi. This observation was confirmed by another trial using four fescue families (tab. 2). Both trials showed a significant interaction between pathogens X endophyte presence, and the second trial also demonstrated a fescue families X pathogens interaction.

Influence of the age of the seed

The results of a trial examining the influence of storage of the seed under favorable conditions of 5°C and low relative humidity on the seedling mortality caused by *F. culmorum* are presented in table 3. After two years of storage, about half of the plantlets were killed by the *Fusarium* inoculation. After six years of storage, only very few plantlets survived the fungal attack, even though the control plants still had an adequate establishment. With the two years old seed, damping-off was again less severe in endophyte free than in endophyte infected seedlings.

DISCUSSION AND CONCLUSION

F. culmorum was the most destructive pathogen prior to emergence. The damage it caused seems to depend very much on the vigour of the seed. Harmful effect of fungal inoculation increased progressively with the age of the seed. It can be imagined that a slight delay in germination exposed the seedlings to the attack of *F. culmorum* for a slightly longer period at a crucial stage for infection.

In most trials, the endophyte infected seed showed a higher mortality than the endophyte-free seed under pressure of *F. culmorum* and other pre-emergence pathogens. In a previous glasshouse trial with meadow fescue, we noted a slight delay in rate of establishment of endophyte infected seed lots compared to endophyte-free ones (Schmidt 1991). Keogh (1987) made the same observation with rye-grass seed infected with *A. lolii*. Other authors, however, found the opposit result (Clay 1987, Reed *et al.* 1985). In the

experiments presented in this paper, the phenomenon was not observed with the uninoculated control plants (tab. 2 and 3).

Within the seed, the embryo is free of endophyte. The endophyte mycelium is concentrated in the aleuron layer, and seedling infection occurs only during germination. In early stages of germination, the fungus is competing for the reserves stored in the grain. It is also imaginable that the establishment of symbioses costs the plantlet some energy. This scenario would explain a slight delay in primary development caused by the endophyte and support the hypothesis that a decreased rate of initial development is responsible for the higher susceptibility to attack by pre-emergence pathogens. This would explain our results for both the endophyte infected seed and the aged seed.

F. culmorum does not seem to cause much post-emergence damage. The surviving seedlings are vigorous enough to compensate yield loss at the age of two months even with about 50% of mortality (tab. 3).

The endophyte could have a positive effect on post-emergence damage. Thus the yield of endophyte infected seedlings was much less affected than the yield of endophyte-free seedlings after inoculations with *D. sorokiniana*, *M. nivale* and *R. cerealis*, even if the latter did not cause mortality (tab. 2). The results shown in table 1 indicate that *R.cerealis* can also cause mortality; here too, *A. uncinatum* had a beneficial effect. Gwinn and Gavin (1992) made similar observations with *A. coenopohialum* in tall fescue (*F. arundinacea*) which reduced seedling damage caused by *R. zeae*.

The highly significant interactions found between endophytes X pathogens as between fescue genotypes X pathogens contribute to the final conclusion that the whole problem is extremely complex, so that no clear answer as to the desirability of endophyte presence can be drawn.

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Table 1. Influence of endophyte level on resistance of *Festuca pratensis* cv. Predix to damping-off caused by different fungi

	Endo-phyte level	Control	*Fusariumculmorum*	*Fusarium graminearum*	*Drechslera sorokiniana*	*Microdochium nivale*	*Rhizoctonia cerealis*	b) Significance between		
								Treatm.	endo. level	interaction
Total emergence (%)	+	95,0	78,5	90,0	91,5	45.0	78,5	***	**	*
	-	96,5	90,0	90,0	88,5	73,5	88,5			
Surviving plants after 1 month (%)	+	90,0	71,5	83,5	83,5	26,5	50,0	***	*	*
	-	90,0	85,0	90,0	70,0	68,5	53,5			
a) Normal. develop. plants, 1 month (%)	+	81,5	70,0	83,5	73,5	10,0	41,5	***	n.s.	n.s.
	-	76,5	78,5	86,5	65,0	36,5	31,5			

a) at least 2 leaves and 6 cm height

b) probability of significance : * p _ 0,95
** p _ 0,99
*** p _ 0,999 n.s. = not significant

Table 2. Influence of endophyte level on resistance of *Festuca pratensis* (means of four ecotypes) to damping-off caused by different fungi

	Endo-phyte level	Control	*Fusarium culmorum*	*Drechslera sorokiniana*	*Microdochium nivale*	*Rhizoctonia cerealis*	Significance between					
							Treatm (T)	endophyte level (E)	fescue cultivar (C)	Interaction		
										T x E	T x C	E x C
Total emergence (%)	+	96,9	64,4	92,5	88,5	96,0	***	n.s.	**	*	***	*
	-	96,9	73,2	91,3	85,7	96,0						
Surviving plants after 2 months (%)	+	91,3	49,3	85,5	73,5	92,8	***	n.s.	n.s.	n.s.	n.s.	n.s.
	-	89,8	51,3	77,8	64,3	90,0						
Yield at the age of 2 months (g)	+	19,8	16,6	19,7	17,7	18,5	***	***	***	***	n.s.	***
	-	18,5	16,0	16,1	12,9	13,5						

probability of significance : * p _ 0,95
** p _ 0,99
*** p _ 0,999

n.s. = not significant

Table 3.
Influence of age of the seed and endophyte infection on damping-off caused by *Fusarium culmorum* on *Festuca pratensis* cv. Predix

Age of seed years	Endo-phyte level	Control	*F. culmorum* strain	
			F 125	F 100
Total emergence (%)				
6	-	78	10	5
2	+	85	58	40
2	-	90	68	55
1	+	98	95	78
1	-	95	75	80
Surviving at 2 months				
6	-	75	8	5
2	+	83	55	38
2	-	78	63	55
1	+	93	93	70
1	-	88	73	75
Yield at 2 months (g)				
6	-	19,0	2,1	1,3
2	+	20,5	18,2	15,8
2	-	18,8	19,8	19,1
1	+	22,2	25,7	23,0
1	-	19,4	18,0	21,8

The effect of endophyte infection in perennial ryegrass on larval infestation and tiller production

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ABSTRACT

Tiller production and larval infestation of perennial ryegrass were assessed in endophyte free and endophyte infested material which had previously shown a yield advantage associated with the presence of endophyte. There were no differences in the number of tillers/m², the number of tillers damaged by larvae, or the numbers of larvae/m² between grass with and without endophyte. However, the former had a significantly higher proportion of living tillers/m². Possible reasons for the effect of endophyte in reducing tiller mortality are discussed.

Keywords: Endophyte; *Acremonium lolii*; *Lolium perenne*; dipteran stem-borers; *Oscinella* spp.; tiller damage; tillering.

INTRODUCTION

The fungus *Acremonium lolii* Latch, Christensen & Samuels is a seed transmitted endophyte which occurs in several New Zealand cultivars of perennial ryegrass *Lolium perenne* L. The endophyte confers resistance of *L.perenne* to Argentine stem weevil *Listronotus bonariensis* Kuschel for mature plants (Gaynor & Hunt, 1983), and reduces seedling damage by adults (Stewart, 1985). Insect feeding deterent chemicals either produced by the grass in response to the intercellular fungus, or produced by the fungus, or by both, are involved (Stewart, 1985). Also, infected *L.perenne* clones of cv. Grasslands Nui have produced more dry matter, with increased leaf area and tiller numbers, than uninfected clones under the same insect-free glasshouse conditions (Latch, Hunt & Musgrave, 1985).

From 1989 to 1991, trials on the effect of endophyte on perennial ryegrass yields were done in four regions of the UK, with plots drilled with seed of cv. Grasslands Nui of either high or low endophyte content. A higher ground cover in high endophyte plots was seen at the end of the first year under simulated grazing, with yield significantly greater by the end of the second at some sites (J Ingram pers. comm.). Weevils are not grass pests in the UK, but dipteran stem borers often infest newly sown ryegrass swards, with larvae of successive generations then present throughout the year, damaging meristems and affecting tiller numbers and herbage yield (Clements, Chapman and Henderson,

1983). Any reduced stem-borer colonisation of the high endophyte plots could contribute to their greater yield. Therefore, in this investigation samples were taken to compare the larval population of high and low endophyte seed origin plots.

METHODS

Samples were taken from the South-western region, where yield differences in 1990 had been high, and from the Eastern region where the lusher growth of endophyte infected plots had been clearly visible. Sample cores were taken on 3 and 5 November 1990 at the respective sites, in order to obtain the overwintering generation of stem-borers. Sampling was not done earlier in the year, because of the probably reduced chance of coincidence of all the dipteran stem-borer species as larval or diapause pupal stages within the plants. 5 m x 1 m plots of cv. Grasslands Nui from high and low endophyte containing seedstocks were sampled at Seale Hayne, Devon and at Cambridge, Cambs. Three samples, on average 70 mm x 60 mm, were taken from each of two plots of each endophyte level at Seale Hayne. Six 60 mm diameter samples were removed from three plots of each treatment at Cambridge. Plant samples including rootballs were frozen, and defrosted as required to record internal tiller condition and larval presence by slitting open each tiller under a low stereo microscope. Tillers were scored for the possession or lack recent meristem growth without larval damage (i.e. healthy or dead), and new or old tissue maceration by larvae (i.e. present or former larval generations). A sample of larvae from each plot were identified. To determine the level of endophyte infection in the samples, ten outer leaf sheaths were removed from each sample and cleared in lactophenol and alcohol before staining with trypan blue for hyphal detection under a compound microscope at 250x magnification.

RESULTS AND DISCUSSION

Two plots of each treatment were sampled at Seale Hayne and three plots of each at Cambridge. From high endophyte plots, hyphae were seen in a mean of 87.79% (SE $\pm$ 7.8) and 78.60% (SE $\pm$ 7.1) of leaf sheaths at Seale Hayne and Cambridge, respectively. In low endophyte plots 3.15% (SE $\pm$ 3.2) and 4.60% (SE $\pm$ 3.8) of sheaths from the respective sites were endophyte infected.

Comparisons within sites of high and low endophyte grass were made using Mann-Whitney analysis (2-tailed) for the samples from each treatment. Results of the tiller assessments at each site are given in Table 1. The tiller density did not differ significantly ($P > 0.05$) between the high and low endophyte plots at each site, with means of 60622 tillers/m^2 and at Seale Hayne and 38262 tillers/m^2 at Cambridge. However, more ($P < 0.05$) tillers from high than from low endophyte plots at Seale Hayne were healthy. Thus, 19% more ($P < 0.01$) of the total tillers in the Seale Hayne high endophyte plots

Table 1. Means of high (+) and low (-) endophyte plots at Seale Hayne (SH) and Cambridge (C) for tiller and larval population characteristics.

	SH +	SH -	C +	C -*
Total tillers/m²	61405.49	59837.40	6670.01	39853.58
± SE	6172.71	2911.07	2374.67	5321.26
Healthy tillers/m²	28151.21	15389.12	25488.21	24250.16
± SE	1283.84	1808.32	1804.16	2486.07
Dead tillers/m²	12375.69	19868.37	7310.42	12852.19
± SE	1410.38	1119.97	988.89	625.80
Senescent (dead) tillers/m²	7345.58	13227.36	3360.44	2829.84
± SE	92.96	266.70	248.31	1003.61
Flowered (dead) tillers/m²	5030.08	7459.40	4181.88	3949.99
± SE	1317.44	2207.51	449.54	1226.02
Old damage tillers/m²	9267.85	12394.12	1238.06	2829.84
± SE	40.77	1760.64	264.39	187.47
New damage tillers/m²	11612.51	12185.83	2829.84	1827.61
± SE	3521.04	1777.88	187.47	581.98
% Healthy of total tillers	46.42	27.75	69.56	60.74
± SE	2.27	1.86	0.10	4.31
% Damaged of total tillers	33.38	41.79	11.54	10.26
± SE	1.91	0.62	1.53	0.96
% of tillers with larvae	11.11	12.40	5.84	6.67
± SE	0.22	1.96	1.21	0.99
Larvae/m²	7020.14	7319.94	2043.78	2790.54
± SE	1006.35	1157.70	347.68	717.51

*df for SE at SH = 1, at C = 2

were healthy than in the low endophyte plots. A smaller difference ($P<0.05$), of 9% more, also occurred at Cambridge. At Cambridge, a higher ($P<0.05$) density of dead tillers in low endophyte plots (27% of total tillers) counterbalanced the fewer healthy tillers. This was also shown less significantly ($P<0.10$) at Seale Hayne, where 33% of total tillers in low endophyte plots were dead, whereas this was 20% in the high endophyte plots.

Dead tillers without larval damage occurred from either senescence or after producing a flowering stem. Neither site differed significantly ($P>0.05$) in the relative densities of either senescent or flowered tillers between treatments.

Dipteran stem-borers, mainly *Oscinella* spp. larvae with some *Geomyza* spp. pupae, occurred at similar densities ($P>0.05$) in the high and low endophyte plots, with means of 7170/m² at Seale Hayne and 2791/m² at Cambridge. There was no difference ($P<0.05$) in the proportion of tillers infested whether or not endophyte was present, so that with the greater tiller density at Seale Hayne there was a mean of 11.75% tillers with larvae, while at Cambridge there was a mean 6.26% infestation. More tillers had larval damage than contained larvae, but without difference between the treatments to give means of 37.59% at Seale Hayne and 10.90% at Cambridge. Of the damaged tillers, there were no differences ($P<0.05$) between the treatments in the numbers of tillers which had received either earlier or recent feeding activity.

Intercellular endophyte hyphae were found to be common in grass grown from infected seed, with hyphae rare in plots of low seed infection. There was no difference in tiller density between the two treatments, but there was a greater proportion of healthy tillers in high endophyte plots, which would have given the lusher plot appearance. The larval population and the number of tillers attacked did not differ between treatments.

There were more tillers damaged than larvae, probably because damage from the stem-borers' summer generation and from earlier instars was included. As old damage did not differ between treatments it was unlikely that the larvae disproportionately influenced yields at earlier cuts. This is in agreement with the results of Lewis and Clements (1986), who found no effect of endophyte against *Oscinella frit* in establishing swards of perennial ryegrass.

The greater proportion of dead uninfested low endopyte tillers suggests a beneficial effect of fungal presence, decreasing senescent tillers in high endophyte plots. It was recorded that the two sites examined gave the largest yield responses to endophyte presence, and that they suffered more severely from drought during the trial period than

the other sites. Greater ability to withstand drought stress has frequently been suggested as a possible benefit of endophyte infection.

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