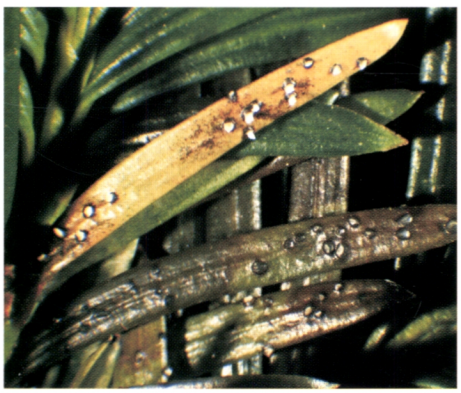
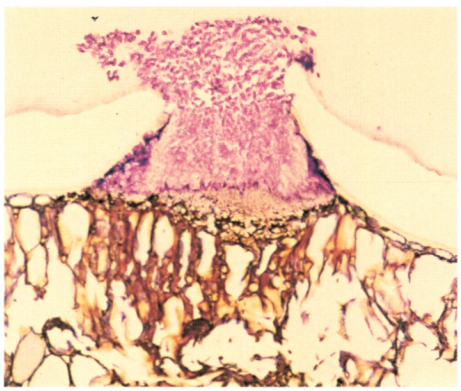
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Proceedings of the IUFRO Working Party 7.02.02 Shoot and Foliage Diseases, Meeting at Hyytiälä, Finland, 17-22 June, 2001


Edited by
Antti Uotila
Vellamo Ahola

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METLA

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Helsinki 2002

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Preface

Forest pathologists specializing in the study of shoot and foliage diseases have met regularly over the last 30 years to report and discuss their findings on the biology and management of important diseases impacting forest and plantation trees. The first big meeting of this group was in Syracuse New York (USA) in 1983 with 66 scientists participating. At that meeting the main topic of discussion was the damage that was occurring at that time as a result of Scleroderris canker in North America, Europe and Japan.

Although there has been extensive research on all aspects of this disease since that first meeting of our group, we learned at this meeting of the current severe epidemic of Scleroderris canker in Sweden occurring in 2001. This underscores not only the need for continued research, but also the importance of environmental factors that can affect the incidence and severity of this disease, limiting our ability to prevent damage in spite of all of our knowledge.

Over the years our working party has evolved from a meeting primarily focused on Scleroderris canker to one that now brings together scientists researching a wide range of shoot, stem and foliage diseases from geographically different areas of the world. This interchange of experiences, information and ideas among scientists is important and is the strength and value of our working party to participants.

During our meeting on the field trips we became familiar with the local diseases and forestry practices at Juupajoki and Orivesi communities. The region around Tampere is called Pirkanmaa where forestry and forest industries are a very important sector of the economy. Even the roots of the Nokia mobile phone company are in forest industries in the Pirkanmaa area. Nokia is the town close to Tampere.

This meeting was held at the Hyytiälä Forestry Field Station of Helsinki University. The role of the field stations is very important in teaching forest sciences. Teaching forestry science has been carried out for the last 90 years at Hyytiälä. Finnish foresters believe that Hyytiälä is the heart of their education. The field station is not only about teaching and research but provides an atmosphere for food, sports, arts and sauna. It was intended that by having the meeting here, that our working party experienced the real Hyytiälä spirit!

Antti Uotila
The Organiser of the meeting



The participants of the meeting at Hyytiälä.

Inoculating trees in nature: a problem of environmental interactions

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Abstract

The study of forest pathogens is frequently hindered by problems in statistical analysis of field trials because host susceptibility and pathogen virulence interact strongly with environmental effects that stress trees differently in different years. ANOVA tests are confounded by significant genotype x environment interactions that preclude mean separation and ranking tests. The Additive Main Effects and Multiplicative Interaction Model is introduced as a method of obtaining valuable information from the complexity introduced by statistical interactions.

Many of the common pathogens of trees are particularly recalcitrant organisms for studying the host pathogen system in nature. This is not due to difficulties in isolation or cultivation of the microorganisms, rather the difficulties are in reproducing the disease symptoms on the host. A basic paradigm in forest pathology is that many pathogens are virulent only on a host suffering stress caused by one factor or multiple factors over time. Environmental factors have profound effects on the incidence and severity of disease symptoms on trees.

The environmental factors that predispose trees to disease are complex and vary with site. Perhaps the best known of the factors causing stress are drought and insect pests that vary seasonally and spatially. Further compounding the complexity of environment on host susceptibility is the variable response of differing host genotypes and host species to a stress. Completing Koch's Postulates with a fungal pathogen on a tree species is not straightforward. A review of older literature shows the surprising fact that many initial inoculation trials yielded only 50-70% successful infection with disease expression. Would today's reviewers accept the claim of completion of the Postulates with such low success levels?

Environmental interactions can have so profound an effect on virulence of a pathogen that the pathogen becomes labeled as weak or opportunistic. Labeling forest pathogens as weak opportunists has damaged the study of forest pathology in recent decades as the label has been used as a reason to reject grant proposals seeking funds to study the organisms. A compounding factor in the study of forest pathogens is the more recent revelations that many fungal pathogens are endophytic residents in waiting. Well-studied examples of forest pathogens that are endophytic and opportunistic yet cause severe disease and great economic damage when interacting

with environmental stresses, include: *Botryosphaeria, Sphaeropsis, Armillaria, Phomopsis,* and *Cytospora.*

Inoculating trees with such pathogens may yield isolate ratings of avirulence, low virulence, and highly variable virulence, but a reaction of high virulence is unlikely. For example, to successfully reproduce a canker symptom on a tree with a *Cytospora* pathogen we find it necessary to inoculate in the fall in the Northern Hemisphere which allows the pathogen to colonize the host when dormancy has disabled the host's defense response. Additionally, we mechanically wound and freeze damage the cambium to aid infection and colonization. In regions without a winter period of plant dormancy we have been unable to induce disease without adding a different stressing agent such as drought stress.

Knowledge that the virulence of a pathogen is altered by interactions of environment on the host has become well established in forest pathology. Additionally, it is well established that host genotypes (i.e., provenances) respond variably to environmental factors. The virulence of a pathogen that can infect several host genotypes and host species will interact in complex ways to quantitative and qualitative differences in environmental stresses.

Dealing with environmental and genotype interactions in inoculation trials in nature remains a major problem in forestry research. The standard method for removing environmental interactions from inoculation trials has been to isolate the plants in an environmentally controlled glasshouse or growth chamber. Unfortunately, inoculations of seedlings do not always reproduce the type of disease symptoms characteristic of trees in nature. More importantly, inoculations removed from nature do not supply information on the behavior of a pathogen in nature. In particular, little can be learned about how the virulence of a pathogen will differ under seasonal changes. The genotype of the pathogen is another complicating factor of significant concern to forest pathologists. Differences among isolates of a pathogen in relative virulence on a host can be the major concern in studies of hypovirulence or of breeding for resistance. To predict the success of hypovirulence in establishing biological control of a forest pathogen, the investigator needs to identify the relative virulence among isolates over several seasons, and over several host genotypes. If the pathogen can infect more than one host species, then the relative virulence among isolates should be examined over different host species, and over several seasons. It is conventional practice in Agronomy to evaluate genotypes a minimum of three years in replicated field trials. In Agronomy, statistical ly significant genotype x environment interactions complicate field trials, frequently. Field trials examining hypovirulence are likely to have significant interactions among isolate genotypes and season (environment) and host genotypes (tree replications or species) and season.

The problem for forest pathologists becomes the statistical analysis of data collected from such field trials. The analysis becomes unusually complicated because of the great variability in disease severity or isolate virulence due to the strong genotype x environment interactions; isolate genotype x seasonal effects (environmental stresses) and host genotype (individual trees or different species) x seasonal effects. In fact, because forest pathologists often are working with a "weak" patho-

gen, analysis is further complicated. Interaction of seasonal effects with host genotype often has a more extreme influence on isolates of lower virulence because relative virulence can swing from avirulence in one season to high virulence in another. Such variation in statistical analysis causes significant nonadditivity in an ANOVA test when comparing isolates of low virulence that exhibit greater variability with isolates of high virulence that are more stable (consistently high in virulence). An example of such a comparison includes the study of hypovirulent and virulent isolates of *Cryphonectria parasitica*.

In an ANOVA test, the investigator seeks highly significant differences between treatments, generally. Significant differences permit mean separation tests that result in the ability to rank the treatments as differing in response. In a field trial, the ANOVA test will reveal whether the isolates are different in virulence, whether the hosts are different in susceptibility, and whether the seasons are different in stressing the hosts. But when the ANOVA test also reveals that statistically significant interactions occurred between isolate genotype x environment or host genotype x environment, it is not legitimate in mathematica to separate means or rank means. Thus, the experiment fails to answer the questions of the investigator. It is the usual case in forest pathology that field trials and even glasshouse trials have statistically significant interactions in the ANOVA test. This situation has hindered forest pathology for decades.

What is most needed for the study of the pathogen/host/environment interaction of forest pathology is a method of separating and ranking treatment means despite having statistically significant interactions in the ANOVA test. For example, we should be able to calculate the mean virulence of an isolate (an adjusted mean) averaged over more than one year, despite the interactions. Additionally, a measure of interactivity is needed for ranking hosts and pathogens in trials extending over years. For example, it would be worthwhile to know that two hosts may be similar in relative susceptibility, but one might radically change in susceptibility in response to stresses while the other may express a consistent level of susceptibility under the changed level of stress. Knowing the level of interactivity response of a host or isolate in each year is also an aide in identifying the season in which the stress occurred, or in identifying the nature of the stress. With such methods available, the mean separation test would answer the investigators questions on the treatment effects. Additionally, the interactivity component would supply a value in relation to the manner in which each treatment responses with environment.

Today, these objectives are obtainable with a relatively new method of statistical analysis, yet no forest pathologist has published an application of the method to our knowledge. The method uses ANOVA combined with principal component analysis (PCA). It is called the Additive Main Effects and Multiplicative Interaction Model (AMMI) (Gauch 1992). The result of employing the AMMI model includes the calculation of adjusted means that are more accurate than the observed means due to partitioning of the effect of interactivity and apportioning the interaction to specific means. In addition, mean separation and ranking tests can be performed in a mathematically legitimate way on the adjusted means.

Let us explain the use of the AMMI modeling with an example of a typical

forest pathogen, *Sphaeropsis sapinea* and a field inoculation trial on various *Pinus* species over two years. The objective of the trial is to compare the virulence of many isolates. The data are from an experiment of ours in which we sought to uncover hypovirulent strains.

Seasonal environmental factors, such as water stress (Blodgett et al. 1997a, b; Swart and Wingfield 1991) and pine spittlebug (Haddow and Newman 1942), have been documented as modifying disease development by *S. sapinea* on *Pinus* species. Therefore, most pathogenicity studies of *S. sapinea* on pine have been confounded by significant interactions between year and isolate (Swart and Wingfield 1991), between trial and isolate (Blodgett and Stanosz 1997), and between *Pinus* species and isolate (Blodgett and Stanosz 1997). The significant interactions have been common in field inoculation trials as well as in greenhouse trials under controlled climatic conditions (Blodgett and Stanosz 1997). These interactions demand closer study.

The relative virulence among 30 isolates was evaluated in field trials by inoculation of three species of *Pinus* over two years, year one (-yr1) and year two (-yr2): *Pinus sylvestris*-yr1, *P. sylvestris*-yr2, *P. nigra*-yr1, *P. nigra*-yr2 and *P. resinosa*-yr2. The experimental design for evaluating virulence on several *Pinus* species was a randomized complete block design with two factors and five replications (five trees of each *Pinus* species). The main plots consisted of the *Pinus* species-years and the subplots consisted of the fungal isolates being tested. The resulting data was analyzed statistically as a two-way factorial ANOVA in the normal fashion. The ANOVA additive model for the two-way factorial was the usual equation **[1]:** $\hat{U}_{isr} = \mu + \alpha_i + \beta_s + \theta_{is} + \varepsilon_{isr}$ where $\hat{U}_{isr}$ is the virulence (isolate by *Pinus* species-year by replication), μ is the overall mean, α_i is the fungus isolate deviation, β_s is the *Pinus* species-year deviation, θ_{is} is the interaction (non-additive residual), and ε_{isr} is the error term. The ANOVA table (additive model) of the randomized complete block design is presented below.

Table 1.

Source	Degrees of Freedom	Sums of Squares	Mean Squares	F Value	Probability
Total	749	16677.8			
Replication	4 (r-1)	18.2	4.5	0.6	
A. *Pinus* species-year	4 (a-1)	4814.2	1203.5	170.4	0.01
B. Isolates	29 (b-1)	4159.7	143.4	20.3	0.01
A x B Interaction	116 (a-1)(b-1)	3475.5	30.0	4.2	0.01
Error	596 (ab-1)(r-1)	4210.3	7.1		

The ANOVA reveals no significant differences among the replicate trees, which is good. *Pinus sylvestris*-yr1, *P. sylvestris*-yr2, *P. nigra*-yr1, *P. nigra*-yr2 and *P. resinosa*-yr2 differ highly significantly in their susceptibility to *S. sapinea* which is

expected. Isolates differed highly significantly in their relative virulence which is desired. Unfortunately, highly significant interactions occurred between isolate and *Pinus* species-years. These interactions prevent statistical separation and ranking of the means of relative virulence of isolates over the combined *Pinus* species or years. Therefore, we cannot make valid conclusions on the mean virulence of any isolate. Additionally, the interactions prevent separation of the means of the susceptibility of *Pinus* species over all isolates in a year, or over combined years. Therefore, we could not make valid conclusions on the mean susceptibility of any tree species. With this standard ANOVA, the considerable effort and expense of conducting the field trials was wasted and usually the result would have been that the data was discarded.

Fortunately, all the value of the field trials, and more, can be uncovered with our use of AMMI analysis to study (model) the effects on virulence of the interaction between isolate (genotype) and *Pinus* species-year (environment). AMMI is a significant statistical resource for understanding genotype x environment interactions (Gauch 1992). The pattern in the interactivity of the isolates is studied in detail by means of principal component analysis of the interaction sums of squares in AMMI analysis. The analysis provides a score (interactivity score) for quantifying the sensitivity of the response of an isolate or of a tree species to the year, in the host-pathogen-year interaction. AMMI analysis is useful in several unique ways. It helps to model interactions, to estimate virulence more accurately, and to permit better selections of hypovirulent isolates. The technique and its application are reviewed by Gauch (1992) and Gauch and Zobel (1996).

AMMI analysis combines ANOVA and principal component analysis (PCA) into a single analysis with both additive and multiplicative parameters. The additive part of the AMMI model uses ordinary ANOVA that leaves the non-additive residual, the interaction. The multiplicative part of the AMMI model uses PCA to decompose the interaction into PCA linear axes. Decomposing the interaction allows a major portion of the interaction to be accommodated in a calculation of a predicted response, for example, the predicted virulence of an isolate. AMMI analysis revealed that the first principal component (PCA 1) accounts for the predictable pattern in the interaction between isolate and *Pinus* species-year (genotype x environment) in our data set, whereas, PCA 2 or PCA 3 might be more important in other data sets.

The following statistical model adapted from Gauch and Zobel (1996) and Eisenberg et al. (1996) is used to obtain a new and more accurate estimate of the mean virulence of each isolate, and similarly a new mean susceptibility of each *Pinus* species of the combination *Pinus* species-year over all isolates. The equation **[2]** is $\hat{\mathbf{U}}_{is} = \mu + \alpha_i + \beta_s + \sum_n \lambda_n \gamma_{in} \delta_{sn}$ where $\hat{\mathbf{U}}_{is}$ is the predicted virulence of an isolate over *Pinus* species-year, μ is the overall mean, α_i is the fungus isolate effect, β_s is the *Pinus* species-year effect, λ_n is the singular value for principal component **n**, γ_i is the fungus isolate vector of scores, δ_s is the *Pinus* species-year vector of scores, and **n** = 1 for the axis of PCA 1. The AMMI ANOVA table (Additive and Multiplicative Model) of the randomized complete block design is presented below. The F test and Probability are not a component of AMMI ANOVA.

Table 2.

Source	Degrees of Freedom	Sums of Squares	Mean Squares
Total	749	16677.817	22.267
Treatments	149	12449.317	83.552
A. *Pinus* species-year	4	4814.153	1203.538
B. Isolates	29	4159.657	143.436
A X B interaction	116	3475.507	29.916
iPCA 1	32	1862.989	58.218
Residual	84	1612.518	19.197
Error	600	4228.500	7.048

The treatment mean square is found to contain 8.43% of the noise related to the experimental design. The AMMI analysis recovers the pattern in its AMMI1 model and relegates the noise to a discarded residual in order to increase the predictive accuracy and thus supply predicted means of virulence for each isolate. The relative virulence of each isolate is predicted on each *Pinus* species each year separately. The predicted virulence represents a calculation of the virulence of the isolate on the *Pinus* species after removing the predictable error due to the influence of the year's effect. Discarding a residual SS of 8.43% of the treatment sums of squares causes the AMMI adjusted (predicted) means to differ from the unadjusted means by a root mean square of 23.93% of the unweighted grand mean.

Results of an AMMI analysis are presented on a type of graph called a biplot, similar but not identical to graphs used in other PCA plots. On the biplot one axis is the adjusted mean virulence and the other axis is the sensitivity of response to the interactivity. The y-axis represents the distribution of interactivity scores of the isolates and *Pinus* species-year, the γ and the δ in equation 2. The virulence of isolates near the zero line (Fig. 1) is characterized as being very much more stable or consistent over *Pinus* species-years (i.e., less interactive) than those further away. One aspect of this type of graph that is not intuitively clear is the negative values on the interactivity axis (y-axis). The negative value does not have a biological meaning, only a mathematical meaning, therefore, negative values do not have less interactivity.

The biplot is informative because it shows both main (additive) and interaction (multiplicative) effects for both genotype and environment. Displacement along the abscissa indicates differences in the additive effects; displacement along the ordinate indicates differences in multiplicative effects. The estimate of the predicted virulence and interactivity of an isolate appears on the biplot (■); and also the susceptibility of a *Pinus* species in a year to the mean virulence of all the isolates combined appears on the biplot (▲). The AMMI calculations and the drawing of the biplots are carried out with the help of the MATMODEL program (Gauch 1993).

For each isolate (■) the x-axis shows a predicted virulence expressed over the combined five *Pinus* species-years. For example, the mean virulence for all the isolates of morphotype A, except A+147, is higher than the mean virulence of all of the

individual isolates of morphotype B, except B+466. The isolates of lowest mean virulence for each morphotype can be selected readily for further study of potential hypovirulence. This selection will be more accurate in prediction of an isolate's virulence over the different *Pinus* species and over different seasons than would selection based on inoculation trials in glasshouses under controlled climatic conditions. Thus, AMMI analysis provides for a major improvement in the quality of research.

Additionally, AMMI analysis has supplied a standard error of the means (SEM) among isolate means. The SEM is 0.53. This value can now be used legitimately for mean separation tests. Our trial and effort are saved! We can now make statistically sound conclusions on which isolate is significantly different in mean virulence than another. The predicted virulence of an isolate expressed over three *Pinus* species and two years (combined as five *Pinus* species-years) are in Table 1 where they are ranked by Duncan's multiple range test, P = 0.05, Least significant difference (LSD) value of 1.475, standard error 0.53.

For the *Pinus* species-year (▲) the x-axis shows a measure of the susceptibility of a *Pinus* species to the mean virulence of all the isolates combined. For example, the mean virulence of all the isolates combined is greatest for *P. sylvestris* in the first year and in the second year, therefore that species is the most susceptible. Conversely, *P. nigra* in the second year was the least susceptible while *P. nigra* and *P. resinosa* were equal in relative susceptibility in the first year. AMMI analysis has also provided valuable information on the sensitivity of the different *Pinus* species differs in response to changes in seasonal effects. For example, *P. nigra* exhibits a relative stability in its interactivity with different isolates and year. *Pinus sylvestris* is highly interactive and will react vigorously with different isolates depending on the year (Fig. 1).

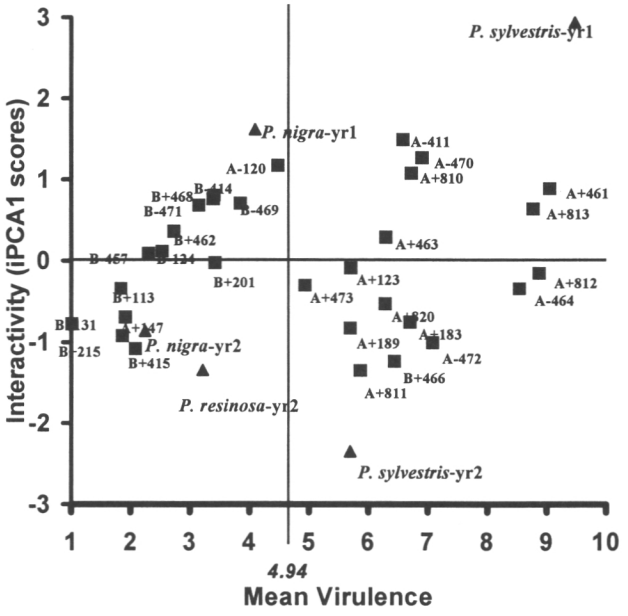


Figure 1.

Additionally, AMMI analysis has supplied a SEM among means of the *Pinus* species-years. The SEM is 0.22. This value can now be used legitimately for mean separation tests. We can now make statistically sound conclusions on which *Pinus* species-year is significantly different than another in mean susceptibility to the mean virulence of all the isolates combined. The *Pinus* species-years can be ranked in the following order: *P. sylvestris*-yr1, 9.48 (A); *P. sylvestris*-yr2, 5.70 (B); *P. nigra*-yr1, 4.09 (C); *P. resinosa*-yr2, 3.21 (D); and *P. nigra*-yr2, 2.25 (E). The letters in brackets following the means are the DMRT rankings at P = 0.05, LSD value of 0.602, standard error 0.22.

In conclusion, in our pathogenicity tests on three species of *Pinus* highly significant statistical interactions occurred between isolate virulence, *Pinus* species, and year. Pine species-year had a profound impact on virulence. The pattern in the interactions was revealed by PCA of the interaction SS of the ANOVA. The interactivity analysis was used to apportion interaction to specific isolates to improve the accuracy of the estimates of virulence. Estimates of the relative virulence of isolates were predicted over five different *Pinus* species-years. Isolates were ranked in virulence and interactivity using the AMMI model. This model permitted mean separation tests of the relative virulence among isolates over the combined *Pinus* species-years. The virulence and interactivity of isolates was summarized in a biplot graph of adjusted means to facilitate selection of isolates that had potential for hypovirulence.

The AMMI method of using PCA within an ANOVA test is a new and revealing mathematics for the study of the interaction of pathogenic fungi with their host plants in changing annual environments. AMMI analysis permits the study of the natural system with *S. sapinea,* rather than moving the host-pathogen system out of the natural environment to reduce the interactions. AMMI analysis provides an interactivity score for quantifying the sensitivity of the response of an isolate or a tree species to the year, in the host-pathogen-year interaction. AMMI analysis also allows for the statistical separation and ranking of means of relative virulence of each isolate over the combined *Pinus* species-years, as well as, the separation of means of the susceptibility of a *Pinus* species to the mean virulence of all the isolates combined.

General experience with agricultural data has found that predicted yield estimates from an AMMI model are as accurate as treatment means based on two to four times as many replications (Gauch and Zobel 1996). Routinely, AMMI models are more accurate in predicting yield than are the unadjusted data from yield trials because of genotype X environment interaction (Gauch and Zobel 1996). AMMI analysis and the resulting biplot is most interesting when the interactivity can be associated with a causal factor such as drought or insect infestation. We hope that this research report will encourage further use of AMMI analysis in pathological studies.

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The mechanism of tracheid cavitation in trees infected with wilt diseases

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Abstract

In trees infected with pine wilt disease, extensive embolism occurs in tracheids. The enlargement of a cavitated and dysfunctional xylem induces a water deficit and mortality in trees. This phenomenon was observed in a wilt disease in oak trees caused by *Raffaelea* sp. To confirm an assumption that this wilt mechanism is fundamentally similar in several wilt diseases, inoculation experiments were conducted on larch and spruce trees. In *Larix kaempferi* trees inoculated with *Ceratocystis lalicicola*, acoustic emission from the trunks increased before the initiation of apex wilting. This indicates that abnormal dehydration from tracheids was occurring in the infected trees. In the trees that rapidly developed symptoms, xylem dysfunction had progressed widely. The leaves of *Picea jezoensis* were partially discolored about one month after inoculation with *C. polonica*. The fungal hyphae were elongating radially through the ray tissue. Before the symptoms were visible, areas that were cavitated and dysfunctional appeared in the xylem. When the outer annual rings became dysfunctional, the sap ascent decreased significantly, and the leaves began to discolor. The dysfunctional areas were much wider than the areas plugged with resin. The fungal activity promoted secondary metabolism in ray parenchyma cells and is responsible for the cavitation. A similar mechanism that promotes embolism and inhibits the refilling of water in pine wilt also occurs in the wilt diseases of larch and spruce trees.

Keywords: Pine wilt, embolism, sap ascent, *Raffaelea, Picea, Larix, Ceratocystis*

Introduction

Pine wilt is caused by the nematode *Bursaphelenchus xylophilus* and is one of the most serious tree diseases in Japan. From the end of 1980s, many oak forests on Honshu Island have been damaged by *Raffaelea* sp. *Ceratocystis* spp. are killing spruce and larch trees in northern Japan. All the pathogens of these diseases are vectored by beetles. The leaves of the trees become discolored about three weeks after inoculation with the pine wood nematode (Kuroda et al. 1988). Usually, healthy trees do not wilt very easily, even when the supply of water is stopped for a certain period. Why these pathogens can kill trees so effectively is a question that remains to be answered. Furthermore, the cause of the swift death of the apical and cambial cells of a huge tree does not yet have a theoretical explanation.

In healthy trees, xylem sap ascends spirally in pine trunks. In trees infected with pine wilt, dehydrated areas emerge in xylem prior to leaf discoloration (Kuroda et al. 1988). Such dysfunctional areas enlarge, and the tree wilts within one or two months. It is clear that some kind of a system rapidly excludes the xylem sap from the tracheids (Kuroda 1989, 1991). In the case of oak trees infected with *Raffaelea* sp., the discoloration of sapwood and blockage of water are well underway before the appearance of other symptoms of wilt in the summer months (Kuroda 2001, Kuroda and Yamada 1996, Takahata and Ikeda 2001).

The mechanism of the symptom development of wilt described here (Fig. 1) is based on research into the pine wilt and oak mortality caused by *Raffaelea* sp. When microorganisms attack a tree, a protection strategy is set in motion. A synthesis of secondary metabolites starts in the tree tissue. With this reaction, cavitation or discoloration occurs in the xylem. Tracheids and vessels become dysfunctional and are filled with air. Unfortunately, the secondary metabolites are ineffective in their efforts to protect the tree against the pine wood nematode and *Raffaelea* sp. As the pathogen is widely distributed, protection occurs in many places in a tree, and, therefore, xylem dysfunction is widely spread. The sap ascent is extensively reduced, and the tree dies because of a water deficit. I considered that these findings might be applicable to other wilt diseases. To check this possibility, wilt processes in larch and spruce trees were monitored after inoculation with the pathogens *Ceratocystis lalicicola* and *C. polonica*, respectively. Investigations were made with techniques involving Acoustic Emission (AE) to detect embolism and cavitation in tree trunks (Kuroda 1995, Kuroda and Kuroda 2000), a dye injected into tree trunks to trace the course of sap ascent, and anatomical analyses to check cytological reactions of host cells and hyphal distribution.

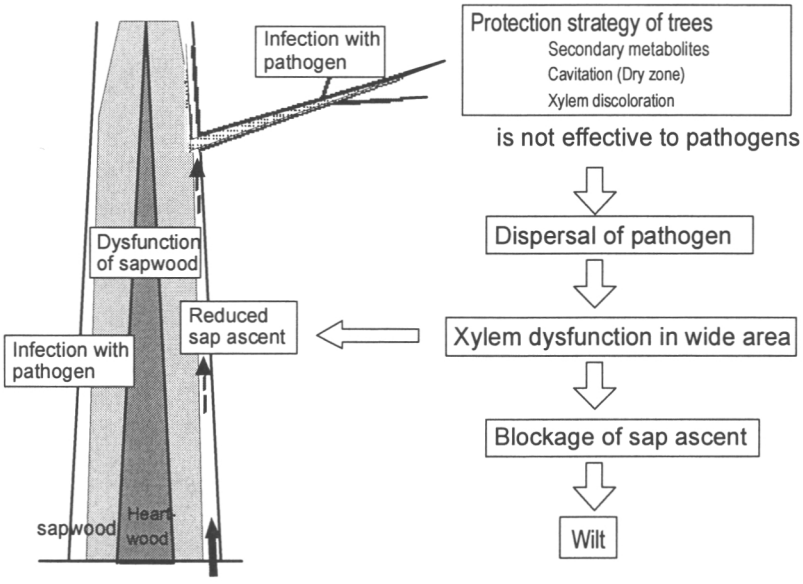


Figure 1. Schematic illustration of wilting mechanisms hypothesized for the wilting disease of pine and oak trees.

Materials and methods

Japanese larch

On July 21, 1998, the pathogen of wilt disease *Ceratocystis lalicicola* Redfern et Minter* was inoculated on the lower trunks of seven-year-old *Larix kaempferi* Sarg. trees. The features of the inoculated and control trees are cited in Table 1. At the start of the experiment, AE transducers (140kHz) were attached to the lower trunks of the inoculated and control trees. This technique is useful for monitoring the embolism in living trees without cutting (Kuroda and Kuroda 2000). In the experiments on pine wilt, the extremely high rate of AEs was confirmed and reflected abnormal dehydration and dysfunction of the tracheids (Kuroda 1995). AE events were monitored until the harvest. The trees were checked for symptoms every week until the end of November.

Yezo spruce

On July 21, 1999, the pathogen *Ceratocystis polonica* (Siem.) C. Moreau** was inoculated into the trunks of seven-year-old *Picea jezoensis* (Sieb. et Zucc.) Carr. trees. Inoculated trees were harvested at one-week intervals. Table 2 shows the harvest dates and symptom development. AE transducers were attached onto the lower trunks prior to the inoculation. To visualize the water conduction, a dye solution was injected at the base of the trunks (1% aqueous acid-fuchsin) at harvest (Kuroda et al. 1988). The main stem was then dissected and processed for an anatomical analysis. Microscopic observations were made to check the reaction of the tree tissues to the fungal activities.

* Pathogen: Isolated by T. Yamaguchi from the blue-stained xylem of a *L. kaempferi* in the experimental forest at the Hokkaido Research Center, Forestry and Forest Products Research Institute.
** Pathogen: YCC- 115. Isolated by Y. Yamaoka from the gallery wall of *P. jezoensis* in the Tokyo Univesity Forest in Hokkaido.

Results and discussion

Wilt of *Japanese larch*

Two weeks after infection, the trees' apices began to droop (Trees No. 4 and 5 in Table 1, Fig. 2). Leaf yellowing and defoliation followed at three weeks after inoculation. These are characteristic symptoms of the disease. Figure 3 shows the frequencies of AEs detected around the time that the apices began to droop, which was the initial symptom. The typical pattern of AEs in healthy trees is indicated by the control data. In healthy trees, the AE pattern increases rapidly when the transpiration rates are high as a result of normal cavitation in the tracheids. There was no AE event at night. In inoculated trees, the frequency of AE was normal until August 24, and then it increased from August 25 just before the apices began to droop.

Table 1. Symptoms in 7-year-old *Larix kaempferi* trees inoculated with *Ceratocystis lalicicola.*

Date	Week	Tree No						
		No.1	No.4	No.5	No.7	No.9	No.10	No.6
Jul-14	0	Inoculated						Control
Jul-21	1	H	H	H	H	H	H	H
Jul-27	2	H	D&F	D	D	D	D	H
Aug-01	3	D	Y	Y	Y	Y&F	Y	H
Aug-18	5	F	F	F	M	M	F	H
Nov-27	4 m	R	M	M	M	M	R	H

H: No symptom, D: Apex drooping, Y: Yellowing, F: Defoliation, M: Mortality, R: Recovery

Table 2. Symptoms on 7-year-old *Picea jezoensis* trees inoculated with *Ceratocystis polonica.*

Date	Week	Tree No								
		No.2	No.9	No.8	No.1	No.5	No.3	No.4	No.10	No.6
Jul-21	0	Inoculated							Controls	
Jul-28	1	H	H	H	H	H	H	H	H	H
Aug-04	2	-	H	H	H	H	H	H	-	H
Aug-11	3		-	H	D	H	D	D		H
Aug-18	4			-	-	D	D	D		H
Sep-01	6					-	D	D		H
Sep-15	8						ED	D		H
May-02	(Next year)						M	R		H

H: No symptom, D: Discoloration, ED: Extensive discoloration, M: Mortality (part),
R: Recovery, -: Felling

In pine wilt disease, it has been confirmed that cavitation occurs extensively in periods of high AE rates. The cavitated tracheids were not refilled with water. The increasing pattern of AE frequencies in larch trees resembles pine wilt, although the rates were lower. Therefore, the abnormal embolism and cavitation were assumed to have occurred in the periods of high AE rates in the trunks of infected larch trees. The shoots drooped because of a lack of water caused by the extensive embolism in the trunks.

Wilt of *Yezo spruce*

The shoots of the spruce trees did not droop. Instead, their needles became discolored as an initial symptom (Fig. 4) about three weeks after the inoculation. In spruce trees, AE events did not increase as drastically, probably because the cavitation pro-

gressed gradually. As a result of dye absorption from the trunk bases, functioning tracheids were stained red. In the inoculated trees, white parts without staining were wider than the controls even before symptom development. Tracheids in those areas were filled with air and had become dysfunctional. Blockage of sap ascent was extensive by the start of leaf discoloration (Fig. 5A). This severe dysfunction caused water shortage and induced leaf discoloration.

Ray tissue in sapwood consists of living cells. Before symptom development, hyphae distributed through ray tissues, some resin canals, and a few tracheids. Vertical distribution was very narrow and limited. Radial elongation was deep and reached the depth of several annual rings. Ray parenchyma cells invaded by hyphae were necrotic. Ray parenchyma cells produced secondary metabolites as a reaction before



Figure 2. Drooping symptom observed on *Larix kaempferi* inoculated with *Ceratocystis lalicicola.*

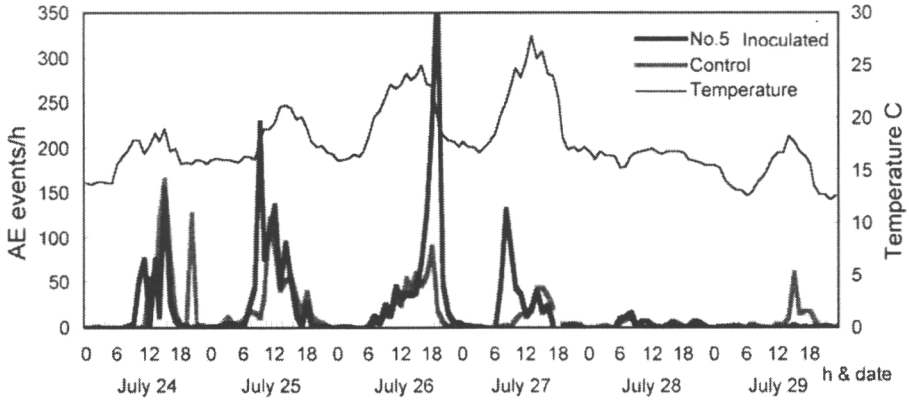


Figure 3. High-frequency AEs detected from a trunk of *L. kaempferi* on July 26 and 27.



Figure 4. Leaf discoloration observed on *Picea jezoensis* inoculated with *Ceratocystis polonica.*

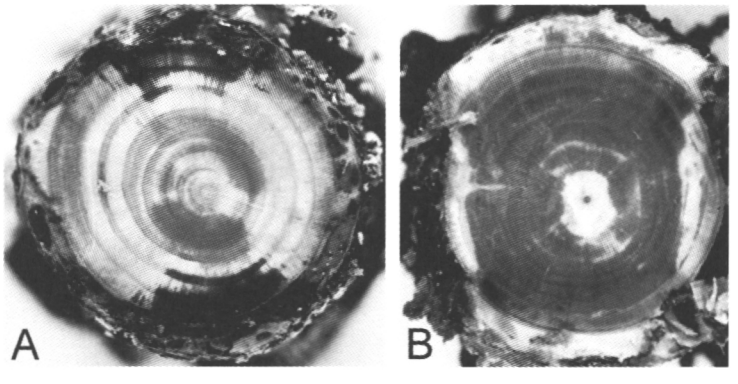


Figure 5. Blockage of xylem sap ascent in *P. jezoensis* indicated by dye injection at the start of leaf discoloration (A) and normal water conduction in the control tree (B).

necrosis, and the cell contents looked yellow. Cambial necrosis was just restricted to inoculated sites and was not serious. All these indicators showed that this fungus is not effective to kill cambium and that the cambial lesion in narrow area does not cause wilt. On the other hand, dysfunction and desiccation occurred in the xylem rapidly and widely. The activity of the fungus was effective to stop water conduction. It was significant that the dysfunctional area was much wider than the area of resin occlusion. Resin occlusion is not an essential factor for dysfunction, although it may also be effective to block sap ascent.

Conclusion

Distribution of two *Ceratocystis* species in larch and spruce trees was both slower and narrower than in the case of pine wood nematode; however, these fungi were effective to stop sap ascent. Complete stop of sap ascent is due to extensive cavitation (or embolism) of water conduits that cannot refill with water. This is the cause of wilt in these two wilting diseases. *Ceratocystis* spp. promoted secondary metabolism in parenchyma cells. As assumed in the case of pine wilt, substances with low surface tension and a hydrophobic character may promote embolism and inhibit refilling with water in the wilting diseases of larch and spruce caused by *Ceratocystis* spp.

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Impact of some crown diseases in Trentino woods

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Abstract

The Forest Trees Damages Monitoring (FTDM) has been carrying out in the Trentino woods since 1990, in collaboration with the Forest Services of the Autonomous Province of Trento, as a tool to support the naturalistic management of forests. In this monitoring program, data were obtained on several crown pathogens. Among them, few seem effective to produce economic damage as like as *Lachnellula willkommii*. Snow fungi proved to be dangerous only in some well defined topographic situation. In the case of *Lirula nervisequia* and some rust fungi, environmental conditions of some sites proved optimal for attacks but no negative effects were observed on the evolution of infected woods. Others pathogens as *Sirococcus strobilinus*, *Lirula macrospora* and *Apiognomonia errabunda* can be occasionally harmful to individual trees, however they are not able to produce sensible damages to the forest. Occasionally outbreaks of crown parasites were reported (attacks by *Mycosphaerella laricina* and *Meria laricis* were recorded on larch and *Rhizosphaera kalkhoffii* on *Picea abies*). The damage were strictly related to particular site conditions and were delimited in space and time. More effective, and related to climatic conditions, appeared the impact of *Sphaeropsis sapinea* and *Cenangium ferruginosum* on *Pinus nigra* artificial stands. The possibility to use these fungi as bio-indicators of host-stress conditions or of wrong forest management choices is discussed.

Introduction

Trentino is one of the most forested provinces of Italy having 345.000 hectares of woodland which cover more than half of the Trento's Province land surface. This mountain area is located in the Northern-east part of the Alps and it is characterised by woodland environment extremely varied because of different climatic conditions, rock formations and soil features. All the alpine tree species, with a clear predominance of Norway spruce (*Picea abies*), are present together with a minor but interesting presence of some Mediterranean ones. Total wood mass in the high forests is estimated around $50x10^6$ m^3 and species that determine this biomass are *P. abies* (62%), *Larix decidua* (16%), *Abies alba* (12%), *Pinus sylvestris* and *Pinus nigra* (6%), *Fagus sylvatica* (3%) *Pinus cembra* (1%). Woods have a strong economical importance not only for their primary production but also because they are the main landscape features, playing a fundamental role in this touristic area.

Since the 50's, silviculture in Trentino aims to enhanced the naturalistic woodland management. The goal is to conserve or restore the natural characteristics of the

woodland which are specific to each environment. The naturalistic management points to maintain a continuos woodland cover and to obtain everywhere it is possible a natural regeneration. The management is pursued by means of the detailed "Woodland Management Plans" which are drawn up for every woodland public property and generally revised every ten years. On this occasion, a census is made of the trees, their development in time is evaluated and decisions are made on appropriate treatments. In this contest the presence of parasites and pests can play a fundamental role influencing or modifying the evolution of wood and nullifying the choices of the forest manager. Knowing and understanding the pathogens role is the target of the Forest Trees Damages Monitoring (FTDM) which has been carrying out since 1990 by the Forest Research Unit of IASMA in collaboration with the Forest Services of the Autonomous Province of Trento (Ambrosi and Salvadori 1998).

Material and methods

Forest Trees Damages Monitoring (FTDM)

Method is based on five distinct phases: observations in the forest, report of the problems, diagnosis, transmission and elaboration of data.

The Forest Service personnel who are specially trained to make the job carry out observations in the forest within a well-organised network (10 forest districts with 45 stations). The ratio of the forest area and personnel is 2350 ha per individual.

The personnel note down observations on five different forms during pre-determined periods of the year. Each of them helps in grouping the various pathologies and other types of damage that manifest during a known period of the year. The form contains a series of information regarding topography of the affected area (forest plan compartment, exposition, altitude, etc.), type of damage and intensity (number of trees affected, defoliation, total affected growing stock, etc.).

When the forest damages are not recognised in field, the Forest Unit of the Institute is informed, field survey and sample examination performed and diagnosis defined. All the diagnosis are completed with indications on management treatment to check the problem and are referred to the Woodland Management Plan compartments.

Data is maintained in a database, related to a Geographic Information System (GIS) based on the woodland management plans compartments; elaboration and conclusions are made available to the organisations in the territory, forest districts and plots that are involved in the study.

For some diseases, specific and more detailed forms can be prepared by the Forest Unit and performed by the Forest Service personnel, previously advised and instructed.

A yearly report compiled by the Forest Research Unit of the ISMAA contains all the causes of damage, pests, pathogens and relationship with weather conditions.

Other data

The values of climatic parameters are collected in several meteorological stations, placed in Trentino Province.

Results and discussion

In more than ten years of continuos monitoring, several parasites were observed and described in the Trentino woods. The list of the observed pest and pathogens in 2000 is reported in Table 1.

Some parasitic fungi may cause economic damage and have a direct impact

Table 1. List of the observed pests and pathogens in Trentino woods during the year 2000. Pathogens were listed following the host species.

DAMAGE AGENTS	HOST TREE
PESTS	
Ips typographus	*Picea excelsa*
Tomicus minor e *piniperda*	*Pinus nigra* e *sylvestris*
Ips acuminatus	*Pinus sylvestris*
Coroebus florentinus	*Quercus* spp.
Rhynchaenus fagi	*Fagus sylvatica*
Thaumetopoea pityocampa	*Pinus nigra* e *sylvestris*
Zeiraphera griseana	*Larix decidua*
Coleophora laricella	*Larix decidua*
Caoptilia syringella	*Fraxinus ornus*
Yponomeuta evonymella	*Prunus padus*
Haematoloma dorsatum	*Pinus nigra*
Heterarthrus cuneifrons	*Acer pseudoplatanus*
PATHOGENS	
Chrysomyxa rhododendri and *C. abietis*	*Picea excelsa*
Heterobasidion annosum	*Picea excelsa, Larix decidua*
Rhizosphaera kalkhoffii	*Picea excelsa*
Sphaeropsis sapinea	*Pinus nigra* e *sylvestris*
Cenangium ferruginosum	*Pinus nigra* e *sylvestris*
Lachnellula willkomii	*Larix decidua*
Meria laricis	*Larix decidua*
Mycosphaerella laricina	*Larix decidua*
Lirula nervisequia	*Abies alba*
Herpotrichia parasitica	*Abies alba*
Cryphonectria parasitica	*Castanea sativa*
Phytophthora cambivora	*Castanea sativa*
Ophiostoma novo-ulmi	*Ulmus* spp.
OTHER DAMAGE	
Defoliation	*Ostrya carpinifolia*
Wilting	*Alnus viridis*

on forest ecosystem evolution. Among them *Heterobasidion annosum, Ophiostoma novo-ulmi* and *Lachnellula willkommii.* The last one is able to produce damage on young regeneration of *Larix* in some areas. This parasite, killing *Larix* young trees, allows the persistence and the predominance of Norway spruce and stops the evolution of forests to mixed woods. Chestnut blight is also present in Trentino's chestnut stands but the disease impact is reduced by the clear prevalence of healing cankers due to hypovirulent isolates. On the contrary Dutch elm disease caused the disappearance of *Ulmus glabra* and induced foresters to define a sanitation program in order to save the few surviving Elm trees.

Other fungi are present in forest on small scale areas. Their impact appear effective although geographically localised. Among them *Phacidium infestans* Karsten and *Herpotrichia juniperi* (Duby) Petrak caused some damage on natural regeneration of *P. cembra* and *P. abies* particularly at high altitude where snow pockets can stand. *Herpotrichia parasitica* Rostrup and *Lirula nervisequia* (DC.) Darker are common on *A. alba* trees but they produce evident symptoms only in few sites: their effectiveness in damaging the growth of infected trees or in influencing the establishment of *A. alba* regeneration is not yet confirmed.

Chrysomyxa rhododendri (DC.) de Bary is reported at timberline in several valleys but the infected surface varied considerably in the different years, probably in relation to the weather conditions during the infection period at the beginning of the growing season. Only recently survey started to estimate the rust influence on the spruce regeneration at the timberline.

Several foliar and needles pathogens and rusts are regularly observed in woods but resulted harmful generally only on single trees and on limited parts of the crowns. Their presence is often unnoticed. Among these parasites: *Sirococcus strobilinus* Preuss is present on cones and shoots and *Lirula macrospora* Darker on needles of *P. abies, Gremmeniella abietina* (Lagerb.) Morelet may colonise shoots and needles on *P. cembra* and *P. sylvestris* and *G. laricina* (Ettlinger) Schlapfer-Bernhard is occasionally found on shoots and needles of larch. Both the fungi were not associated to evident damage till now.

In the last years sudden and widespread outbreaks of some foliar disease were reported in all the Trento Province. During the summer 1999, in many larch woods severe foliar defoliation by *Mycosphaerella laricina* (Hartig) Neg were observed. The pathogen is common in central Europe but scarcely considered or unnoticed until now in Italy probably for the contemporary presence of the crown insects *Coleophora laricella* and or *Adalges laricis*. Damage was noticed, mainly on mixed woods, at around 1000 m a.s.l. of elevation on the southern slopes of valleys. Fungus caused needle necrosis and early defoliation starting from the beginning of September. The climatic conditions of summer 1999, characterised by relatively high temperatures and high humidity during the conidial dispersal period (August) was the main factor involved in the epidemics (Maresi et al. 1999).

At the end of winter 1999-2000, in several valleys it was observed an anomalous reddening of *Picea abies* related to the presence of *Rhizosphaera kalkhoffii* Bubàk on necrotic needles. Trees showed complete necrosis of 1-3 years old needles. Damages were concentrated on the bottom of small valleys, generally above

1200 m. a.s.l., on the exposed part of the crown and on trees placed at the margin of forests. Symptoms were related to climatic stress conditions during winter, enhanced by the longer vegetative season in 1999, characterised by rain and temperatures higher than usual (Maresi et al. 2001).

During July 2000, in some mixed larch forests, plants showed a complete crown yellowing, showing tips of needles first brown then whitened. On infected foliage it was detected *Meria laricis* Vuill. The fungus was not described before in Trentino although was probably undetected. Its pathogenic role appears limited. After the sudden appearance the disease stopped and no more effective damages were observed. Lower temperatures and heavy precipitation during July may be the predisposing factors for the attacks (Maresi et al. 2001).

Climatic condition during the last few years and particularly several spring-summer drought periods induced spreading of *Sphaeropsis sapinea* in *Pinus nigra* stands which in the past were planted in poor soil areas of the Province. The attacks of the parasite were sometimes associated with *Cenangium ferruginosum.* Starting from 1995 damage were so heavy (more than 11000 cubic meters were felled during 1998) that the survival of some stands resulted very difficult (Maresi et al. 1999). Both the fungi can be considered as bio-indicator of decline of "pioneer" pine stands which are at the end of their life cycle (Ambrosi et al. 2000). Silvicultural management is needed to reduce impact of damage and to improve natural evolution of stands towards more resilient broadleaf woods.

Conclusion

Forest Tree Damages Monitoring proved to be an useful tool both to collect data on pathogens and to support the managing choices. From the recorded data it is possible to combine information on the presence of pathogens, site conditions and transfer them on maps to use in the Woodland Management Plans. The improvement of a GIS dedicated to forest health problems is one of the future goals.

In this contests knowledge on the role of several "minor" parasites in forest ecosystem resilience must be enhanced both by a better mapping of their presence and spread and working on their relationship with the eco-physiological status of trees. Most of the recent outbreaks, including those by minor or unknown pathogens, appear strictly related to weather conditions particularly favourable to the micro-organisms or to climatic factors that induced stress on hosts trees as in the case of *S. sapinea* attacks or as supposed for the observed wilting of *Alnus viridis* (Maresi and Ambrosi 1999).

In this contest fungal pathogens may be considered as bio–indicators both of wrong past silvicultural choices and-or climatic changes that can modify forest stability and evolution. A better knowledge on these topics is desirable.

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Taxonomy of the genus *Gremmeniella*, causal agent of scleroderris canker

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Abstract

The conifer disease known as Scleroderris canker is caused by ascomycetes of the genus *Gremmeniella*. The taxonomy of these pathogens has undergone many changes from the rank of order down to the rank of species, since the first description of the teleomorph in Sweden under the name *Crumenula abietina* Lagerberg, in 1913. A second species found in Switzerland on larch, *C. laricina* Ettlinger, was described in 1945. In 1975, three serovars or races were recognized for the species *G. abietina*. In 1989, a reassessment of the genus was made based on results of morphological, cultural and biochemical studies. Thus, in addition to the var. *abietina*, representing the three serovars, the var. *balsamea* was proposed for the pathogen infecting spruce and balsam fir in Canada. New information on the epidemiology of these fungi infecting different hosts, in combination with results of sequencing the isolates representative of the host range of the disease, provides a better understanding of this disease on its respective hosts. A review of the genus *Gremmeniella* is presented, including a proposal for several new species.

Keywords: Scleroderris canker, *Gremmeniella*, taxonomy

Introduction

Pathogen fungi belonging to the genus *Gremmeniella* cause serious diseases of conifers in Asia, Europe and North America (Donaubauer 1972, Karlman et al. 1994, Laflamme and Lachance 1987, Ohman 1966, Setliff et al. 1975, Yokota 1984). In the genus *Gremmeniella*, morphological characteristics are not very helpful for microscopic identification and overlaps are common between measurements of specimens from different hosts (Petrini et al. 1989). The diagnostic of the disease on pine was limited to the identification at the genus level which was believed for quite a long time to be monospecific with *G. abietina*. This is why the pathogen identification at the species level was not considered to be a problem even if fungal taxonomy is a key element in the understanding of a pathosystem. For example damage caused by the so called European race, which can kill large trees, is very different from that caused by all other *Gremmeniella* types.

Symptoms of the disease are quite well known, starting in early spring with brown coloration at the base of the previous year's needles, followed by a shoot

blight in the summer (Laflamme 1991). Cankers can be produced a few years later on twigs and on the main stem. The English name of the disease comes from the fungus'scientific denomination *Scleroderris lagerbergii*, used in the late 1960s when the disease became an epidemic in western Europe, in eastern North America and in Japan. This disease has been found on several conifer species of the northern hemisphere but the most severe damage has been recorded on pine.

Classification from the order to the genus

These *Ascomycetes* were classified in the order *Helotiales* and in the family *Helotiaceae* by Dennis (1968); these fungi are now ranked in the order and family *Leotiales* and *Leotiaceae* by Hawksworth et al. (1995). *Gremmeniella*, the genus denomination, is now widely accepted, but this was not always the case; the denomination went through several changes over the last century. When discovered in 1913 by Lagerberg in Sweden, this pathogen was first included in the genus *Crumenula.* Forty years later, Gremmen decided to integrate this fungus into the genus *Scleroderris.* Several fungal taxonomists did not agree with this last change and from 1969 to 1971 three different genera were proposed by three different authors. Schläpfer Bernhard (1969) proposed integrating this fungus into the genus *Ascocalyx*. At the same time, Morelet (1969) and Reid (in Dennis 1971) created two different new genera, respectively *Gremmeniella* and *Lagerbergia. Gremmeniella* was the valid name because it was published first. But it took some time before everyone agreed on that name because *Ascocalyx* was proposed again (Müller and Dorworth 1983). Based on a study using chemical, biochemical, cultural and morphological information on these fungi, Petrini et al. (1989) supported the name *Gremmeniella.*

Table 1. List of genera used for the teleomorph denomination during the last century for the causal fungi of Scleroderris canker.

Crumenula abietina	1913
Scleroderris	1953
Ascocalyx	1969
Gremmeniella	1969
(*Lagerbergia*)	1971
Ascocalyx	1983
Gremmeniella	1989

Species in the genus *Gremmeniella*

Morelet (1969) created the new genus *Gremmeniella* with *G. abietina* as the type species and the only species of the genus. *Crumenula laricina* was then moved to the genus *Encoeliopsis*. A few years later, Dorworth and Krywienczyk (1975) recognized three pathological races based on serology: the North American race, the Eu-

ropean race and the Asian race. These races or serovars do not have any taxonomic value.

Petrini et al. (1989) studied isolates and their corresponding herbarium specimens of *Gremmeniella* spp. from pine, spruce and balsam fir as well as specimens of *Ascocalyx laricina* from larch and of *Ascocalyx abietis* from balsam fir. For that study, morphological, cultural and chemical characteristics were used. All the specimens from larch were very distinct from the specimens of all other hosts but had the characteristics of the genus *Gremmeniella*. Therefore, a new combination was created, *G. laricina* (Ettlinger) Petrini et al. The species *Ascocalyx abietis* Naumov, a saprophyte on fir, was retained as it is distinct from *G. abietina*. All the specimens on the other hosts had overlaps in their morphological measurements and the authors could not create new species. Then two varieties were recognized: the variety *abietina* for the type species including the three serovars, and the variety *balsamea* for the specimens from spruce and balsam fir from Canada.

New information on the disease and on the fungi give a better understanding of the genus *Gremmeniella.* It was believed that the European race was more virulent than the other two races on all pine species, but that was not true. In fact *P. banksiana* and *P. contorta* are strongly resistant to the European race (Laflamme and Blais 2000, Laflamme et al. 2000, Simard et al. 2000) and probably other pine species are too. The isolates from pine, balsam fir and spruce in North America show host specificity reactions (Laflamme et al. 1996). Phylogenies based on sequencing show that the three races of the type species are not closely related (Hamelin and Rail 1997). The Asian race is closely related to the variety *balsamea*. The divergence between *G. abietina* and *G. laricina* is as great as between the varieties *balsamea* and *abietina*. Finally, in the variety *balsamea*, it is possible to differentiate isolates from fir and spruce by their colour in culture on PDA. In Europe, the so-called European race of *G. abietina* has been divided into three entities. The Fennoscandian amplitype (Hamelin et al. 1996) is the equivalent of the small tree type in Sweden (Hellgren 1995) or type B in Finland (Uotila 1983). The second one, the Alpine amplitype, is restricted to the Alps and damage caused by the pathogens is similar to the previous one and to the North American race in North America. The third one, the European amplitype, is the equivalent of the large tree type in Sweden (Hellgren 1995), type A in Finland (Uotila 1983) and the European race in North America. The species *G. juniperina* L. Holm & K. Holm has not been studied.

Discussion

From this review, it is becoming more and more evident that the genus *Gremmeniella* is a complex of several species. When Morelet (1980) studied the type species *Crumenula abietina,* he did not have access to the type specimen collected by Lagerberg on *Picea abies*. As the type specimen was lost, he had to propose a neotype, which was another specimen that Lagerberg collected on *Pinus sylvestris*. But the first specimen on spruce could have been the European amplitype and the one on Scots pine could have been the Fennoscandian amplitype. If the first Lagerberg specimen could be found, further observations could clarify this point.

For the past several years, we have studied *Gremmeniella* on fir and spruce in Canada (Laflamme 1988a, 1988b). We are now confident that we are dealing with two different species and a paper is in preparation to describe them.

We did not have enough material from Japan on *Abies sachalinensis* but it seems possible that the Asian race could prove to be a new species after further studies. Finally, more observations should be done on isolates from *G. juniperus*.

Conclusion

In short, the complex of suggested species is as follows:

1. *G. abietina* (or a new species?) found mainly on *Pinus* spp.
   = NA race
   = small tree type
   = type B
   = Finoscandian amplitype
   = Alpine amplitype, which is very similar to the Fennoscandian one
2. *G. abietina* (or a new species?) found mainly on *Pinus* spp.
   = European race
   = large tree type
   = type A
   = European amplitype
3. *G.* n.sp. 1 on *Abies balsamea* in Canada
   = *G. abietina* var. *balsamea*
4. *G.* n.sp. 2 on *Picea* spp. in Canada
   was included in *G. abietina* var. *balsamea*
5. *G.* (new species?) on *Abies sachalinensis* in Japan
   = Asian race
6. *G. laricina* on *Larix* spp.
   Is the species in Europe different from the one in North America?
7. *G. juniperina* on *Juniperus*: to be further studied.

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Size of ascospores in the A and B types of *Gremmeniella abietina*

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Abstract

To find morphological differences, ascospores of A- and B-types of *Gremmeniella abietina* were measured and compared. A significant difference between A- and B-types was found in the length of ascospores. Width and roundness of the spores did not differ or varied irregularly between samples and both types. However, the frequency distributions in the length measurements were overlapping too much to be useful as a taxonomic key. If still used, to get reliable data at least one hundred spores have to be measured.

Introduction

The fungus *Gremmeniella abietina*, which causes scleroderris canker of pines and some other conifers, is known to be a complex of several geographical strains or races. Dorworth and Krywienczyk (1975) suggested to divide the fungus into North American, European, and Asian races, based on physiological and immunological characteristics. By comparing morphology and electrophoretic patterns, Petrini et al. (1989), segregated *G. abietina* into two varietis, *G. abietina* var. *abietina* (including European, North American, and Asian races) and *G. abietina* var. *balsamea* which occurs on *Picea* and *Abies* in Quebec. Later Petrini et al. (1990) found also some evidence for the existence of one more variety occurring on *Pinus cembra* at high altitudes in the Alps and on *P. sylvestris* in the boreal forests of northern Europe. DNA-tests conducted in Sweden by Hellgren and Högberg (1995) revealed that *G. abietina* had two ecologically different strains (LTT and STT) which seemed to have reflection with A and B types of the fungus described by Uotila (1983, 1992) in Finland. Finally, this ecotypic variation was found to correlate in Europe with three distinct DNA amplification profiles (Hamelin et al. 1996), from which two, northern European and Alpine types were adapted to long-lasting snow and the third type was spread throughout Europe. In further studies Hamelin and Rail (1997) showed that two varieties, *G. abietina* var. *abietina* and *G. abietina* var. *balsamea* are divergent at species level. Results from hybridization experiments allowed Uotila et al. (2000) to conclude that A and B biotypes are genetically different populations and should be described as separate species as soon as sufficient information is available.

The aim of this study was to find out possible morphological differences in the size of ascospores between A and B biotypes of *G. abietina*.

Materials and methods

A-type samples were from southern Finland and the B-types were from both southern and northern Finland (Table 1). The type of each sample was tested with DNA markers (Hantula and Müller 1997). Spores from mature apothecia were allowed to drop to microscopic objective glasses on which they were mounted. Only specimens with mature apothecia were chosen for the study. We concluded that apothecia were mature, if high number of spores were released shortly (in few hours) after moistening. Ascospore images were obtained in microscope with a digital camera using 10x40 magnification. Image analyser (Leica Qwin) was used to measure the dimensions of ascospores. In this system one pixel was equivalent to 0.21 μm. The software measured automatically the area, length, width, perimeter, and roundness of the spores. Roundness was determined with the following equation:

$$\text{Roundness} = \frac{\text{Perimeter}^2}{4 \times \pi \times \text{Area} \times 1.064}$$

ANOVA was used for statistical treatment of the data.

Results

Ascospores representing B-type were longer and slightly wider than those of A-type (Fig. 1, Table 2). The differences in both dimensions were statistically significant ($p<0.001$). In the average length of spores the difference was 3.7 μm. That caused significant difference also in the area and roundness. The width differed only very slightly, 0.2 μm. The spores were the longest in Solo B, which differed significantly also from other B type specimens ($p<0.05$). The appearance of spores was also variable. In some specimens the ascospores had rounded ends while in some the ends were much more acute. However this difference was not possible to reveal by roundness measurements. This character differed between some specimens but appeared to vary quite much in both types.

Table 1. The specimens of *Gremmeniella abietina* used in the study and the number of spores measured.

Specimen	Type	Location	Coordinates	Number
Solo	B	Inari, Solojärvi	N68°49', E26°47'	104
Arctic circle	B	Rovaniemi, Arctic circle	N66°33', E25°38'	84
Siika	B	Ruovesi, Siikakangas	N61°52', E24°10'	120
Huikko	A	Juupajoki, Huikko	N61°51', E24°19'	77
Valkea	A	Ruovesi, Valkeajärvi	N61°52', E24°16'	117

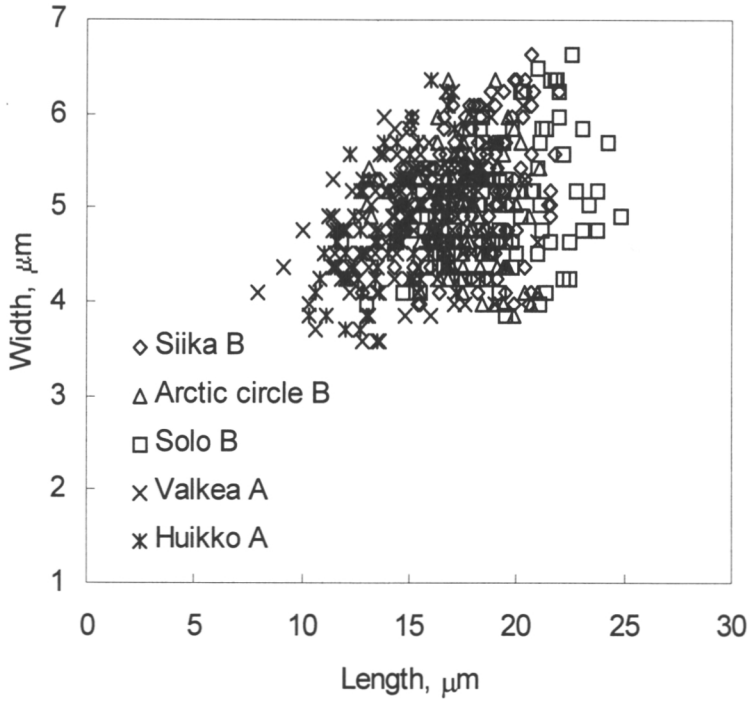


Figure 1. The ascospore length and width in A and B type specimens.

Table 2. Mean values of the characteristics of ascospores.

Strain	Area, µm^2	Length, µm	Width, µm	Roundness
A	50.2	14.6	4.9	1.9
B	61.4	18.3	5.1	2.3

Discussion

We found spore size difference between A and B types of *G. abietina*, which could be a useful clue for describing these two types as distinct taxa. However, the difference could hardly be used as an identification key, since the frequency distributions are too much overlapping on the scale. The maturity of apothecia could also be a limiting factor when one is using spore size as distinguishing character. We do not know when the spores in an ascus reach their maximum size. Is it when they are ready to be released, or is it long before that phase. Usually spores are measured from smashed apothecia on microscopic glasses, in which case they could be released by an external force prior to maturity. The number of spores measured is also important, if we try to distinguish these two types using spore sizes. It seems that at least 100 spores have to be measured for obtaining reliable data, instead of 5–10 that is common practice in identification (Hawksworth 1974).

If we like to describe A and B types as different species, we will meet another difficulty. We have to separate the new proposed species also from other related

strains, such as North American strain and alpine strain in Central Europe, both occurring on pines, and other ones occurring on firs in North America and Japan. For describing a new species in this complex, more reliable data have to be obtained. It has been shown genetically (Hamelin et al. 1996, Hamelin and Rail 1997, Uotila et al. 2000) that *G. abietina* should be divided to several distinct species. However, to establish new species in this complex is very difficult until the evidence obtained with tests using DNA-markers is not accepted at the same level as morphological characteristics. Therefore, we still need morphological evaluations of all strains of *G. abietina*. In the present study, we found hints that some morphological differences could be detected between these strains.

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Factors affecting the risk of *Gremmeniella abietina* infection in Scots pine stands

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Abstract

Field inventory data from the temporary plots of the 8th National Forest Inventory (NFI) in southern Finland, digital elevation models, modelled air pollution deposition and models for monthly temperature and rain were used in this study. *Gremmeniella abietina* was the most frequently identified cause of damage in Scots pine stands in southern Finland. The disease was clearly spatially clustered.

On mineral soils, disease frequencies were highest on paludified plots or on the most fertile plots. On peatland plots, the disease was most common on drained, transformed peatland plots. Naturally regenerated stands were affected more than artificially regenerated stands. The proportion of diseased plots increased with an increase in stand density and in absolute elevation in mineral soils. The relative altitude of the plot had a stronger influence.

The risk of *G.a.* infestation was modelled with various techniques. CART (classification and regression trees) analysis was used for data exploration. Already at this stage, it was possible to recognize groups of very high or very low risk. 77.5% of the diseased stands were correctly classified. Stand density, drainage stage, relative elevation, regeneration method, division between mineral soil/peatland plots and weather factors were the most important classifying factors. A preliminary risk model with multi-level logistic regression gave support to the importance of transitional stages after drainage on peatland, rich site type on mineral soil and, most of all, stand density.

Keywords: *Gremmeniella abietina, Pinus sylvestris,* Finland, national forest inventory, stand density, peatlands, topography, climate, risk modelling

Introduction

The ascomycete fungus *Gremmeniella abietina* (Lagerb.) Morelet is the causal agent of a canker and die-back disease of *Pinus* and many other coniferous genera. The disease has been known in Europe for more than a century. In southern parts of Finland severe outbreaks have been reported in the mid-1970's, the early 1980's and in 1988 (Aalto-Kallonen and Kurkela 1985, Nevalainen and Yli-Kojola 1990). In southern Finland, it is the most common fungal disease in forests dominated by Scots pine *(Pinus sylvestris* L.). According to the 8th National Forest Inventory (NFI), disease symptoms were recorded in 10.5% of Scots pine stands, on more than 690 000 hectares during 1986-1992.

Several environmental factors may affect the risk of forest stands to infection by *G. abietina*. Planting of poorly adapted provenances is one of the most important reasons for severe epidemics of Scots pine in Europe, although physiogenic diseases are also important in this contex (Dietrichson, 1968, Uotila 1985, Roll-Hansen 1972).

When pines are of suitable origin, epidemics can be favoured by meteorological factors and site conditions. Epidemics usually begin after cold and rainy growing seasons (Aalto-Kallonen and Kurkela 1985, Uotila 1988); and on the other hand, warm growing seasons can slow down the spread of the disease (Karlman et al. 1994). Frost damage is an important reason for the differences in susceptibility to *G. abietina* (Dietrichson 1968). Steep valleys, north-facing slopes and kettle holes are among the most vulnerable sites (Dorworth 1974, Uotila 1988, Sairanen 1990). Susceptibility of pines is increased in shaded or dense stands (Read 1968, Niemelä et al. 1992). Some epidemics have occurred on areas with considerable pollution (Bragg and Manion 1984), but in Scandinavia the effects of air pollution have not been confirmed with experiments or field observations (Kaitera et al. 1995, Vuorinen and Uotila 1997).

Most of the information about the factors that can affect the risk of infection come from experiments or small scaled field studies. Experiments are normally aimed at testing one specific hypothesis at a time. Local field studies, on the other hand, have most often been conducted in known diseased centres. Thus, they may overemphasize some predisposing factors. Only large-scaled, statistically sound samples make a simultaneous examination of the several predisposing variables and their relative importance conceivable. The Finnish National Forest Inventory (NFI) has produced information on forest resources over large areas of the country for more than 70 years. The Eighth NFI was the first to include detailed information on health of forests, including diseases and pests. The NFI dataset allows for the simultaneous comparison of several environmental and silvicultural factors affecting the occurrence of diseases and pests. It also provides raw data for risk modelling. Furthermore, it is possible to combine other spatial data and/or models with the NFI field measurements.

The purpose of this study is to describe the occurrence *Gremmeniella abietina* in relation to several environmental, site and silvicultural factors, based on a large representative field sample, and to develop models for estimating the susceptibility of Scots pine stands.

Material and methods

The 8th National Forest Inventory (NFI) was a systematic field sampling of the forest resources covering the entire area of Finland. Field plots were arranged in detached 21-plot clusters, called tracts, the plots forming a half-square. In southern Finland the distance between the tracts was 8 km x 7 km (south-north x west-east directions) and the distance between the plots was 200 metres. Altogether 18999 Scots pine dominated stands (without recent cuttings) on forest land, from the years 1986-1992, in southern Finland were included in this study. Each "stand" was actually the forest compartment in which the centre point of the plot was situated.

Forest damage was assessed in every plot situated on forest land. By definition, on forest land, the mean annual increment of wood should be at least 1 m^3/ha. Forest damage was assessed using three different codes: the symptom, the cause and the apparent severity of the damage (damage degree). Only stand level damage assessments were used in this study. Only one, the primary damage, was recorded for each stand. Description of the variables and codes used for assessing forest damage in this study are presented in Table 1. In the analysis, the disease degrees were recorded into three classes: 0=no Gremmeniella infection 1=slight infection 2=at least moderate infection (disease degree more than 1). About one hundred variables describing the stand and site conditions were recorded in the NFI. A more detailed description of the inventory system and of the most important variables assessed in the field in the NFI can be found in Nevalainen (1999).

In addition, some external data was merged to the NFI dataset. A nationwide elevation data set, provided by the National Land Survey of Finland, was used to study the effects of elevation. The digital elevation model (DEM) was calculated from the contour lines of the basic map by triangulation network interpolation onto a grid model. The original grid cell size was 25 m x 25 m, but in this study the DEM was mean filtered to a 1 km x 1 km grid. This grid was used to compute the elevation of the 1 km x 1 km cells in the tract area and the relative elevation of the plot, i.e. the absolute elevation minus tract area elevation.

The average sulphate and total nitrogen deposition on the sample plots was based on a model applied at the Finnish Meteorological Institute. The long-range deposition in the model calculations was obtained from the EMEP model (Tuovinen

Table 1. Description of the variables and codes used for assessing forest damage in this study.

Description	Codes
Visual symptom of injury	0) no damage 1) dead standing tree 2) fallen tree or standing stem broken below the crown 3) decay 4) stem or root damage within 1 m from the stem 5) broken or dry top (in the upper half of the crown) 6) other crown malformations 7) defoliation 8) discoloration of the needles or leaves Letters A-F are used for symptoms 1-6, if the injury is older than five years
Primary cause of injury	0) unknown 1) wind 2) snow 3) other meteorological or soil factors 4) competition between plants 5) harvesting scars 6) other human activity 7) voles 8) moose 9) *Tomicus* sp. 10) Other insects 11) *Cronartium* sp. 12) *Gremmeniella abietina* 13) Other fungi
Importance (degree) of the damage	0) slight damage, symptoms observed, but the damage does not reuce the silvicultural quality of the stand 1) moderate, the stand quality is reduced by one class 2) severe, the stand quality is reduced by more than one class 3) complete, artificial regeneration is required

et al. 1994), and the domestic deposition was based on the HAKOMA (Finnish integrated acidification) model (Johansson et al. 1990).

The chemical composition of till at depths of 0.5-2 m was obtained from the data of the Geochemical Atlas of Finland (Koljonen et al. 1992). In this study, concentrations of calcium, magnesium and potassium were computed for each field plot of the NFI by the Geological Survey of Finland.

Climatic data were generated using the models of Ojansuu and Henttonen (1983). The monthly mean temperature, effective temperature sum and precipitation sums for each plot were computed for the period of ten years prior to the inventory year, and as a grand mean for the 30-year normal period (1960-1990).

Classification and regression trees (CART) analysis (Breiman et al. 1984, Steinberg and Colla 1997) was used for data exploration, e.g. for selecting the variables to be included in logistic regression analysis, and for determining the cut-points of continuous variables.

A preliminary risk model was built using the MlwiN software (Goldstein et al. 1998). It can be assumed that the binary responses of the stands (occurrence of damage) are correlated within for example different spatial levels, year level, measurement team level etc. In modelling the probability of damage, ignoring these correlations leads to underestimated standard errors of estimated regression coefficients. The problem can be mitigated by the introduction of random variables to the model, estimating the residual variances at different hierarchical levels of the data (Goldstein 1995). Therefore, a multilevel logit-model was used in the model construction. The model can be expressed as

$$\text{logit}(\pi_{ijk}) = \ln(\pi_{ijk} / (1-\pi_{ijk})) = \beta_{0ijk} + \beta_{1ijk} x_{1ijk} + \ldots + \beta_{nijk} x_{nijk} + u_{jk} + v_k$$

$$y_{ijk} \sim \text{Binomial}(n_{ijk}, \pi_{ijk})$$

where y_{ijk} is the observed response of stand i in cluster j measured by team k, π_{ijk} is corresponding response probability, x_{ijk} are explanatory variables of the model, β_{ijk} are the coefficients for the explanatory variables and u_{jk} and v_k are normally distributed errors at cluster and measurement team levels, respectively.

The predictors for the fixed parts of the probability models were selected stepwise based on the Wald test (Hosmer and Lemeshow 1989), in addition to the CART procedure mentioned earlier.

Results

Relationships between the disease and some environmental and silvicultural factors

Stand density, here expressed as basal area (m^2/ha) was most clearly correlated with the disease degree (0-2), and the relation was almost linear up to 28 m^2/ha (Table 2, Fig. 1a). The dummy variable "artificial regeneration" correlated negatively with the disease degree, indicating more disease in naturally regenerated stands (Table 2).

The disease was more common on peatland than on mineral soil plots. Drained peatlands were most liable to Gremmeniella infection, but the difference between undrained (natural state) peatland and mineral soil plots was not significant. The more the original peatland site type had changed after drainage, the more common was the disease. The correlations between the two drainage stages that had changed the original type the most and the disease degree were very significant, although mineral soils and peatlands were treated together. The fertility of the site correlated positively with the disease degree. Paludification (in mineral soil plots) as well as the variable indicating the need for drainage showed a positive correlation with the disease degree (Table 2).

Damage due to *Gremmeniella abietina* increased slightly with an increase in absolute elevation (Fig. 1b). However, the relative altitude of the plot compared to the elevation of the tract area was most strongly correlated with the disease. The disease was most common (and severe) in the plots situated lower than the mean elevation of the tract area (Fig. 1c, Table 2).

The modelled total S- and N-deposition were not correlated with the disease. The modelled total concentration of the base cations K, Mg and Ca in the till were slightly positively correlated.

Of the monthly weather factors 10 years prior to infection, only rain sum during the summer months (June-August) correlated significantly with the disease degree. On the other hand, almost all the variables depicting the difference between 30 years normal period and the period 10 years prior to field survey, were significant. Of these, the difference in rain during spring quarter (March-May) and during the most probable infective period (May-June), were the most significant (Table 2).

Classification of the disease stands with CART was not very successful. 77.5% of the diseased stands were correctly classified as diseased with CART. The corresponding number in the 'healthy' plots group was 66.0%, which gave the overall accuracy of 67.3%. Despite this fact, the CART-analysis was able to separate some groups with a very high or a very low disease frequency out of the data. For example, the disease frequency was as high as 25.0% within the 776 plots which shared the definitions "stand density >12.5 m^2/ha and drainage stage something else than undrained mineral soil". On the other hand, the disease frequency was only 3.8 % within the 299 plots sharing the definitions "stand density <12.5 m^2/ha and drainage stage undrained mineral soil, drained mineral soil or recently drained peatland".

The most important variables (based on the relative importance of variables as primary splitters) varied slightly in different CART runs. In general, the most important were stand density (expressed as stand basal area), stand age, peatland (0/1), the regeneration method, drainage stages, the need for drainage, difference in winter temperature, autumn temperature, winter temperature and the relative elevation of the plot.

Table 2. Non-parametric correlations (Spearman's rho) between the disease degree (0-2) and some silvicultural and environmental variables. Significant correlations in each variable group are printed in bold.

Variable	Coefficient	Significange	Number of plots
Proportion of pine	-.026	.000	18999
Stand age	.177	.000	18999
Density (basal area)	**.217**	**.000**	**18998**
Artificial regeneration (0/1)	-.112	.000	18999
Mean elevation in tract area (1 x 1 km pixels)	.055	.000	18956
Elevation of the plot	.022	.003	18999
Relative elevation of the plot	**-.097**	**.000**	**18956**
Total S-deposition	-.004	.573	18999
Total N-deposition	-.004	.593	18999
Aluminium in till	.016	.034	16950
Base cations (K, Ca, Mg) in till	.019	.015	16950
Peatland plot (0/1)	**.117**	**.000**	**18999**
Site needs drainage (0/1)	.103	.000	18359
Palufied mineral soil (0/1)	.045	.000	13682
Transition stage after drainage (peatlands)	.073	.000	5317
Drained mineral soil (0/1)	-.001	.915	18999
Recently drained peatland (0/1)	.006	.385	18999
Transforming drained peatland (0/1)	.092	.000	18999
Transformed drained peatland (0/1)	.073	.000	18999
Undrained peatland plot (0/1)	.003	.699	18999
Site type (1=richest)	-.053	.000	18999
Grove or grove-like site type (0/1)	.029	.000	18999
Fresh site type (0/1)	.026	.000	18999
Dryish site type (0/1)	-.006	.397	18999
Dry or poorer site type (0/1)	-.047	.000	18999
Effective temperature sum	-.016	.028	18379
Spring rain (March-May) 10 yrs prior survey	.008	.278	18999
Spring temperature (March-May)	-.010	.185	18999
Infective period rain (May-June)	-.006	.381	18999
Summer rain (June-Aug)	**.037**	**.000**	**18999**
Summer temperature (June-Aug)	-.015	.037	18999
Autumn rain (Sept-Nov)	.009	.218	18999
Autumn temperature (Sept-Nov)	-.009	.204	18999
Winter rain (Jan-Feb)	.020	.006	18999
Winter temperature (Jan-Feb)	-.002	.799	18999
Difference in spring rain (30 yrs-10 yrs)	**-.040**	**.000**	**18999**
Difference in spring temperature	-.030	.000	18999
Difference in infective period rain	-.039	.000	18999
Difference in summer rain	.009	.230	18999
Difference in summer temperature	-.016	.031	18999
Difference in autumn rain	.030	.000	18999
Difference in autumn temperature	-.027	.000	18999
Difference in winter rain	-.022	.002	18999
Difference in winter temperature	-.033	.000	18999

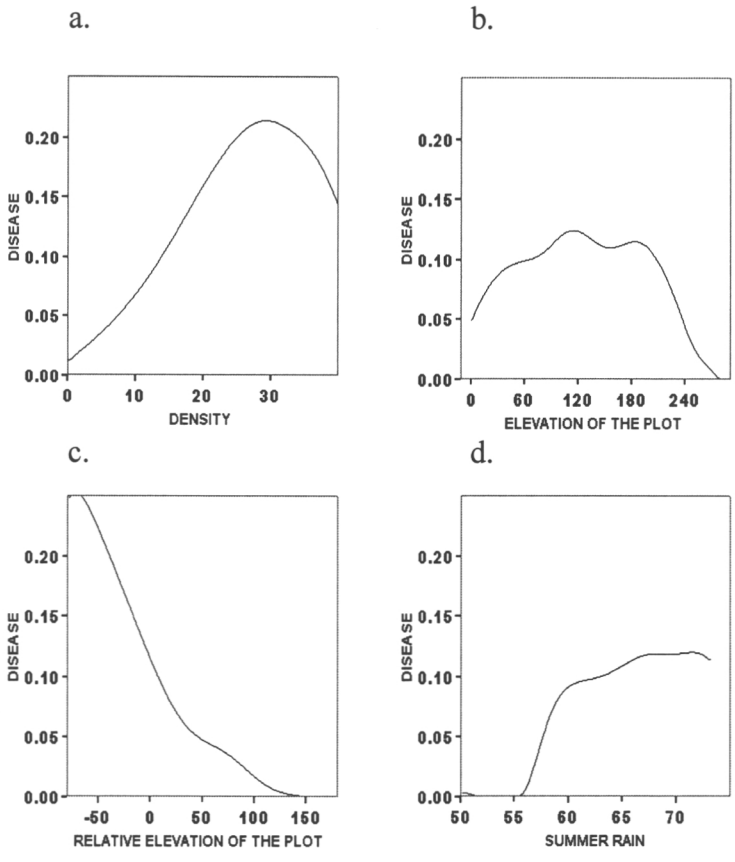


Figure 1. Schematic presentation of the relationships between the disease and some explanatory variables. The curves are smoothed in SPSS-interactive graphics using local linear regression, normal kernel and the bandwidth multiplier=1
a) stand density (expressed as stand basal area m^2/ha)
b) the elevation of the plot (m)
c) relative elevation of the plot (plot elevation – elevation of the tract area, dm)
d) precipitation sum during June-August for 10 years before field survey (mm).

A preliminary risk model for the susceptibility of Scots pine stands

The hierachial multilevel logit-model gave results that were quite similar than the ones reported in the earlier paragraph. However, several of the explanatory variables used earlier (Table 2) were dropped out of the model. For instance, the absolute elevation of plot was not included in the model. According to our preliminary model, the probability of damage was higher
-In dense stands (density expressed as stand basal area here)
-In stands situated lower than the mean altitude in the tract area (7 x 8 km)
-In areas, where it has rained much during May-June 10 years before the field survey (compared to a long-time average)
-In drained peatlands (compared to undrained peatlands and mineral soils)
-In grove-like, fresh, and dryish stands (compared to poorer site types) (Table 3).

On the other hand, the probability of damage was lower on artificially regenerated stands on fresh forest sites (compared to artificially regenerated dryish and poorer sites and to naturally regenerated fresh sites (Table 3). Statistically significant residual variation was found on cluster level (σ^2 =1.114, s.e. 0.077) and on measurement team level (variance σ^2 =0.328, s.e. 0.127).

The effects of fixed variables are shown in terms of population averaged odds ratios, which are calculated by taking the exponent of the estimated parameter value of the fixed part of the model (odds ratio = exp(b)). The population averaged odds ratio approximates, for example, how much the ratio of the frequency of damaged stands to undamaged stands changes when the fixed variable increases by one unit. For example, ratio of the frequency of damaged stands to undamaged stands is 1.07 times higher when the basal area of the stand increases by one m^2/ha. Accordingly, ratio of the frequency of damaged stands to undamaged stands is exp(10*0.068) = 1.97 times higher when the basal area of the stand increases by ten m^2/ha (Table 3).

Table 3. Estimated parameter coefficients and odds ratios for the preliminary risk model.

Variable	Estimated coefficient (β)	s.e.	Odds ratio expβ	
Relative elevation	-0.014	0.002	0.98	(1 m)
Difference in infective period rain	-0.020	0.006	0.98	(1mm)
Stand density (basal area)	0.068	0.004	1.07	(1 m2/ha)
Recently drained peatland	0.813	0.174	2.25	(0/1)
Transforming drained peatland	0.927	0.070	2.53	(0/1)
Transformed drained peatland	0.834	0.099	2.30	(0/1)
Grove & grovelike site	0.393	0.131	1.48	(0/1)
Fresh site type	0.431	0.103	1.54	(0/1)
Dryish site type	0.301	0.091	1.35	(0/1)
Stand age	0.006	0.001	1.01	(1 year)
Artificial regeneration	-0.240	0.117	0.78	(0/1)
Intercept	-5.068	0.299		

Discussion

In terms of sampling errors, the National Forest Inventory produces accurate results. However, this data set has many disadvantages regarding monitoring of forest diseases. The most important of these is that the field work covered two or three administrative areas (forestry board districts) per year, and thus, when epidemic diseases are concerned, the assessments from different years are not directly comparable. Further, only one injury per stand (the economically most important) was recorded, which leads to an underestimation of the overall occurrence of a disease. The authors think, however, that the most important stand damage (in an economic sense) was recorded reliably in the routine NFI inventory.

77% of the residual variation in the preliminary logit model occurred on sampling cluster (tract) level. This could be a result of the cluster-wise systematic sampling. In our opinion in merely indicates that the disease itself is clustered (Nevalainen 1999). Residual variation on measurement team level includes partly variation on larger spatial level than cluster and variation on year level. It, however, also suggests that in practice, the teams have not assessed the stands as damaged or undamaged with similar criteria.

As to *Gremmeniella abietina*, the field inventory cannot have any data the occurrence of various races of the fungus. Although the age structure of affected stands and the disease symptoms refer to type A (or LTT), in the present study the presence of the B-type (STT) (Uotila 1983, Petäistö et al. 1996) cannot be completely ruled out.

Gremmeniella abietina occurred more frequently in naturally regenerated stands than in artificially regenerated stands, in contrast with the earlier findinds of e.g. Kallio et al. (1985). This is due to the higher proportions of slight damage in naturally regenerated stands. On the poorest forest types on mineral soils, the proportion of the disease has been higher in artificially regenerated stands than on naturally regenerated stands (Nevalainen 1999).

The effect of the estimated climatic factors area partly questionable, due to the effect of elevation, which was included in their computation. The difference between the grand mean of 30 years and 10 years should remove artifacts like these. Then, however, almost all the weather variable differences became significant. These factors should be studied more closely in the future.

The effect of topography was not studied in detail in this paper. However, the effect of e.g. aspect of the slope and microtopography around the plot were not as important as the relative elevation of the plot (as compared to the mean elevation in the tract area) (Nevalainen 2001).

In our vision, disease risk models will be incorporated into forestry planning systems, although at the moment it is still unclear, how this should be done. Maybe they should form a part of "natural process models" (birth, growth and death models).

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Formation and growth of stem cankers caused by *Gremmeniella abietina* on young *Pinus contorta*

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Abstract

From 1990-1995, the formation and growth of stem cankers caused by *Gremmeniella abietina* (Lagerb.) Morelet on *Pinus contorta* Dougl. ex Loud. var. *latifolia* Engelm. was studied in three stands in northern Sweden. The stands were planted in 1976-1980. The total number of cankers on 756 trees that were individually followed increased from 233 to 477 during the five-year period. With 42.0% of the cankers, the pathogen entered through or from the base of diseased branches, and 33.6% through visually undamaged bark. Most of the cankers were within 100 cm of the ground. In one of the three areas, the cankers were evenly distributed within 180 cm of the ground. The frequency of cankers facing north exceeded those facing south. The average vertical length of cankers had increased, 55.6% of cankers had increased their percentual coverage of the stem girth; 13.8% had fully girdled the stem.

Introduction

In a large-scale study of damage to commercial plantings of *P. contorta* in northern Sweden 1987-1991, we found that the frequency of stem cankers caused by *G. abietina* increased during the seasons 1989-1991 (Karlman et al. 1994). North of latitude 64°N, 20% of the trees had cankers, of which 7% were severe (Karlman et al. 1992).

The symptoms of *G. abietina* on *P. contorta* in northern Sweden are similar to those of the North American race of the fungus, with canker formation and production of apothecia on trees 1-3 m high (Laflamme 1991). Investigations of genetic variation in *G. abietina* in northern Sweden recorded, however, only the Northern amplitype, identical to type B (Uotila 1983) or small tree type (Hellgren and Högberg 1995), of the European race in *P. contorta* (Hamelin et al. 1996, Hansson et al. 1996).

The aim of this study was to describe the formation and growth of cankers caused by *G. abietina* on *P. contorta* in northern Sweden, focusing on canker development on younger trees, 10-15 years old.

Materials and methods

From 1990-1995, the formation and growth of stem cankers caused by *G. abietina* was studied on *P. contorta* in the following localities of northern Sweden. In total,

756 trees were included in the study.

1. Grannäs (lat. 64°56'N, 510 m. a.s.l.). The area is located on an east-facing slope originally regenerated by *P. abies*. The stand forms a provenance test containing the most northern provenances available of *P. contorta*, the majority originating north of lat. 62°N in the Yukon Territory, and thus treated as a homogenous material. It was planted with *P. contorta* in 1976 after patch scarification. A total of 226 trees were included in the study.

2. Allejaur (lat. 65°54'N, 500 m. a.s.l.). The area is located on a slope with a slight east exposure that was originally regenerated by *P. abies*. It was planted with *P. contorta*, provenance Rusty creek (63°30'N) in 1980. A total of 385 trees were studied.

3. Akkajärvi (lat. 66°55'N, 425 m. a.s.l.). The area is located on a north exposed slope originally regenerated by *P. abies*. It was planted with *P. contorta*, provenance Rusty creek (63°30'N) in 1980. One-hundred forty-five trees were examined.

Tree height was measured and the trees were examined each year for shoot blight and cankers caused by *G. abietina*. Every year, each canker was measured for vertical length and horizontal width. For each canker, the pathogen's entrance to the stem was noted. The aspect of each canker in terms of compass direction (N-E, E-S, S-W and W-N) was recorded. The occurrence of apothecia of *G. abietina* were also recorded.

Results and discussion

The frequency of trees with shoot blight caused by *G. abietina* increased in Allejaur and Akkajärvi from 1990 to 1995. However, both sites showed a slight decrease in the frequency of damaged trees during 1992 due to trees assigned as moderately (<50% crown damaged) or lightly damaged (a single branch damaged) in 1991 and assigned as recovered or lightly damaged in 1992 (Fig. 1). In Grannäs, the number of damaged trees was constant during the period. The frequency of severely damaged trees and trees killed by *G. abietina* increased at all three sites during the period (Fig. 1).

The total number of cankers increased from 233 to 477 during the five-year period. The number of cankered stems increased in Allejaur and Akkajärvi, but was constant in Grannäs. However, the number of multi-cankered stems increased at all three sites during the period (Fig. 2).

In Allejaur and Akkajärvi, 18% of the stems investigated had cankers in 1990 (Fig. 2). This was in accordance with our earlier investigations in northern Sweden, where the *P. contorta* plantations severely damaged by *G. abietina* in 1988 showed a high frequency of stem cankers in 1989 (Karlman 1989, Karlman et al. 1994). However, in Grannäs 36% of the investigated trees had cankers in 1990 (Fig. 2). This was probably a result of the somewhat older and higher stand in Grannäs compared to Allejaur and Akkajärvi.

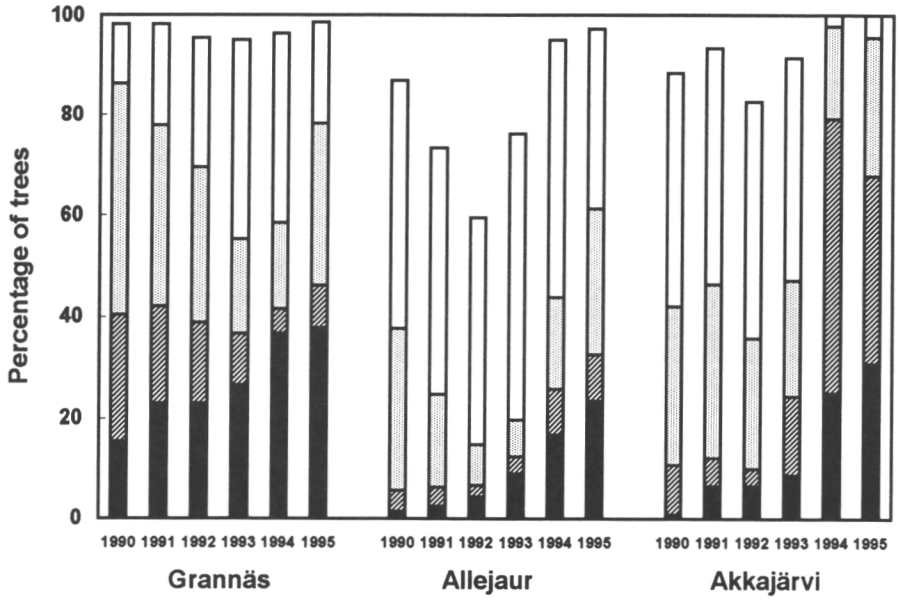


Figure 1. The percentage of trees with shoot blight caused by *Gremmeniella abietina* during 1990-1994. Killed or dying trees are shown by black, severely damaged trees (>50% of crown damaged) by stripes, moderately damaged trees (25-50% crown damaged) by dots and lightly damaged trees (a single branch damaged) by white.

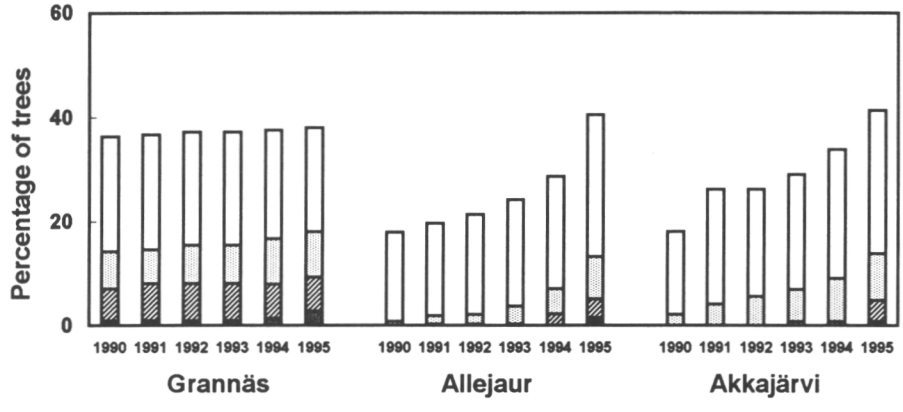


Figure 2. Percentage of trees with stem cankers caused by *Gremmeniella abietina* during 1990-1994. Trees with one canker are shown in white, those with two cankers by sparse dots, those with three cankers by stripes, those with four cankers by dense dots, and trees with five cankers are shown in black.

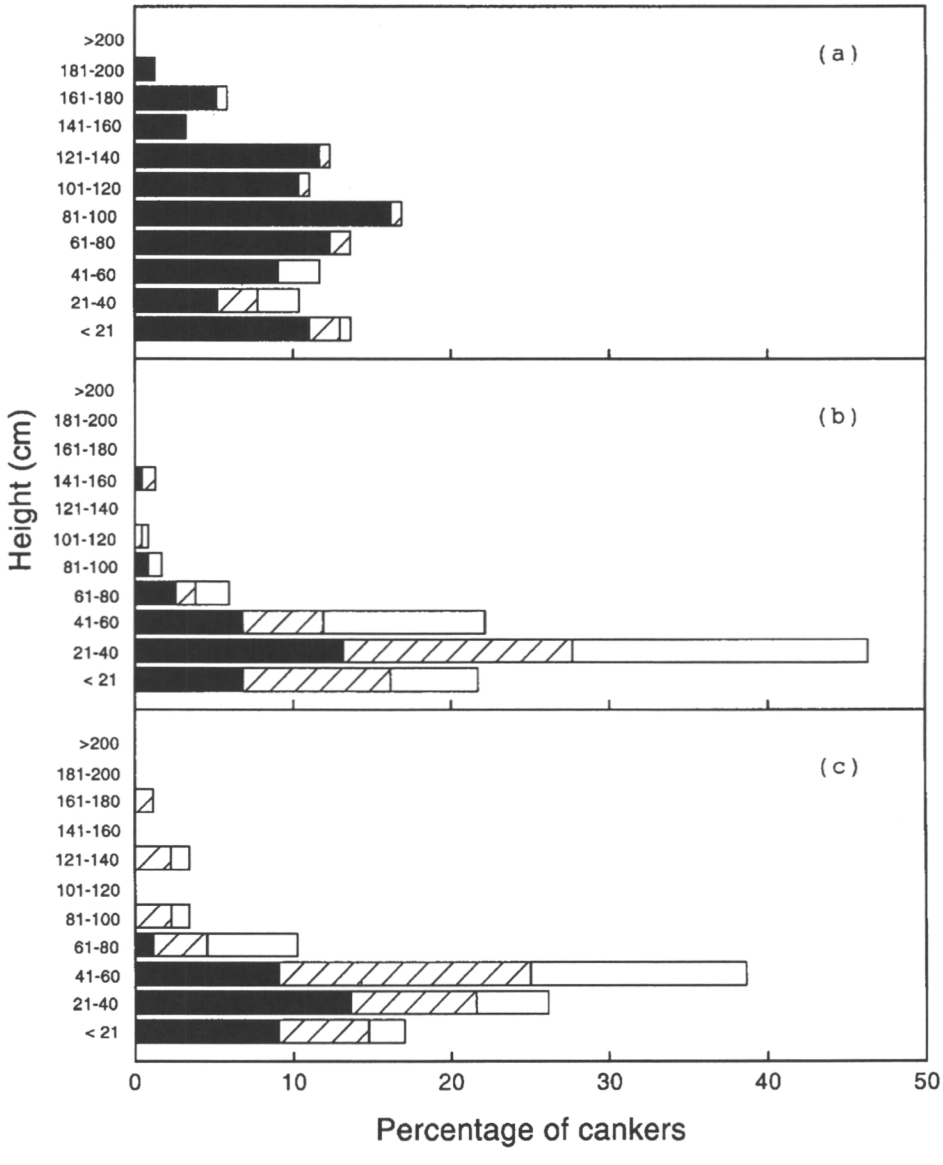


Figure 3. The percentage of cankers caused by *Gremmeniella abietina* in a) Grannäs, b) Allejaur and c) Akkajärvi, with respect to height above ground (cm) and year of formation. Cankers formed in 1990 are shown in black, cankers formed from 1991 to 1994 are shown in stripes, and cankers formed in 1995 are shown in white.

In 1990, the average height of the four years older trees in Grannäs was almost twice that in Allejaur and Akkajärvi. During the winter of 1988, with a 1.5-1.6 m snow cover, twice as thick as average, in areas between latitudes 64°N and 66°N in northern Sweden (Karlman 1993), the trees in Grannäs were high enough to be bent over into the snow, providing highly favourable temperature and moisture conditions for the fungus (cf. Laflamme 1991, Yokota et al. 1974, Tanaka 1988, Marosy et al. 1989).

In Allejaur and Akkajärvi, 97.9% and 95.5% respectively of the cankers were formed within 100 cm of the ground (Fig. 3). In Grannäs, however, the cankers were evenly distributed between 180 cm of tree height and the ground (Fig. 3). Most cankers above 100 cm of tree height were formed in 1990 or earlier. Cankers formed between 1991-1995 were mostly situated below the height of normal snow depth. These results support Dorworth (1973), who found that 95% of *Gremmeniella*-infected branches were within 0.8 m of tree height, and thus covered by snow during winter.

Cankers were formed seven different ways (Table 1). Cankers appeared around the base of diseased branches, as also found by Dorworth (1973). On *P. resinosa*, he found that the fungus advanced along the branch approximately one internode per year. Once the fungus had entered the bole, a canker was formed. However, *G. abietina* can also infect through injuries resulting from snow bending the branch (Kurkela 1981). Cankers occur between the whorls of branches, and form around mechanical damage as well as on visibly undamaged bark (Table 1). According to Roll-Hansen and Roll-Hansen (1973) and Kurkela (1981), bark damage caused by frost and ice formation or by snow bending, may serve as an entrance for the pathogen directly into the bole. Furthermore, Kurkela (1984) suggests that visible damage to the bark is not always necessary for successful infection. Further experimentations are, however, necessary to clarify the importance of bark damage as a way of entrance for the pathogen.

The frequency of cankers facing north (58.8%) exceeded those facing south (41.2%) ($p<0.05$). Most cankers were formed on the north side of the stem; 35.5% in the N-E sector ($p<0.05$). This was in accordance with Kurkela and Norokorpi (1979), who observed that formation of cankers was somewhat more frequent on the NW-E sectors compared to the SE-W sectors of the stems, but found that the position of the canker in terms of compass direction did not affect its size. Dorworth (1973) found that the frequency of infected branches facing north exceeded those facing south by approximately 25%.

In 1995, apothecia by *G. abietina* were present in 64.6% of the cankers. Twenty-five per cent of the cankers were formed in the final year of the study, why apothecia could not be recorded in those cankers. The high canker development in 1995 might be a result the cold and wet weather in northern Sweden during the autumn of 1993 (Anon. 1993, cf. Kaitera and Jalkanen 1992). For most cankers, however, fruit bod-

Table 1. Percentage of cankers with regard to different ways of entrance for the pathogen (values with different letters are significantly separated at 0.05 level)

Entrance	Percentage
Infected branch	42.0 A
Undamaged bark	33.6 A
Other/unknown damage	21.2 B
Vole damage	1.8 C
Infected lateral stem	0.5 C
Moose damage	0.5 C
Mechanical damage	0.4 C

ies of *G. abietina* were recorded in affected branches adjacent to the cankers. No pycnidia was found in the cankers.

The average vertical length of the cankers increased during the period. For cankers formed in 1991-1995, the average vertical length increment the first year after formation was 85.5 mm, and from year two 23.4 mm/year. The fast growth during the first year might be explained by limited chemical defence and absence of callus tissue in the bole during the first winter after infection. During the following years, the fungus must exceed the mechanical and chemical defence of the tree in order to expand (Blanchette 1992, Manion 1991, Shigo 1984).

For 55.6% of the cankers, the average coverage of stem circumference increased from the first measurement to that of 1995; 13.6% of the cankers had fully girdled the stem. For 44.4% of the cankers, the average coverage of stem circumference decreased or was constant between the first measurement and 1995; 10.1% of the cankers were completely occluded in 1995. For 40% of the cankers, the average coverage decreased or was constant between the first measurement and that of 1995. Some of the cankers were completely occluded in 1995. Kurkela (1981) also recorded healing of cankers, but found it possible for the fungus to survive in the wood of eight-year-old, completely occluded cankers. There might be a risk that the pathogen will start to grow again in periods of weather conditions favourable for the fungus.

Occluded cankers can also hide severe damage to the wood, substantial resin exudation, fibre aberrations, bark pockets and compression wood, though some of the occluded cankers are hardly visible on the trunk surface (Karlman and Witzell 1991).

Gremmeniella-damaged wood has a considerably higher basic density compared with undamaged wood (Ahlqvist et al. 1996). This can partly be explained by the very high extract content of the former, and by the occurrence of reaction wood. The use of trees with larger contents of *Gremmeniella*-damaged wood leads to serious processing problems. Thus, such wood should be sorted out and classed as low-grade raw material (Ahlqvist et al. 1996).

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Prof. Margareta Karlman initiated this study. Financial support was received from the Swedish Forest Research Foundation and the Swedish Environmental Protection Agency.

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Needle size and needle nutrient contents of Scots pine after *Gremmeniella abietina* infection and green pruning

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Abstract

Scots pine trees may have several long-term foliar responses to defoliation caused by *Gremmeniella abietina* or green pruning. Two years after pruning treatments the needles were longer, heavier and contained significantly more nutrients (per individual fascicles) than the needles of the unpruned trees. Even 10 years after *Gremmeniella* infection needles of the affected trees were still slightly longer and contained more boron and calcium than the needles of the healthy control trees. On the other hand, the foliar contents of few other elements and dry weight of the individual fascicles were lower in the diseased trees than in the control trees.

Introduction

Scleroderris canker (*Gremmeniella abietina* (Lagerb.) Morelet) may rapidly reduce the size of the living crowns of Scots pine (*Pinus sylvestris* L.) by killing their shoots and the whole branches during the epidemics. When this type of defoliation concentrates on the lower parts of the crown of the trees, it may alter the chemical composition of the healthy needles which have grown after the needle loss in the remaining upper and green part of the crown (Nuorteva and Kurkela 1998). In our previous study (Nuorteva and Kurkela 1993) we have described altered nutrient concentrations in healthy needles of Scots pine trees which had been seriously defoliated few years earlier by *Gremmeniella abietina* or artificial green pruning of the lower branches. In this study we report about the possible compensatory changes in the length and dry weight of those same upper-crown needles of the affected trees (Nuorteva and Kurkela 1993). We were also interested, whether there were any changes due to defoliation in actual amounts of needle nutrients *in situ* (eg. per needle or fascicle).

Material and methods

The needle material (youngest needles, uppermost lateral top shoot) of this experiment was collected during winter from six Scots pine stands in southern Finland. Four of the stands (tree age 25-30 years) had suffered from *Gremmeniella abietina* 5-10 years and in two healthy stands part of the trees had been pruned 1-2 years earlier (tree age 15-20 years). Twenty sample trees per stand (10 diseased or pruned and 10 control trees with an unaffected crown) were selected based on the length of the living crown. The crown ratio (length of the green crown / tree height) of the diseased or pruned trees were on average over a half smaller than in the control trees.

The length and dry weight of 100 needle fascicles per each sample shoot were measured (a total of 12 000 fascicles). In addition, we calculated the actual needle nutrient contents per individual needle fascicles based on the earlier analyses of foliar nutrient concentrations (for more detailed description of the sample trees and the chemical analyses see Nuorteva and Kurkela 1993).

Results and discussion

The needle length was slightly increased both in the diseased (+7%) and pruned trees (+24%) as compared to the needles of the adjacent control trees with an unaffected crown. The dry weight of the individual needle fascicles in the pruned trees were also higher (over 20%) than those of the unpruned trees. However, in the diseased trees, the dry weight of the fascicles were still lower (-10%) than in the control trees in spite of slightly longer needles of the affected trees. The amounts of foliar boron and calcium per needle fascicles were increased both in the diseased and pruned trees. Other element contents (per fascicle) were in the diseased trees mainly lower (Fe, Mg, K, C and H) and in the pruned trees higher (N, S, P, K, Mn, Cu, Zn, Na, C and H) than in the healthy undefoliated trees.

According to this experiment, reduction in the living crown by *Gremmeniella abietina* or green pruning induces long-term changes in the needle length and weight of recovering Scots pine trees. Further, we noticed significant changes in the amount of nutrients per individual needles (=nutrient content), and that these changes were larger, expressed in percentages, than those of the concentrations in the foliage.

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The genomes of dsRNA viruses of *Gremmeniella abietina*

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Abstract

Two fairly different, independently occurring, virus particles were found from plant pathogenic fungus Gremmeniella abietina. GaRV-L particle had one ~6000 by long dsRNA molecule and GaRV-MS particle had three dsRNA molecules (~2000 bp, ~1900 by and ~1300 bp) in its genome. The sequence of GaRV-L1 was determined and analysed. GaRV-L 1 belongs to the Totiviridea family according its nucleic acid sequence. The genome of GaRV-L 1 contained two large open reading frames which code for putative coat protein and putative RNA dependent RNA polymerase. GaRVMS belongs to the Partitiviridae family. The viruses discussed here seem not have any effect on the pathogenicity of their hosts.

Pine dieback by *Sphaeropsis sapinea* in Northern and Central Italy

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Abstract

Pine dieback, shoot blight and needle disease associated with *Sphaeropsis sapinea* are quite common symptoms on *Pinus* spp. in both natural stands and plantations, after a relatively mild winter followed by a dry summer. During the last few years damage was quite severe in Northern and Central Italy, particularly on *Pinus nigra* but also on *P. halepensis, P. pinea,* and *P. sylvestris*. Fungal isolates were analysed with both morphological and molecular methods and were assigned to the type A. The role of an insect, the cone bug *Gastrodes grossipes*, as epidemiological factor in the spreading of conidia, was evidenced. Weather patterns and site conditions seemed the most effective factors in enhancing the symptom development. The fungus was quite common in pine forest and stands and it can be considered as a bio-indicator of natural decline of pine plantations which are at the end of their "pioneer role".

Keywords: *Sphaeropsis sapinea*, *Gastrodes grossipes*, *Pinus,* fungal population, epidemiology

Introduction

Sphaeropsis sapinea (Fr.) Dyko & Sutton is a parasitic fungus known and studied in Italy since the early '900 (Petri 1913, Petri et Adani 1916, Goidanich 1933). It is considered responsible of many symptoms and may affect different parts of the plant. The fungus has been reported on various host species mainly from the genus *Pinus* but also on *Pseudotsuga* and, recently, *Cupressus* (Frisullo et al. 1997). The most affected hosts are *Pinus nigra* (Fig. 1) and *Pinus pinea*, the latter especially at cone level (Capretti 1956, Maresi et al. 1999, Vagniluca et al. 1995, Tainter and Baker 1996).

During the last century, considering the occurrence and damage by *S. sapinea* on different host species and environmental conditions in Italy, many attempts were done in order to differentiate the fungus and "new species" or varieties were re-

ported. The organism was described as:
- *Sphaeropsis ellisii* Sacc. (Petri 1913)
- *Sphaeropsis necatrix* Petri and Adani (1916)
- *Sphaeropsis ellisii* var. *cromogena* (Goidanich 1933).
Later all these "species and varieties" were included in *S. sapinea* (Fr.) Dyko & Sutton.

Leonello Petri (1913) described a disease of shoots on young trees of *Pseudotsuga douglasii* planted nearby *Pinus sylvestris* affected by the fungus. As the conidial morphology and size did not correspond to those reported on pines the organism was described as a "new species": *Sphaeropsis ellisii* Sacc. (Petri 1913).

A different causal agent was considered responsible of *Pinus pinea* cones' disease, the so-called "Malattia delle pine pagliose" *Sphaeropsis necatrix* (Petri et Adani 1916). The fungus was associated with intense necrosis on immature cones and seed abortion, with economic damage due to the commercial use of seeds. Petri et Adani (1916) judged *S. necatrix* different from *S. ellisii* mainly because of conidial morphology.

One of the fungus' peculiarities (to cause blue stain of colonised wood) induced Gabriele Goidanich (1933) to describe a new variety as *Sphaeropsis ellisii* var. *cromogena*. The fungus produces old hyphae with pigmented substances and colonises both medullar rays and tracheids.

Recently, as a consequence of heavy damage, particularly in northern Italy (Maresi et al. 1999), the main epidemiological factors such as fungal characters, insect association and environmental conditions related to *S. sapinea* disease have been evaluated.

Materials and methods

i) Studies on the fungal pathogen have been focused on population variability detected by both morphological and molecular methods. The fungus was collected from different hosts species and localities. Conidia were checked according to the



Figure 1. Pycnidia of *Sphaeropsis sapinea* on a cone of *Pinus nigra.*

criteria suggested by Wang et al. (1986) and Palmer et al. (1987) with both optical and scanning electronic microscope (SEM).

DNA profiles from a selection of 9 Italian isolates collected mainly from *Pinus nigra*, *P. sylvestris* and *P. pinea* have also been compared utilising RAPD markers produced by DS9, DS10 and DS19 primers following Smith and Stanosz (1995). Electrophoretic patterns have been compared with those of typical referenced A and B isolates kindly provided by G. Stanosz.

ii) As regard to the dissemination of the conidia, the possibility that the fungus colonises the seed cone of the pine hosts (Vujanovic et al. 2000), producing a considerable quantity of pycnidia, suggested to assess the presence of the fungus on *P. nigra* cones and evaluate the role of cone insects as potential dissemination agents in a stand of *P. nigra* of Monte Morello, Firenze, Italy. The cone bug *Gastrodes grossipes* (De Geer) (Heteroptera Lygaeidae) (Fig. 2) was the most frequent insect species and appeared to be a good candidate. Four hundred open cones were collected from 40 trees and inspected for the presence of both the fungus and the insect in spring 2000. The presence of the fungal conidia on the insect body was ascertained by a washing technique already established for a cone bug attacking cypress cones and disseminating fungal conidia (Battisti et al. 1999). Each living insect had been washed in 400 ml sterile water with 1% detergent (Tween 80), shaking for 1 min at 40 Hz. The presence of conidia in the suspension was assessed at the microscope on a slide carrying 10 ml of the suspension. An insect was considered free of conidia when five replicates were negative.

iii) Forty-one *P. nigra* plots, all severely infected by *S. sapinea* in 1998 in Trentino (Northern Italy), have been considered to analyse site characters. Data were obtained from Woodland Management Plans in which woodland property was subdivided in homogeneous compartments: for each of them about 120 different environmental parameters were recorded every ten years following standards adopted in all the



Figure 2. *Gastrodes grossipes* on *Pinus nigra* cone scales.

Trentino woods. In this context, the following characters were considered: facing, morphology, slope, soil type, soil depth and soil humidity (Maresi et al. 1999).

Weather patterns were obtained from temperature and rainfall data collected at S. Michele all'Adige: the monthly mean of temperature and total monthly rainfall of the period 1997-2000 were compared with the mean of the period 1983-1995.

Results and discussion

i) *S. sapinea* isolates have been collected from different localities and host species, mostly from *P. nigra* but also from *P. halepensis, P. pinea, P. sylvestris;* characteristics of the fungal isolates are reported in Table 1.

Conidia of these samples varied within 34-41 x 10-15 µm in size, their surface at SEM observation was smooth while the mycelium was white to grey-green on PDA media. When compared with molecular makers all the isolates tested were assigned to the type A by the presence of characteristic bands (respectively 600 bp with DS9, 1600 bp Ds10 and 350 bp with DS19 RAPDs primers).

ii) The cones collected in the stand of Monte Morello were highly infected with both the fungus and the insect (Table 2). *G. grossipes* is a common insect which inhabits mature and open cones of pines but feeds on needles and shoots. The association between the cone bug *G. grossipes* and the pathogenic fungus *S. sapinea* in cones of *P. nigra* appears to be occasional, as the insect exploits the cones only for shelter during its whole development. A possible advantage for the insect would follow from the extended persistence of the fungus-infected cones on the branches of *P. nigra* trees, which has been observed in sampled stands. The high frequency of insects loaded with conidia of *S. sapinea* may suggest to consider their potential role as fungus' dissemination agents. Nymphs can spread the fungus at tree level from the cones to the pine needles where they feed, whereas adults after exploiting open

Table 1. Selection of *Sphaeropsis sapinea* isolates utilised for fungal characterisation.

Site location and province	Latitude and longitude	Host species	Length x width (mean µm)	Type [1]
Prissiano (Bolzano)	46°.33' - 11°.11'	*Pinus sylvestris*	38.7 x 12.4	A
Valternigo (Trento)	46°.10' - 11°.10'	*P. sylvestris*	40.2 x 12.8	A
Alassio (Savona)	44°.11' - 8°.10'	*P. halepensis*	34.7 x 10.8	A
Monte Senario (Firenze)	43°.54' - 11°.20'	*P. nigra*	41.2 x 14.7	A
Artimino (Firenze)	43°.47' - 11°.22'	*P. nigra*	38.3 x 14.4	A
Firenze	43°.46' - 11°.15'	*P. nigra*	34.2 x 11.1	A
S. Rossore (Pisa)	43°.43' - 10°.20'	*P. pinea*	34.2 x 14.8	A
S. Severino (Macerata)	43°.14' - 13°.11'	*P. nigra*	40.6 x 14.4	A
S. Cataldo (Lecce)	40°.23' - 18°.18'	*P. halepensis*	39.1 x 11.2	A

[1] Smith and Stanosz, 1995

cones can be responsible for the dissemination of the conidia among trees. Adults of *Gastrodes* are able to fly over distances of several kilometres in alpine valleys, reaching also the upper timberline (Nägeli 1934). These findings may suggest to include the insect-fungus association in the management of the disease in the stands of *P. nigra*.

iii) Although the fungus is quite common in Italy, the occurrence of heavy damage and death of adult trees by *S. sapinea* can be considered as a indicator of pine decline. This was particularly undertaken after field observation in Trentino (Northern Italy). Here the disease was more intense on sunny slopes, poor soil and dry conditions. Most of the damage (76.0%) was registered on southern and western facing slopes; 83.0% on superficial soils and 98.0% on well drained and dry soils. As showed in Table 3 all the damaged stands vegetated in site where drought problem can be easily enhanced by morphological and soil characteristics.

Analysis of meteorological data emphasised a heavy reduction of rainfall in February and March both in the years 1997 and 1998 when compared with the period 1983-1995. Adrought period was also recorded in the autumn of 1997. In the same year the temperature from January to March was higher than in the reference period (Figs. 3, 4). Following these climatic conditions, an evident and dramatic increase of damage was recorded in 1998. However in 1999 and 2000 the damage was lower probably due to an increase of rainfall and lower temperature.

Table 2. Results of the analysis of 400 *Pinus nigra* mature cones from the stand of Monte Morello Firenze, Italy.

Cones with the fungus	Cones with insects	Cones with fungus and insects	Insects loaded with the fungus
41.7%	42.9%	24.4%	58.2%

Table 3. Characters of 41 damaged stands in Trentino as obtained from Woodland Management Plan following the standard classification adopted by the Forest Service of the Province.

Facing	22% N; 2% E; 39% S; 37% W
Morphology	30% bottom of valley; 48% slope; 7% terrace; 15% edge
Slope	15% flat; 80% steep; 5% very steep
Soil type	58% brown soil; 42% rendzina soil
Soil deep	83% superficial; 17% medium deep
Soil humidity	55% arid; 43 % dry; 2% wet

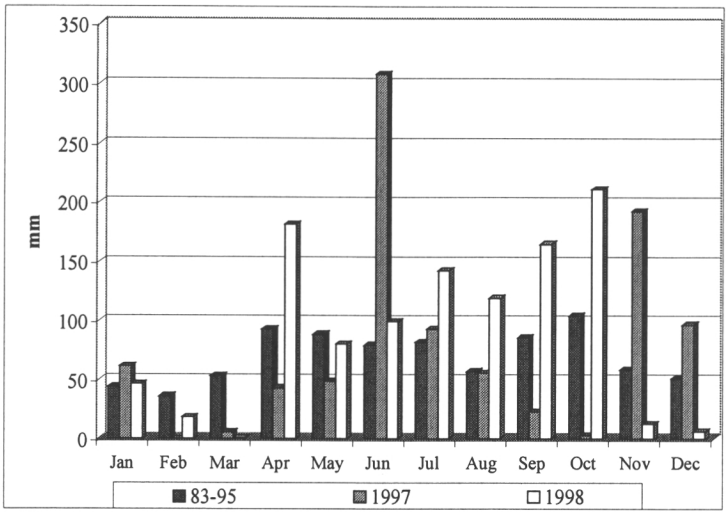


Figure 3. Mean of monthly precipitation data for the periods 1983-1995, 1997 from S. Michele a/ Adige (Trento).

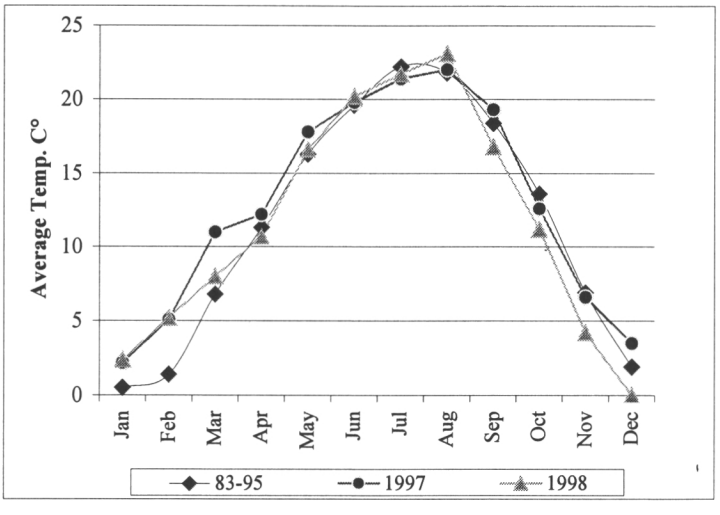


Figure 4. Monthly average of temperature of S. Michele a/Adige (Trento) for the period 1983-1995 and 1997.

Conclusion

S. sapinea is a quite common fungus in pine forest in Italy. It can be easily detected on cones even when there is no visible damage on shoots. *P. nigra* was the most affected tree species especially in the North of the country, but *P. sylvestris* was also badly affected in South Tyrol and *P. halepensis* in Southern Italy (Danti and Capretti 1997). In these stands the disease was effective in changing the wood composition and in leading the evolution of the stand towards a mixed wood in which *Pinus* species are absent or secondary (Ambrosi et al. 2000).

Water stress may pre-dispose trees to the attack of the fungus at crown level and tree mortality can be often observed. In this context, Blodgett et al. (1996) and Paoletti et al. (2001) after inoculation tests showed that the damage was higher on water stressed trees of *P. halepensis* and other *Pinus* species.

All the randomly collected isolates analysed in this study belong to the morphotype "A" which is considered the more pathogenic (Smith and Stanosz 1995). The differences observed in conidial size suggest that a certain variability among the isolates may exists.

A contribution to the epidemiology of the fungus was given by the observation on the role of *G. grossipes* in dissemination of conidia.

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Observations of *Sirococcus conigenus* and its pathogenicity

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Abstract

The occurrence of *Sirococcus conigenus* on spruce was observed in Southern Finland. In addition the pathogenicity tests were established on spruce and pine seedlings or pine germlings. Sixty samples of the fungus were collected mainly from spruce cones. The fungus was also found on spruce shoots earlier infected by *Chrysomyxa abietis* or wounded by frost. The fungus was isolated from pine germlings growing below spruces. When pine seedlings were grown in petri dishes the point inoculation of conidia produced 36% infection. In spruce seedlings the inoculation produced 14% infection. It seemed that *Sirococcus* is quite weak pathogen. Special conditions are needed for infections.

Introduction

Sirococcus conigenus (DC.)P. Cannon&Minter, syn. S. strobilinus Preuss, (Desm.) Petrak, *Ascochyta piniperda* Lindau, *A. parasitica* (Hart.) Rostr., was first reported by Hartig (Hartig 1894). It could cause shoot blight to spruce (Kujala 1950, Sutherland et al. 1981, Halmschlager et al. 1998), seed-borne disease (Motta et al. 1996), and damping off and dieback on larch (Motta et al. 1994, Harrison 1997). This species occurs widely in Europe and parts of Canada and USA. In Britain it has been recorded on cones of lodgepole pine (Ellis and Ellis 1985).

Conidia are fusiform, two celled, 12-16 x 3 μm (Ellis and Ellis 1985), 12-15 x 3.5 μm (Kujala 1950) in dimension. Mature pycnidia, dark-brown to black, usually multilocular appearing on the dead bark of shoots, needles or cones. Hyphae are colourless or whitish, sparsely forked.

S. conigenus can infect young seedlings in the early summer at the base or the middle of the current shoots, where the needles turn brown and shrank, and soon fall. The disease tends to spread upwards from lower needles towards the ends of the shoots and some movement may also take place downwards into the shoots of previous year's growth, leading to dealth of some of side shoots (Hartig 1894). For a time the shoots tips remain green, but later wilt, droop and finally die, often curling over and assuming the shepherds'crooks (Funk 1972). Most cases spring or autumn frosts can significantly promote the fungus to infect shoots (Hartig 1894). Temperature and humidity are important factors to the infection. A daily temperature cycle of 16-

21°C could create the great infection (Wall and Magasi 1976).

The aim of this study was to introduce some basic characteristics of *Sirococcus conigenus*. The pathogenicity of the fungus was studied by doing artificial inoculations.

Materials and methods

Isolates

The fungus was isolated from symptomatic shoots, seedlings and spruce cones. In summers 1996 and 1997 over 60 samples of this fungus on diseased seedlings, spruce cones and frosted shoots were collected in southern part of Finland (Table 1).

Table1. List of collected samples of *Sirococcus conigenus.*

Location	Geographic coordinates	Collection time	Host	Samples
Juupajoki	24°17′E and 61°50′N	June 5-7, 1996, July 1997, July 1998	Spruce cones and shoots, pine germlings	30
Kuru	23°30′E and 61°45′N	June 13, 1996	Spruce cones	2
Orivesi	24°00′E and 61°30′N	June 12, 1996	Spruce cones and shoots	8
Orivesi	24°19′E and 61°48′N	July 1, 1997	Pine germlings, spruce cones	8
Ruovesi	24°15′E and 61°50′N	July 1, 1996	Spruce shoots and cones	10
Savonlinna	29°30′E and 61°50′N	July, 1997	Spruce cones	2

Life cycle of *Sirococcus conigenus* in Vitro

On June 10th, 1997, the single spore isolation method (Korhonen and Hintikka 1980) was used to isolate conidium of *Sirococcus conigenus* to MA petri dish. Eight Petri dishes were left in the growth chamber (Termaks 5260) of light (Airam 15W, 35-X) at 15°C, and another 8 petri dishes were stored in the growth chamber in darkness at the same temperature. Observation was carried out every second day.

The infection experiment of *Sirococcus conigenus* in Vitro

The origin of pine seed (*Pinus sylvestris*) was seed orchard No. 246 (Tammela, Äijoenniitty) and it was collected in the autumn of 1992. Spruce seeds (*Picea abies*) were from the seed orchard No. 179 (Inkoo, Svartbäck) collected at same time. The seeds were stored in the cold storage at 5°C, in the dark. In vitro tested seed germination was 95% of pine and 96% of spruce.

Pine seeds were sown in 10 Petri dishes of malt agar on May 19th in the transfer chamber, which have been disinfected by 30% of H_2O_2 for 30 minutes, then rinsed 3 times by using sterile water and placed the seeds on sterilised filter paper to get rid of water.

In each petri dish 8 pine seeds were sown under aseptic condition. Then Petri dishes were wrapped by polyethylene and stored in the growth chamber at 15°C in light. The inoculation was carried out on June 17th, 5 petri dishes for inoculation and another five for control. The average germination was 88%, and no seedborn damping off was detected.

Following stage we designed a point inoculation with those seedlings in the petri dishes. The inoculum was prepared by increasing sterilised water on petri dish where the fungus has produced already pycnidia and conidia. The suspension was mixed with sterile glas bar. A small drop of suspension was transferred to needles or stem of the seedlings growing in Petri dishes. The suspension was transferred so that it was not dropped to the agar. After inoculation Petri dishes were kept in growth cabin at 15°C and light.

Inoculation onto spruce seedlings

On June 12th, 1997, 480 spruce seeds disinfected by H_2O_2 30% and 480 undisinfected spruce seeds were sowed in 24 containers (ϕ13cm x 10cm) filled with peat (TURVE BIOLAN.AV). The initial idea was to test whether this spruce had seed-borne disease, then they could be inoculated as tested objects by using inoculum *Sirococcus* conidial suspension.

After six week growth 60 seedlings were treated as irrigating distilled water; 120 seedlings were sprayed with conidial suspension and left in the open area (where the day temperature changed naturally), and 60 seedlings inoculated by same conidial suspension were grown in the greenhouse (the average temperature was around 26°C).

Before inoculation, on July 17th the seedlings were irrigated. To ensure the moisture was high enough the containers were put in plastic bags one week after inoculation. The conidial suspension was produced by adding distilled water into *Sirococcus* culture in Erlenmeyer flask and shook the bottle until the liquid became turbid, then transferred the suspension (160 000 conidia/ml) into houseplant atomizer. In each plastic bag packed one container of seedlings, sprayed the suspension onto seedlings until liquid drops existed on needles, then sealed the plastic bags immediately with rubber strings and kept them in such condition for one week, and then took off the plastic bags and incubated the seedlings as normal treatments.

The fresh, surface sterilised spruce cones were inoculated with *S. conigenus* conidia. The cones were incubated in plastic boxes in light and 15°C.

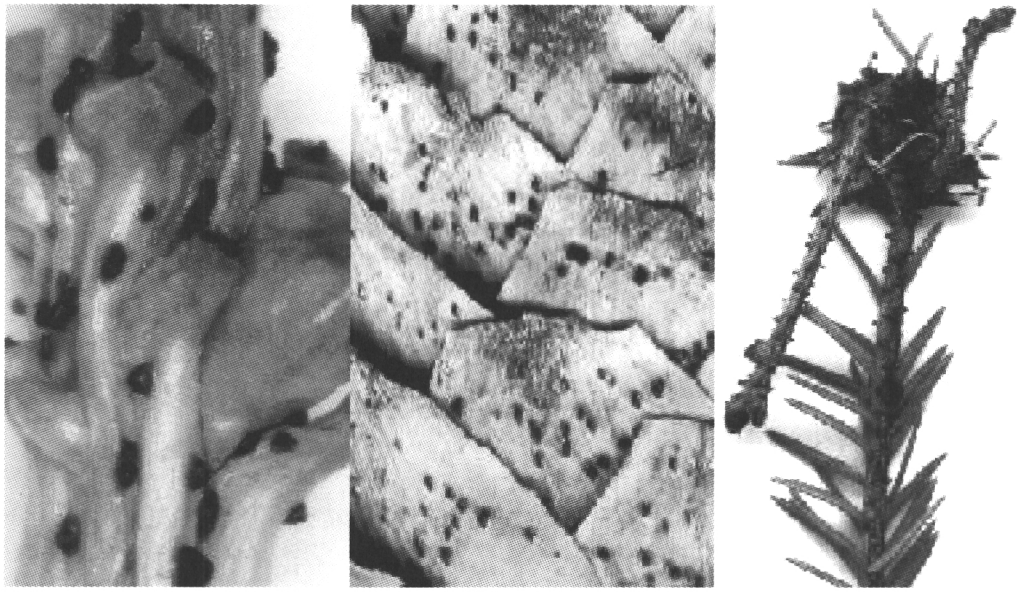


Figure 1. *Sirococcus conigenus* black pycnidia formed on a dead Norway spruce shoot, cone scales and aphid gall.

Table 2. The dimensions of conidia, hyphae and pycnidia.

	Conidia[a]		**Hyphae**[b]	**Pycnidia**[c]
	Length (μm)	Width (μm)	Diameter (μm)	Diameter (mm)
Mean	11.9 ± 1.7	4.4 ± 07	5.3 ± 1.2	0.3 ± 0.1
Max.	15	5	10	0.5
Min.	7.5	2.5	2.5	0.1

[a]: 150 conidia obtained from the pycnidia formed on aphid galls of Norway spruce shoot.
[b]: 100 hyphae were measured from a single strain growing in malt agar.
[c] : 150 pycnidia obtained from aphid galls of Norway spruce shoots.

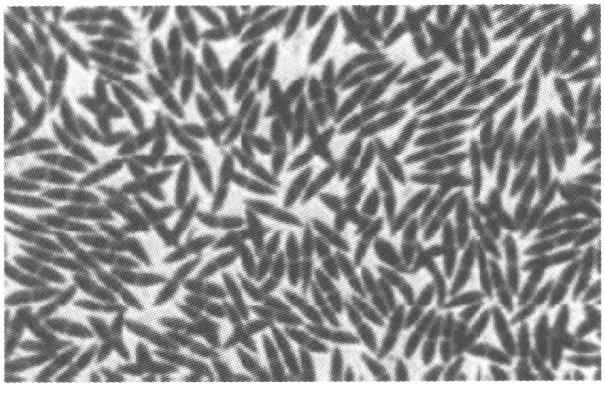


Figure 2. Conidia of *Sirococcus conigenus* (x 400) stained with anilin blue.

Results

Characteristics of the fungus

Hyphae were long and sparsely forked, colourless, thickness 5 μm. Pycnidia was dark-brown to black on spruce cones or shoots, 0.1 to 0.5 mm in dimension (Fig. 1). In wet conditions ripened pycnidia opened irregular exits and released spores. Conidia, colourless, 2-celled, 12 x 3.3 μm, spindle-like or fusiform (Fig. 2, Table 2.)

Conidial production of the fungus in Vitro

After five days incubation hypha grew in petri dishes in light and darkness. After one week hypha composed mycelia in dark and light. It seemed that light had no influence to the growth of *S. conigenus*. However two days later some mycelia in light chamber started to form pycnidia and released conidia. But in dark chamber pycnidia were not developed and no conidia were formed. The fungus could complete its asexual life cycle within two weeks in Vitro (light, 15°C).

A micro-point inoculation to pine seedlings in Vitro

Eight days after inoculation the first symptoms appeared. Firstly diseased needles or stems shrank and wilted. The infected tissues started to rot, the shoot tops were bending, and finally the seedlings died. Whitish and woolly mycelia were growing on the diseased tissues and spreading towards the healthy tissues. Soon brown pycnidia of *S. conigenus* emerged on the diseased seedlings and conidia were formed on the pycnidia (Fig. 3). It takes one week time for the fungus to kill a germling. The mean infection rate was 36%. No infections were in control seedlings.

Inoculation to spruce seedlings and cones

After one week incubation symptoms appeared. On needle tips or in the middle of needles appeared brown points, extended towards stems, then the tissues of the stem and needles wilted and died. But the infection frequency was not high, the average infection was only 14%. In the greenhouse the temperature during the incubation was 22-32°C (accordance with the daily data recording in Hyytiälä Forestry Field Station of Helsinki University), the fungal infection was 13.5%, however the natural temperature at same moment was concentrated at 14-26°C, and the infection was 14.6% (Table 3).

Two weeks after inoculation pycnidia were developed in all inoculated spruce cones (Fig. 4). One week later the conidial mass was liberated from pycnidia.

Table 3. The number of diseased seedlings in experiments. The symptoms of control seedlings were caused by unknown factors.

Group	Mean-extreme temperature (°C)	Seedlings	Symptomatic %
Control	14 – 26	134	4.5
Open area	14 – 26	303	14.6
Greenhouse	22 – 32	120	13.5



Figure 3. Disease symptoms on Scots pine seedlings in the point inoculation experiment. Note the woolly mycelia and pycnidia of *S. conigenus* on the needles and stems.

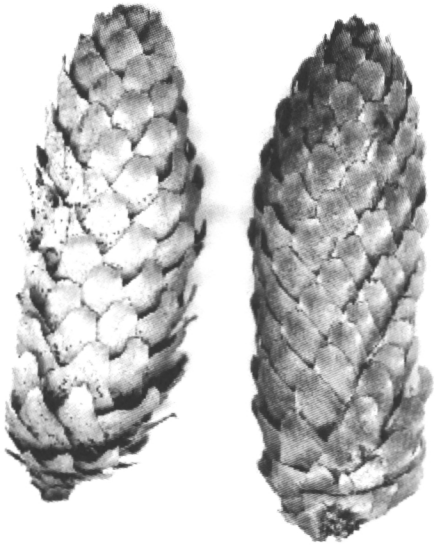


Figure 4. Pycnidia of *S. conigenus* on spruce cones. Left: Black pycnidia occurred on scales of the cone. Right: Uninoculated control.

Discussion

Sirococcus conigenus is common fungus on spruce in Finland (Kujala 1950). On Norway spruce it was found on shoots, cones and aphid galls. On pine it killed germlings in shady conditions. The fungus could cause seed born damping off (Motta et al. 1993), but this was not detected in one tested seedlot in this study. The infection seem to be successful in moist and shady conditions. In Austria the fungus has caused damage in foggy zone spruce forest (Halmschlager et al. 1998), which support the importance of moisture as predisposing factor. It seemed that *S. conigenus* is weak pathogen and predisposing factors as moisture, shading, frost, nutrient imbalances or infection of *Chrysomyxa abietis* are needed for successful infections on spruce shoots. In Austria *S. conigenus* destroys the spruce stands suffering magnesium deficiency (Halmschlager et al. 1998). In spruce cones the fungus was present regularly and in these infections no special predisposing is needed.

The dimensions of conidia were almost same size as mentioned in literature (Ellis and Ellis 1985, Kujala 1950). Kujala (1950) collected the fungus also from *Pseudotsuga menziesii* and *Abies concolor.*

The possible genetic variation of the fungus is unknown. Propably intra species genetic variation is detected if the isolates from different geographic origins are compared. This kind of research could be important with this pathogen due to broad distribution area and several host species in Europe and North America.

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Effect of growth stage and microclimate on *Botrytis cinerea* outbreak in *Picea abies* container seedlings: Preliminary results

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Introduction

Grey mold (*Botrytis cinerea*) is a very common fungus, which can have saprofytic, pathogenic or endofytic character. This fungus causes damages also in forest tree nurseries especially on spruce (*Picea abies*) and birch (*Betula pendula*). Most of the spruce seedlings in Finnish nurseries are grown in containers in greenhouses. This growing method seems to favour the outbreak of the grey mold disease compared to the growing of bareroot seedlings outdoors. The microclimate inside container seedling canopy is supposed to be the main reason.

The aim of our work was to study possible changes in the resistance of *P. abies* seedlings to *B. cinerea* and in microlimate factors within seedling canopy during the growing season and interactions between them. The final purpose was to find possibilities to predict the outbreak of the disease and to control it.

Experiments

P. abies seedlings were sown in spring into containers filled with light peat growth medium. They were for two greenhouse experiments (Exp. 1 and 2) and for a growth chamber experiment with simulated growing season (Exp. 3). Sensors for temperature, relative humidity (RH), surface moisture and light intensity were set to measure conditions near the surface of the growth medium in containers (Fig. 1). Daily mean temperature was commonly within 10 and 25°C, relative humidity (RH) above 60% and vapour pressure deficit (VPD) within 0.2 and 0.95 kPa during the growing season. Temperatures below 10°C, RH above 85-90% and VPD below 0.2 kPa were recorded from the beginning of September.

Inoculations with *B. cinerea* were made on the seedlings when they were 1, 2, 3, 4, 5 or 6 month-old. Conidia in water suspension (conidia density 1 x 10^6 /ml, 3.2 ml/seedling) were sprayed on the seedlings. Four seedling trays (with 36 inoculated and 36 uninoculated seedlings in each tray) were used on each inoculation time in

each experiment. In Exp. 3, seedlings were put into a dew chamber after inoculations (VPD close to zero). In Exp. 1, seedlings stayed in the greenhouse and in Exp. 2 seedlings were moved into a small plastic house, where the surface of seedlings was kept continuously wet by using atomizer nozzles. The seedlings were checked daily during the 11-12 day-period after inoculations to find symptoms caused by the mold.

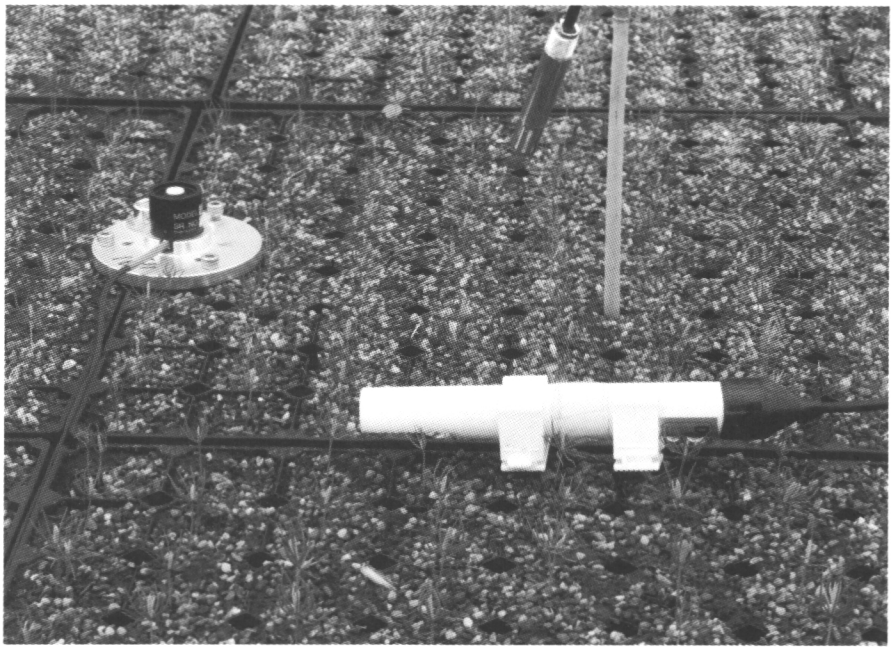


Figure 1. Measurement sensors used in the experiments.

Results

Microclimate was affected by the growth of the seedlings and especially by the closure of the canopy causing e.g. decreasing saturation deficit (data not shown). The first symptoms in the seedling needles were small spots with color changes. In the new needles, the whole needle might change color and decay, while in the older needles a clear dead strip in the middle part of the needle was common (Fig. 2). The speed of disease outbreak was measured with the duration. The effect of seedling growth stage and microclimate on the disease outbreak was considered using the duration when 50% of seedlings was diseased (days to 50% diseased = DD_{50}) (Fig. 3, Fig. 4).

In the beginning of the growing season the disease broke out slowly, DD_{50} was more than 7 days in all the three experiments. Nearly the same situation was at the end of the growing season in Exp. 1 and 2 but not in Exp. 3. The conditions in the dew chamber (Exp. 3) were rather optimal for the disease outbreak, e.g. the saturation deficit was continuously very low. So, it seemed that at the end of the growing

season conditions close to optimal are needed for the disease outbreak. In the middle of the growing season (2 to 5 months) the seedlings got disease more quikly, DD_{50} was only 1-3 days, except in the inoculation time 3 in Exp. 1. An additional experiment in the dew chamber showed clear symtoms in 3-month-old seedlings after only a couple of hours from the inoculation.

Further experiments and data handling are to be carried out concerning the grey mold outbreak in tree nurseries.



Figure 2. Grey mold symptoms in the needles of the seedling.

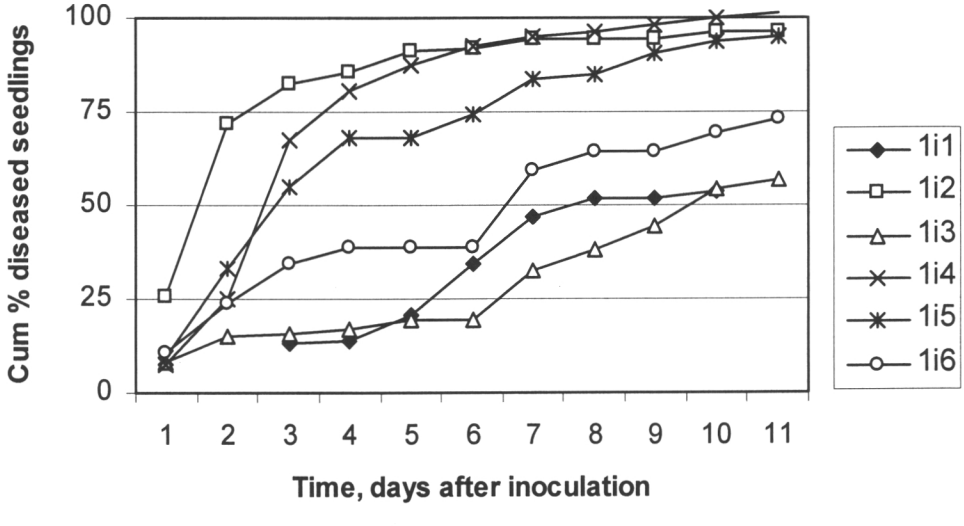


Figure 3. Cumulative proportions of the seedlings having the grey mold outbreak after different inoculations (1-6) in Exp. 1.

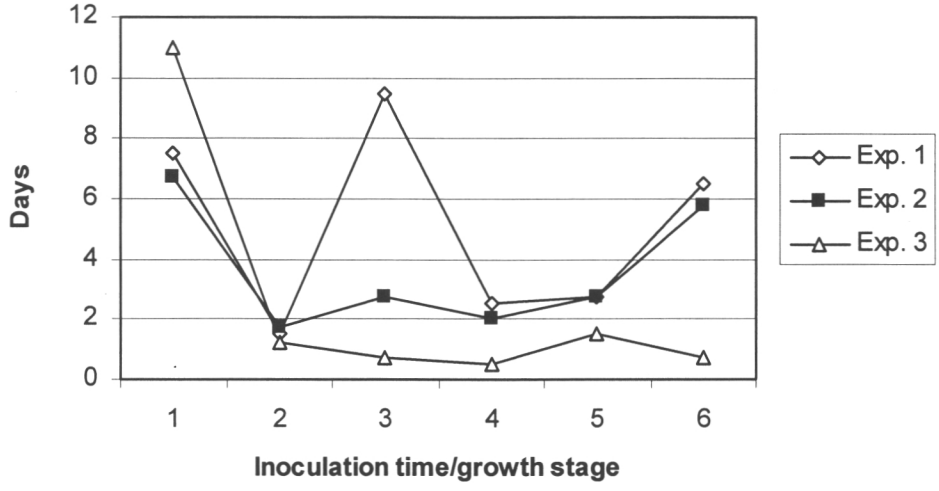


Figure 4. Duration in days when 50% of the seedlings had the grey mold outbreak.

Changes of xylem pressure potential in *Quercus serrata* saplings inoculated with *Raffaelea* sp., a possible causal fungus of oak mortality in Japan

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Abstract

From the end of the 1980s, mass mortality of *Quercus* spp. has increased in Japan. All dead trees show numerous entry holes bored by the oak pinhole borer, *Platypus quercivorus* (Coleoptera: Platypodidae). A new species of *Raffaelea* has been isolated from both the beetle's body and the tree tissue attacked by this insect. After mass attacks by *P. quercivorus*, oak trees exhibit rapid-wilt symptoms. In order to study the pathogenicity of *Raffaelea* sp. and evaluate the changes in the water status of the *Quercus* trees after a fungal infection, *Q. serrata* saplings (5-7 years old) were inoculated with *Raffaelea* sp., and some water physiological parameters were measured in 1996, 1998, 1999 and 2000.

The transpiration rate and stomatal conductance to water vapor were greatly reduced in the saplings after the inoculation. Midday and predawn shoot xylem pressure potentials were higher after the inoculation than those in the control saplings. These potentials suddenly and rapidly declined in some of the inoculated saplings. The length of the periods before the decline in the xylem pressure potential was highly variable. Not all saplings died as a result of the inoculation. In the 2000 experiment, five of 16 trees died. *Raffaelea* sp. was reisolated from the dead saplings.

Raffaelea sp. showed ability to reduce the water conductance of the xylem and kill *Q. serrata* saplings. These results support the hypothesis that the fungus causes mass mortality of the *Quercus*, which has been increasing in recent decades in Japan.

Introduction

From the end of the 1980s, mass mortality of oak trees (*Quercus* spp.) has increased on the Honshû and Kyûshû islands of Japan (Ito and Yamada 1998). The dead trees are deciduous and evergreen oak species, mainly *Quercus crispura*, *Q. serrata*, *Q. salicina*, and *Q. acuta*. All dead trees showed numerous entry holes bored by the oak pinhole borer, *Platypus quercivorus* (Coleoptera: Platypodidae). This insect was assumed to be an ambrosia beetle, but its associated (ambrosia) fungi were not known. Similar mass mortalities of oaks had occurred from the 1930s (Ito and Yamada 1998), but no forest pathological research was conducted at that time. Recently, however, a new species of *Raffaelea* was isolated from the bodies of *P. quercivorus* (body surface and mycangia) and tree tissues attacked by this beetle (Ito et al. 1998, Kubono and Ito, under submission). This fungus was proposed to be the cause of the recent

mass mortality of oak trees. Indeed, according to inoculation tests, the pathogenicity of this fungus against *Q. crispura* was suggested (Ito et al. 1998). However, it is still not clear whether *Raffaelea* sp. has pathogenicity against other *Quercus* species in Japan. After the mass attacks by *P. quercivorus*, oak trees exhibited rapid-wilt symptoms. We speculated that oak trees inoculated with *Raffaelea* sp. would show rapid changes in their water status if the fungus is a causal agent of this mortality.

In order to inquire the pathogenicity of *Raffaelea* sp. and evaluate the changes in the water status of the *Quercus* trees after a fungal infection, *Q. serrata* saplings were inoculated with *Raffaelea* sp., and some water physiological parameters of water relations were measured.

Materials and methods

The inoculation tests were conducted from late June to early July in 1996, 1998, 1999 and 2000. Saplings of *Q. serrata* (5-7 years old) growing at an experimental nursery at the Kansai Research Center, Kyoto, Japan (35°1' N, 135°44' E) were used for inoculation. The saplings were the same age for each year.

The *Raffaelea* sp. isolate for inoculation was NA9. This isolate was isolated from the bark of an unidentified *Quercus* (*Q. crispura* or *Q. serrata*) tree. At each inoculation area, bands and plugs of bark were removed, and the mycelium of the isolate NA9 grown in a rice bran and wheat bran medium was placed on the wound. Thereafter, bands and plugs were replaced, and the inoculated area was sealed with parafilm and cloth adhesive tape to avoid contamination and desiccation (Fig. 1).

Three types of control were used to evaluate the effects of wounding because the wound in these experiments was potentially harmful to the saplings. Type 1 control saplings were inoculated with a sterile medium. Type 2 control saplings were wounded as inoculated saplings, and no treatment was done on the type 3 control saplings.

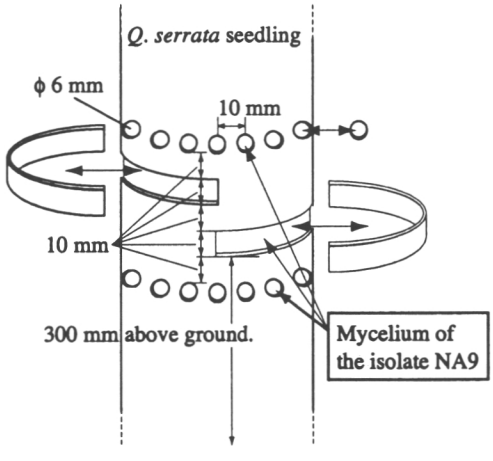


Figure 1. Diagram of the method of an inoculation.

To evaluate the water status of saplings, the transpiration rate, stomatal conductance to water vapor, and midday and predawn shoot xylem pressure potentials were measured. The transpiration rate, stomatal conductance to water vapor, and midday xylem pressure potentials were measured around noon on sunny days. On the following days, predawn xylem pressure potentials were measured. The transpiration rate and stomatal conductance were measured with a portable gas exchange system (Shimadzu SPB-H3), and xylem pressure potentials were measured with a pressure chamber (Soilmoisture Equipment Co.). These parameters were measured one or two times before inoculation and four to eleven times after inoculation. The intervals of measurements varied from one day to about two weeks. The last measurement was conducted by late September in each year.

Results

In 1996 and 1998, no sapling died after inoculation (Table 1). Two of 12 inoculated saplings died in 1999; five of 16 inoculated saplings died in 2000; however, none of the control saplings died during these two years. *Raffaelea* sp. was re-isolated from the tissues of some dead saplings. The length of the time periods before the death of the inoculated saplings was highly variable.

In each year, after inoculation, the means of the transpiration rates and stomatal conductance to water vapor dropped greatly, except in the type 3 control saplings, which were untreated. The type 1 and 2 controls recovered values equivalent to those of the type 3 control saplings by the end of the experiments. Although the values of the living inoculated saplings also recovered, more time was required than for the type 2 and 3 controls. The values did not recover in the dead inoculated saplings.

During 2-7 weeks after inoculation, midday and predawn shoot xylem pressure potentials in the inoculated, type 1 and 2 control saplings were higher than those of the type 3 control saplings. However, in some of the inoculated saplings, midday and predawn shoot xylem pressure potentials dropped suddenly and rapidly to extraordinarily low values, followed by death within several days.

Table 1. Survival of *Q. serrata* seedlings inoculated with *Raffaelea* sp.

	1996*		1998		1999		2000	
Treatment	No. trees	No. Dead	No. trees	No. dead	No. trees	No. dead	No. trees	No. dead
Raffaelea sp.	9	0	12	0	12	2	16	5
Sterilized medium	4	0	3	0	4	0	3	0
Barked	4	0	3	0	4	0	3	0
No treatment	4	0	3	0	4	0	3	0
Mean diameter at ground level (cm)	2.8 ± 0.59**		4.2 ± 0.86		3.8 ± 0.48		2.4 ± 0.44	

* In 1996, plugs of bark were not removed

** Mean value ± standard deviation

Discussion

The results in which some inoculated saplings wilted after inoculation and *Raffaelea* sp. was re-isolated from dead saplings show that this fungus can kill *Q. serrata* saplings. Because inoculation of *Raffaelea* sp. caused closure of stomata and reduction of shoot xylem pressure potentials, it was thought that the fungus was related to xylem dysfunction of *Q. serrata* saplings. These results suggest that *Raffaelea* sp. has pathogenicity against *Q. serrata*. The rapid progress of wilt in some of the inoculated saplings in these experiments was similar to that observed in mature oak trees in mass mortality

In conclusion, the results of this research support the hypothesis that this new *Raffaelea* species is the causal agent of the recent *Quercus* mass mortality in Japan.

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Phytophthora cactorum on silver birch seedlings

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Abtract

In 1991, *Phytophthora cactorum* was first isolated from necrotic stem lesions of nursery seedlings of *Betula pendula* in Finland. Inoculations of birch stems with this Oomycetous Chromista in class Pseudofungi, resulted in necrotic lesions identical to those on birch seedlings in nurseries.

In this study we monitored the effect of *P. cactorum* infection on the development of container-grown, silver birch seedlings in nursery and after outplanting. In nursery stem lesions affected the height growth of birches, the shoot height of seedlings was related to the disease severity. Asymptomatic seedlings were taller than the diseased seedlings and the shortest seedlings were those with stem leasons covering over half their stem diameter. After outplanting stem lesions did not affect seedling mortality or on the number of leader shoot changes. The height growth of seedlings in the reforestation site did not decrease with the disease rating. The diffenrences in the shoot heights related to the disease rating almost disappeared. It is still too early to conclude that silver birch seedlings will recover totally from *P. cactorum* infection although the differences in shoot heights present in the nursery between disease and apparently healthy seedlings have after two growing season in the field reduced.

Keywords: Stem lesions, *Betula pendula*

Introduction

Since the early 1980's the production of container-grown *Betula* seedlings in Finland has increased from 5 to 85% (Finnish Statistical... 2000). The use of greenhouses with controlled temperature and light, low –humified Sphagnum peat as the growth medium, and controlled irrigation and and fertilization in greenhouse and later on outdoor areas has resulted in good seedling growth in a shorter time. However the rapid seedling growth and high seedlings growing density may favor fungal diseases.

Numerous problems such as stem lesions and cankers have occurred during nursery production and subsquent outplanting of silver and pubescent birch seedlings (*B. pendula* Roth and *B. pubescens* Ehrh.). Several fungi cause these lesions. The most extensively studied in Finland is *Godronia multispora* J. W. Groves which affects both natural birch saplings (Kurkela 1974) and nursery seedlings (Petäistö 1983). Other fungi isolated from birch stem lesions in nurseries include *Fusarium*

avenaceum (Corda, Fr.) Sacc., *Alternaria* spp. and *Botrytis cinerea* Pers. ex Nocca & Balb (Petäistö 1983, Lilja et al. 1997). *Anisogramma virgultorum* (Fr.) Theiss. & Sydow, Syn. *Plogwrightia virgultorum* (Fr.) Saccardo also causes lesions on young silver birch seedlings in clearcut areas (Kujala 1942).

In 1991, *Phytophthora cactorum* (Lebert & Cohn) was first isolated from necrotic stem lesions of silver birch seedlings in Finland (Lilja et al. 1996). Inoculations of birch stems with this Oomycetous Chromista in class Pseudofungi (Baldauff et al. 2000) resulted in necrotic lesions identical to those on birch seedlings in nurseries (Lilja et al. 1996, Hantula et al. 1997, 2000).

The aim of the present study was to monitore the effect of *P. cactorum* infection on the development of container-grown, silver birch seedlings in nursery and after outplanting on a reforestation site.

Material and methods

During the rainy summer of 1998 *P. cactorum* commonly affected birch seedlings in several Finnish forest nurseries. The following spring diseased and healthy silver birch seedlings were collected from two nursery fields in mid-Finland. In total 480 seedlings were collected. Half of the seedlings had been grown in peat pots, and the other half in hard plastic containers. The stem-lesion disease severity on each seedling was assessed using a scale of 1 to 4 where: 1= no lesion , 2 = lesion < 5 mm^2, 3 = lesion > 5 mm^2, but not covering over half of the stem diameter, and 4 = lesion spread over half of the stem diameter, but not girdling the stem (Fig. 1).

On 4 May, after one-week storage at + 4°C, the seedlings were outplanted in a field in Southern Finland. The field (on former agricultural land had been ploughed one week earlier) was divided to 12 blocks. The seedlings grown in peat pots were planted to blocks 1-6 (experiment 1) and those grown in hard plastic containers were planted in blocks 7-12 (experiment 2). There were 10 seedlings in each of the four disease categories in both experiments in each block. There was 1 meter between rows and seedlings within rows and the rows were randomized within each block. A fence was built around the field to exclude moose. To avoid vole damage each seedling was protected with a treeshelter. The shoot height of each seedling was meas-

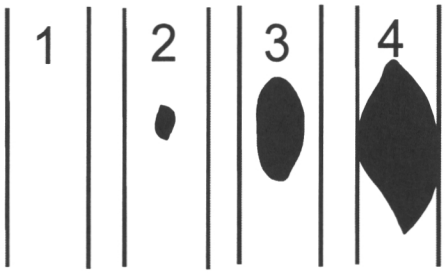


Figure 1. The stem-lesion disease severity on each seedling was assessed using a scale of 1 to 4 where: 1= no lesion , 2 = lesion < 5 mm^2, 3 = lesion > 5 mm^2, but not covering over half of the stem diameter, and 4 = lesion spread over half of the stem diameter, but not girdling the stem.

ured after planting and later on 22 September in 1999 and on 5 September in 2000. On these latter two dates dead seedlings and those in which a new leader had formed were also counted.

On 21 June, 2000, 50% glyfosate (Roundup Bio, Monsanto, USA) was applied to weeds around each birch seedling using a Weedwiper 40 (Lasco, USA).

Results

Nursery experiment

Stem lesions affected the height growth of birch seedlings both in peat pots and in hard plastic containers. In both cases the shoot height of seedlings was related to the disease severity. Asymptomatic seedlings were taller than the diseased seedlings and the shortest seedlings were those were lesions had spreaded over half of the stem diameter, but had not girdled the stem (Fig. 2). The effect of disease severity on the shoot height was more evident on seedlings grown in peat pots than on seedlings grown in hard plastic containers with seedlings in peat pots being about 40% taller than the seedlings in hard plastic containers.

Outplanting experiment 1

After outplanting the healthy seedlings grown in peat pots in the nursery grew less than diseased seedlings, especially during the second growing season (Fig. 2). Height growth of seedlings increased with disease severity. Growth of seedlings with stem leasons covering over half their stem diameter grew more than healthy control seedlings or seedlings with smaller stem lesions. However, after the first summer diseased seedlings were still shorter than the asymptomatic controls. After the second growing season category 4 seedlings were the shortest, but the differences in the shoot heights related to the disease rating at time of outplanting had almoust (Fig. 2).

Stem lesions did not affect seedling mortality or on the number of leader shoot changes after outplanting. All seedlings were alive in September, 1999. After the second growing season in the field the seedling mortality (mean ± SE) for disease categories 2-4 was .7 ± 1.7, 3.3 ± 2.1 and 11.7 ± 6%, respectively (Fig 3). In 1999 the percentage of seedlings which developed a new leader was 5.0 ± 2.2, 10 ± 3.6, 11.7 ± 4 and 3.3 ± 2.1 for disease categories 1-4. The corresponding percentages in 2000 were 8.3 ± 3.1, 5 ± 2.2, 8.3 ± 4 and 13.3 ± 4.9 (Fig. 4).

Outplanting experiment 2

The height growth of control seedlings and diseased seedlings grown in hard plastic containers in the nursery did not differ after outplanting (Fig. 1). After the first growing season in the field, differences in shoot heights among seedlings rated in different categories at the time of planting were still visible, however, after the second growing season these diffenrences had almost diseapeared as in the experiment 1.

Stem lesion severity did not affect seedling mortality after outplanting. During the first growing season 1.7 ± 1.7% of the seedlings in category 2 died while 3.3 ± 2.1% died in category 4. After the second growing season the mortality of seedlings were according to the disease rating 1-4 :11.6 ± 5.4, 16.7± 8, 10 ± 4.5 and 16.7 ± 4.9 %, respectively (Fig. 3). The percentage of seedlings which changed the leader shoot were 1 = 3.3 ± 2.1, 2 =3.3 ± 2.1, 3 = 1.7 ± 1.7 and 4 = 5 ± 2.2 in 1999. The corresponding percentages in 2000 were 6.7 ± 3.3, 15 ± 5.6, 8.3 ± 4.8 and 5 ± 2.2 (Fig. 4).

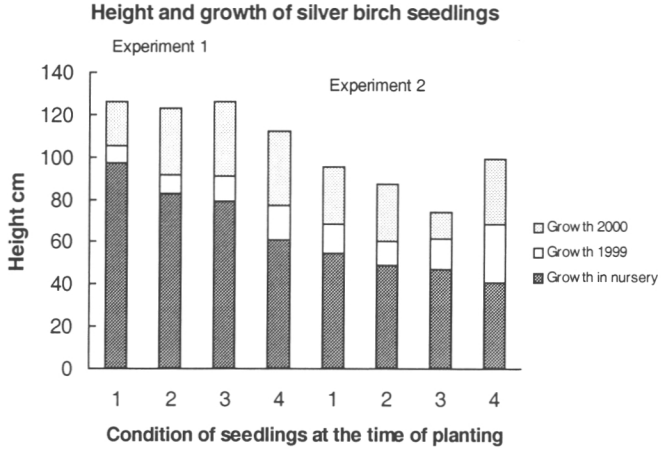


Figure 2. The height of seedlings at the time of planting and growth after outplanting. Seedlings grown in peat pot in nursery (Experiment 1). Seedlings grown in hardplastic containers in nursery (Experiment 2). 1= no lesion , 2 = lesion < 5 mm², 3 = lesion > 5 mm², but not covering over half of the stem diameter, and 4 = lesion spread over half of the stem diameter, but not girdling the stem.

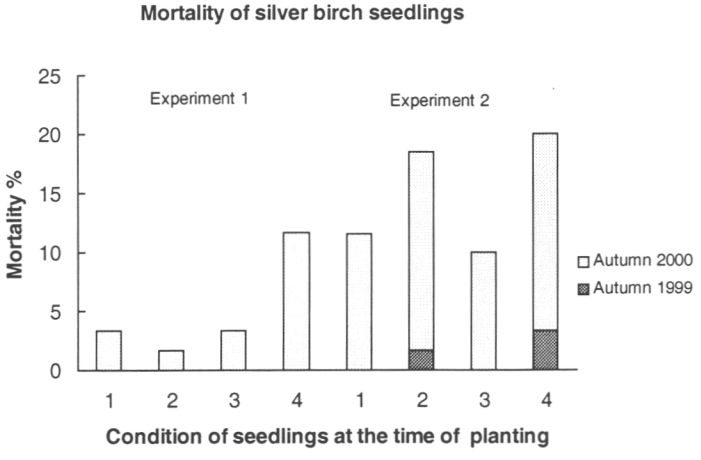


Figure 3. The mortality of silver birch seedlings after outplanting. Seedlings grown in peat pot in nursery (Experiment 1) Seedlings grown in hardplastic containers in nursery (Experiment 2). 1= no lesion, 2 = lesion < 5 mm², 3 = lesion > 5 mm², but not covering over half of the stem diameter, and 4 = lesion spread over half of the stem diameter, but not girdling the stem.

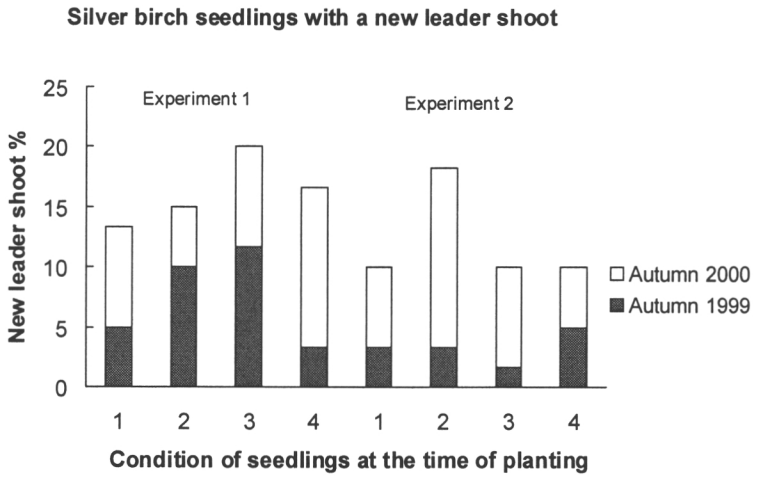


Figure 4. The percentage of silver birch seedlings, which changed leader shoot after outplanting. Seedlings grown in peat pot in nursery (Experiment 1) Seedlings grown in hardplastic containers in nursery (Experiment 2). 1= no lesion , 2 = lesion < 5 mm^2, 3 = lesion > 5 mm^2, but not covering over half of the stem diameter, and 4 = lesion spread over half of the stem diameter, but not girdling the stem.

Discussion

In this study nursery the stem lesion caused by *P. cactorum* decreased the height growth of birch seedlings. This is accordance with the previous studies with nursery seedlings (Lilja et al. 1996). After outplanting the height growth of seedlings did not decreased with the disease rating. The diffenrences in the shoot heights related to the disease rating which were present at time of outplanting had almoust diseappered in two years in the field.

Vigorous growth of healthy seedlings in hard plastic containers in nursery might explain the poor growth of control seedlings after outplanting in experiment 1. The seedlings were too tall compared with the size of container cavity. After planting the rooting process might have been less successfull because of large transpiration area.

Today the production of container seedlings to a large extent have replaced production of bareroot seedlings in Finland (Finnish Statistical... 2000). The environmental factor on which soil-borne *Phytopthoras* have a obligatory dependency is moisture (Grove et al. 1985, Erwin and Ribeiro 1996). The microclimate within birch stands in containers is not easy to keep dry and well aerated, because regular irrigation is needed. During last tree years excess rainfall in midsummer, in a phase when coverplastics have been removed from greenhouses, has been found to create favourable conditions for severe *P. cactorum* infection.

The clonality of *P. cactorum* on strawberry inside Europe shows that it easily can be spread on infected seedlings (Hantula et al. 1997, 2000). It is also commonly brought into apple (*Malus domestica* Borkh) orchards on seedlings or rootstocks

(Harris 1991). A *Phytophthora* sp. (Brasier et al. 1999) causing widespread alder (*Alnus glutinosa* (L.) Gaertn.) mortality on riparian populations (Cech and Brandstetter 1999, Gibbs et al. 1999, Streito et al. 1999) is also supposed to be spread on nursery seedlings.

The potential for *Phytophthora*s to survive and spread after outplanting depends on that how these water depedent pathogens can adapt on new environmental conditions. *Phytophthora* spp., including *P. cactorum*, causing root rot on Douglas fir (*Pseudotsuga menziesii* (Mirb.) Franco) caused mortality in forest, but surviving trees regenerated healthy roots even though *Phytophthora* persisted in old lesions (Hansen et al. 1980). In this study stem lesions did not effect on mortality of birch seedlings after outplanting. And even those lesions healed well which have spread over half of the stem diameter in the nursery. Usually under suitable conditions, high moisture and cool temperatures, new *Phytophthora* inoculums are producted continously when lesions produce sporangia and zoopsores which can be splahed by rain (Erwin and Ribeiro 1996). The summer 1999 was exceptional dry and warm in the South part of Finland. There was no need to control weeds before summer 2000. Dry growing season in 1999 and weed control in 2000 perhaps kept the relative humidity of air around the seedlings under the level optimal for the pathogen growth and new infections.

It is still too early to conclude that silver birch seedlings will recover totally from *P. cactorum* infection although the differences in shoot heights present in the nursery between disease and apparently healthy seedlings have after two growing season in the field reduced.

Acknowledgments

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Tubercularia ulmea canker: effect of pathogen, host, and cultural practice

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Abstract

Several systemic fungicides were tested as treatments for stem cankers caused by *Tubercularia ulmea* on *Ulmus pumila*, *Eleagnus angustifolia*, and *Gleditsia triacanthos*. None was able to prevent infection but benomyl most consistently reduced canker size. Further work is warranted.

Keywords: chemotherapy, *Nectria cinnabarina*, tree wound dressings

Introduction

In windbreak and other tree plantings in North Dakota, USA, cankers caused by *Tubercularia ulmea* Carter have caused considerable damage to *Ulmus pumila* L. (UP), *Elaeagnus angustifolia* L.(EA), and *Gleditsia triacanthos* L. (GT) tree plantings (Dooling 1973, Sengpiel 1977, Walla and Stack 1987, Walla and Stack 1988). In this otherwise treeless region of windswept prairies, planted trees are constantly subject to stress by wind, drought, winter cold, summer heat, and attack by defoliating insects (Stack et al. 1990).

Tubercularia ulmea was identified as causing a stem canker disease of *Ulmus pumila* by Carter in Illinois, USA (Carter 1947). We identified the teleomorph of *T. ulmea* as *Nectria cinnabarina* Tode ex Fries (Sengpiel and Stack 1985). That would make *T. ulmea* synonymous with *T. vulgaris*; however, we continue to use the anamorph name *T. ulmea* for the present because our strains of this fungus differ somewhat from typical *T. vulgaris*.

Stack et al. (1990) applied Manion's disease spiral concept to the decline of Siberian elm windbreaks on the northern Great Plains. They cited *T. ulmea* and *B. hypodermia* as contributing factors in decline of *U. pumila*. Since most facultative canker fungi require a weakened host, maintaining tree vigor is extremely important in prevention of canker diseases. Drought and freezing stress have been recognized as two of the main environmental factors in canker disease development (Schoeneweiss 1981).

Treatment of pruning wounds was suggested for many years, based on a long-accepted belief that painting wounds could prevent decay. Shigo and Shortle (1983)

disproved that assumption; however, their findings in regard to decay have been misinterpreted by some to mean that wound dressings are of no value under any circumstance. Wound dressings are still recognized as valuable for prevention of canker diseases (Bedker and Blanchette 1983, Riecken 1985).

Researchers continue to search for fungicide-containing dressings that will reduce the effects of some canker fungi. Rosenberger et al. (1983) found that neither 6.0 g/ml captafol nor 0.3 g/ml benomyl were effective in controlling twig dieback of apple caused by *N. cinnabarina*. Bedker and Blanchette (1984) found that *N. cinnabarina* cankers on GT treated with 0.1 g/ml benomyl were smaller than those not treated. Benomyl has been shown to reduce *Nectria galligena* Bres. canker on apple (*Malus pumila* Mill.) (English et al. 1979). Riecken (1985) showed that a wound treatment containing 3% thiophanate-methyl was effective in reducing *N. cinnabarina* canker development. Peterson and Helmer (1992) demonstrated that applying a wound treatment containing 20 g/l imazalil would reduce *N. cinnabarina* penetration in several species.

The studies reported here were done to determine the variability in pathogenicity of *T. ulmea* isolates in North Dakota, and to investigate the potential value of wound dressings to prevent *T. ulmea* infections in species often planted in ND.

Materials and methods

Seventeen *T. ulmea* isolates from seven host species were tested for pathogenic variability on EA and UP. Ten wound dressings were applied in field tests to determine effectiveness in preventing *T. ulmea* canker development.

Isolation and inoculum production

Dead stems and cankered bark with sporodochia were collected, cleaned with distilled water, and induced to sporulate by incubating for 24 hours over moist filter paper. Conidia were removed from sporodochia and cultured on PDA. After two to four days, colonies were transferred to new PDA plates. After six weeks, sporodochia developed on the agar surface. Isolates were transferred to fresh media approximately every 60-90 days.

Colonized wheat kernel inoculum was used throughout these tests and was prepared by placing sterilized wheat kernels onto the surface of *T. ulmea* cultures for two to five weeks. When thoroughly colonized, kernels were removed and applied to the wounds.

Pathogenicity tests

Seventeen *T. ulmea* isolates were collected. Greenhouse-grown UP and EA trees were inoculated with *T. ulmea* isolates in two experiments. Isolates confirmed as *T. ulmea* were tested for the ability to cause disease by inoculating plants in the greenhouse; each test had three replicate inoculations of each isolate on UP and EA. An

inverted V-notch wound 5 mm by 8 mm was used in greenhouse tests. A colonized wheat kernel was placed between the bark and the wood in the inverted v-notch wound. The bark flap was pressed against the kernel, wrapped in parafilm®, and covered with aluminum foil. Cankers were measured at 3 wk or 5 wk after inoculation.

Wound dressings

Wound dressings were tested in field studies to determine if they would suppress *T. ulmea* cankers in a natural environment. Several commercially available fungicides were used; these included benomyl, fenarimol, propiconazole, tebuconazole, thiabendazole, and thiophanate-methyl. Other products were a commercial tree paint ("Ortho") and alcohol-based shellac. Choice of wound dressings was based on greenhouse trials not reported here (Jackson 1997).

For tests on UP and EA, trees approximately 30 years old were used. Sixty stems 5-8 cm diam were selected at random for each host. A 27 mm disk of bark and vascular cambium was removed from each stem with a hole saw and two seeds of wheat kernel inoculum were placed in opposite sides of the wound. Two isolates were used on each host. The wounds were immediately dressed with one of eight treatments at 1% active ingredient or left untreated. Fungicides were carried in dormant oil except benomyl and thiophanate-methyl which were in water. Stems were wrapped with a 30 cm x 30 cm piece of plastic food wrap.

Non-inoculated controls consisted of wounded stems covered with plastic wrap. Three replicate inoculations for each treatment x isolate combination and control were done. Canker length was determined at four months after inoculation for EA or 6 months for UP.

For GT, methods were similar except that 20-year-old trees were used, the wound was 10-mm diam, the dressings were applied immediately at 5% active ingredient, four inoculum kernels were used per wound, and canker length was measured at two months after inoculation.

Disease evaluation and data analysis

To determine the extent of cankering, the bark on each inoculated and control stem was cut and removed above and below the inoculation point. Canker length measurements were taken above and below the wound and measurements combined. For the pathogenicity tests, the two experiments for each host were combined for analysis.

For the tests of wound dressings, similar measurements of canker length were taken. The isolate by treatment interaction was not significant in any of the experiments so the results for the two isolates for each host were combined for analysis.

Results

Pathogenicity tests

In all, 17 *T. ulmea* cultures were evaluated by inoculation to EA and UP. In two experiments, all of the *T. ulmea* isolates tested caused cankers on both hosts. A few isolates were present in only one of the two experiments. There was no relationship between the source (host) of the isolate and lesion size on either test host (data not shown). A combined analysis using data from both experiments and both hosts showed that isolates differed significantly in the size of cankers produced (Fig. 1). Canker size was taken as a measure of generalized disease causing ability = pathogenicity.

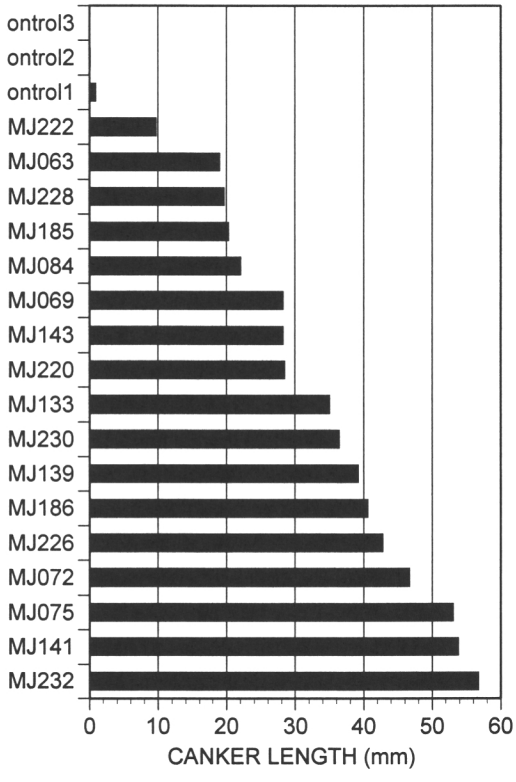


Figure 1. Pathogenicity of *T. ulmea* isolates to *U.pumila* and *E. angustifolia*. Values are means of two experiments and two hosts. Canker length determined at 3 or 5 weeks after inoculation with *T. ulmea* by placing a colonized wheat kernel into a stem wound in greenhouse grown saplings. Fisher's F-protected Least Significant Difference (FLSD) @(p=.05) = 33.0.

Wound dressings

Wound dressings were tested in field trials to determine if they could reduce or prevent cankers caused by *T. ulmea*. In the UP and EA tests, cankers developed from all wounds in the inoculated controls. Cankers also developed from many non-in-

oculated wounds as well probably because of the abundance of natural inoculum in this nursery.

The effect of the wound dressings on canker size at 6 months following inoculation with *T. ulmea* in the field test on UP is shown in Figure 2. The canker size caused by the two isolates was not different so only the combined values are shown. None of the treatments reduced canker size compared to the inoculated control but benomyl and Ortho tree paint did have cankers shorter than the non-inoculated controls.

The effect of the wound dressings on canker size at four months following inoculation of EA in the field test is shown in Figure 3. Again, the canker size caused by the two isolates of *T. ulmea* did not differ. In this test, two treatments, benomyl and thiophanate-methyl, reduced canker length compared to the inoculated control. Two additional treatments, thiabendazole and propiconazole, differed significantly from the non-inoculated control.

The effect of the wound dressings on canker size at two months following inoculation of stems on the 20 yr old GT in the field test is shown in Figure 4. The canker size caused by the two isolates of *T. ulmea* differed but there was not a significant isolate by treatment interaction so the measurements for the isolates were combined. In this test, no treatments differed from the inoculated check although the benomyl treatment showed a strong trend toward reduced canker length.

Despite variation, some of the wound dressings did reduce canker development; benomyl, in particular, tended to be effective in reduce canker length. None of the dressings, however, was really effective in preventing infection, even though they were applied at an optimum time.

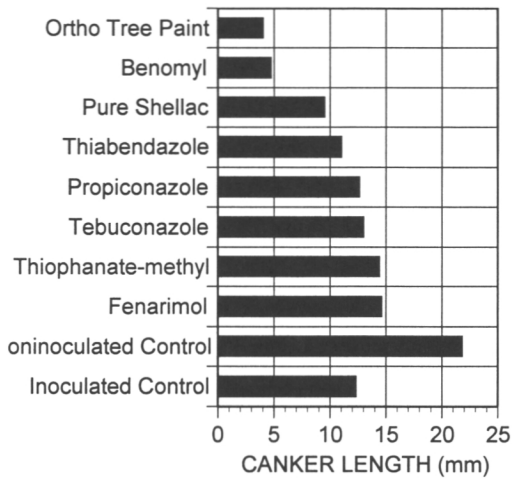


Figure 2.. Effect of wound dressings on canker size in *U. pumila* inoculated with *T. ulmea* in field tests. Canker length was determined at 6 months after inoculation with *T. ulmea* by placing colonized wheat kernels into a wound on stems of 30 year old trees. Mean of two isolates. Fisher's F-protected Least Significant Difference (FLSD) @(p=.05) = 15.2.

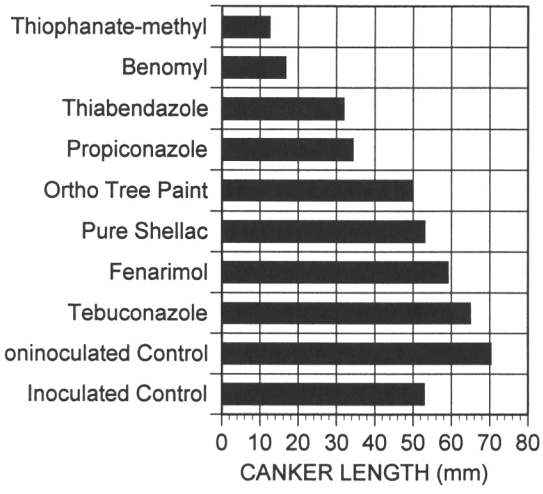


Figure 3. Effect of wound dressings on canker size in *E. angustifolia* inoculated with *T. ulmea* in field tests. Canker length was determined at 4 months after inoculation with *T. ulmea* by placing colonized wheat kernels into a wound on stems of 30 year old trees. Fisher's F-protected Least Significant Difference (FLSD) @(p=.05) = 25.2.

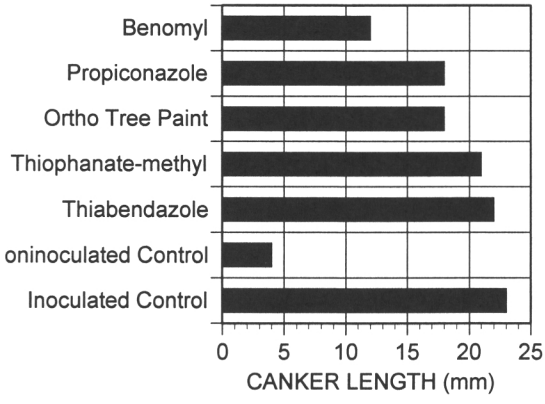


Figure 4. Effect of wound dressings on canker size in *G. triacanthos* inoculated with *T. ulmea* in field tests. Canker length was determined at 2 months after inoculation of stems of 20 year old trees with *T. ulmea* by placing a colonized wheat kernels into a stem wound. Fisher's F-protected Least Significant Difference (FLSD) @(p=.05) = 12.9.

Discussion

Pathogenicity tests

We observed during the pathogenicity tests that *T. ulmea* sporodochia occurred more often on killed stems than on live, cankered stems, suggesting that saprophytic growth of *T. ulmea* favored sporulation.

Cankers that caused wilting of tops beyond the inoculation site were much larger than cankers that did not cause wilting. A major factor appeared to be the rate at which the cankers grew in width across the stem. It appeared that some *T. ulmea* isolates caused more extensive lateral cankering even though their cankers were not as long as some other isolates which caused extensive growth lengthwise along the stem. Unfortunately, the numbers of plants were not sufficient to form any conclusion about this. Additional study might show whether isolates differ in how they form cankers.

Wound dressings

Cankers developed from many non-inoculated wounds. This nursery with its many 30 yr old trees had a long history of broken branches and tops from storms, etc. While many old cankers had been removed prior to this study, some probably remained and provided natural inoculum of several potential pathogens - including *T. ulmea*. When combined with a severe wind/rain storm that occurred in this nursery 10 days after the wound treatments were done, that natural inoculum proved more than sufficient to infect the control wounds.

Wound closure Index. On EA and UP, small but significant reduction in width of wounds (wound closure) occurred in some treatments. Those treatments that showed reduced canker development also tended to show wound closure. Since the two measures, canker length and wound closure, appear correlated, the latter was not analysed further.

On GT, an additional comparison was made of stem wounds dressed with only petroleum oil (not shown in Figure 4). These developed larger cankers than the inoculated controls, suggesting this product was toxic to freshly cut tissue. The choice of oil as a carrier in the EA and UP trials was probably unfortunate, and may have interfered with any potential activity of those fungicides, especially propiconazole, that were carried in it.

The variable and somewhat inconsistent results noted in this study of wound dressings is similar to some of the results reported by others. In our case we believe the large coefficient of variation noted in analysis of these experiments indicates a high level of experimental error. This variability may have been due to differences in the individual host plants (grown from seed collected in the wild), to changes in the *T. ulmea* isolates over time, or the environment, or more likely a combination of all these. Reducing the variation in future studies will be needed to better discriminate among treatments. This might be done by using clonal trees and improving maintainence of cultures, and substantially increasing the degree of replication within experiments.

As was seen in previous research (Bedker and Blanchette 1984, Riecken 1985, Peterson and Helmer 1992), none of the chemicals tested was curative. The reduced cankering produced by benomyl and possibly by thiophanate-methyl and propiconazole were nevertheless encouraging and warrant further work. When combined with improved cultural practices, application of benomyl could be an important component of an integrated program for the control of cankers caused by *T. ulmea*.

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Frequently isolated endophytes from the Japanese beech -Endophytic fungal composition, their temporal variations in colonization rate and distribution in Northern Japan

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Abstract

To determine the dominant fungal endophytes of the Japanese beech (*Fagus crenata*) and to monitor their isolation frequency, we isolated fungi from symptomless organs of beech including leaves, petioles, and current and old (1- to 5- year-old) twigs after surface sterilization. Of the thirteen fungal taxa obtained, three were isolated most often. An unidentified species of *Discula* and an unidentified sterile fungus, Lb, were isolated frequently from leaves, and an unidentified species of *Phomopsis* was isolated most frequently from twigs. The isolation frequency over the growing season varied for the two dominant fungal species in the leaves, *Discula* sp. and Lb. These two species had similar patterns of isolation, even in petioles and current-year twigs, although isolation frequencies of a given species varied with organs. An organ-specific distribution of the fungal species in the host plant was apparent. The three fungal species noted above were considered to be the dominant endophytes of the Japanese beech.

In addition, to confirm whether the two endophytic fungi in the beech leaves are ubiquitous at different sites, leaves of the beech were collected monthly during the vegetation period at five sites in the Tohoku district, the northeastern part of Japan. Two dominant endophytic fungi were *Discula* sp. and Lb. This result strongly suggests that these two fungi are generally associated with leaves of the Japanese beech at different sites. Spores of *Discula* sp. were released for a very short time in late May, just after the disappearance of the snow cover on the forest floor. These spores may be important for the infection of newly flushing leaves.

Keywords: *Fagus crenata*, fungal endophyte, isolation frequency

Introduction

Endophytic fungi, which induce asymptomatic infections of healthy plant tissues, have been the subject of many studies. These studies, which have focused on endophyte communities in woody plants mainly in Europe and in North America, have covered such subjects as methods of detection, taxonomy, species composition, distribution at a variety of scales, biological, ecological and physiological aspects, and interactions and mutualistic symbiosis between endophytes and host plants (e.g.,

Bills 1996, Carroll 1986, 1988, 1995, Petrini 1986, 1991, 1996). In Japan, although there have been some reports on endophytic fungi in conifers (Hata and Futai 1993, 1995, 1996, Kaneko et al. 1997), little is known about the species composition of endophytic fungi in deciduous broad-leaved trees. Moreover, limited information is available on temporal changes in the endophyte assemblages. Clarification of the fungal species composition, variations on a temporal scale of endophyte isolation, and their distribution among different sites is an important step in understanding the biology, ecology and physiology of endophytes and host-endophyte interactions. The objectives of this study were (I) to isolate fungal endophytes from various organs of the Japanese beech (*Fagus crenata* Blume), (II) to monitor their temporal variations in isolation success and (III) to confirm whether the endophytic fungal composition of the beech are ubiquitous at the different sites in the Tohoku district, Northern Japan.

Materials and methods

Isolation of fungal endophyte

Several organs of beech which included healthy and disease-symptomless leaves, current- and 1- to 5-year-old twigs, and petioles (Fig. 1) were collected twice a month in 1995 (for old twig, from may, 1995 to April, 1996) at Tazawako. Symptomless leaves were also collected monthly from five different sites (Fig. 2) in 1996. These samples were cut into small pieces, surface sterilized and incubated on potato dextrose agar (PDA) at 15°C for 3 weeks or more. The isolation frequency (IF) of a single endophyte taxon was calculated by the following formula:

$IF=N_i/N_t \times 100$

where, N_i and N_t are the number of segments from which the fungus was isolated and the total number of segments cultured, respectively.

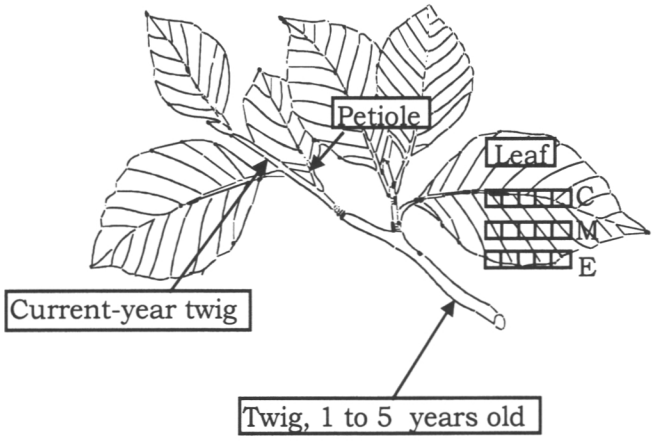


Figure 1. Organs of Japanese beech used for isolation of fungal endophyte.

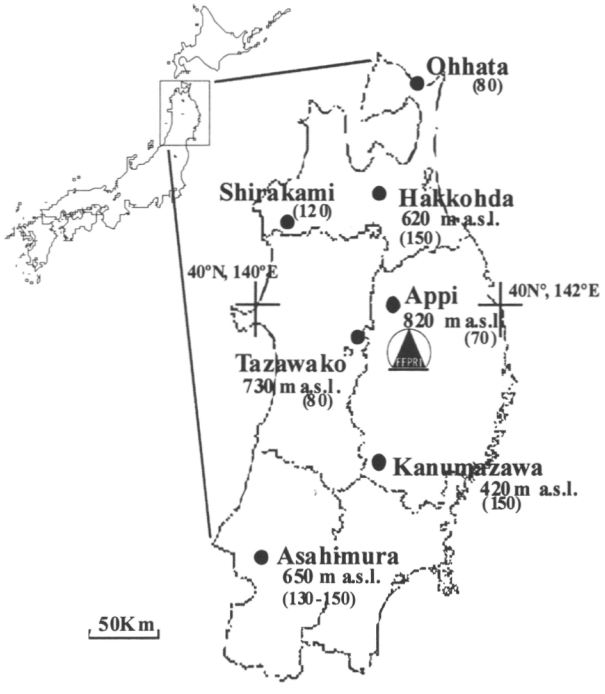


Figure 2. Map of sample collection sites for isolation of endophytic fungi from beech leaves. Black circles indicate main five collection sites. Numbers in parentheses show the stand age (years) in each experimental sites.

Detection of air-borne spore of *Discula* sp.

To confirm whether spores of *Discula* sp. are dispersed as air-borne inocula and, if dispersed, to determine the dispersal time of spores, spore trapping experiments using Petri-plates containing PDA was carried out in one of the main experimental sites, Tazawako. Ten trapping plates were exposed weekly for 1, 3, 5 and 10 min. at 1 m above ground under the canopy in the beech forest from mid-April to August. The plates were taken to the laboratory within 2 h, incubated at 15°C for 2 weeks and the number of *Discula* sp. colonies were calculated. Snow depth at the same site was also recorded under the canopy inside the forest.

Results and discussion

Endophytic fungal composition

Discula sp. and an unidentified isolate, Lb, which had *Ascochyta*-like colonies, were frequently found in the leaves, and *Phomopsis* sp. was frequently found in the twigs after surface sterilization. In early May, just after expansion of the current leaves, no fungal isolates were detected in the leaves. Also, no fungal isolates were detected in leaflets flushing from terminal buds. *Discula* sp. was detected for the first time on May 25 and continued to be isolated with a relatively high frequency until July 11

(Fig. 3A). The isolation frequency of the fungus then decreased gradually until August 7, and was 10 to 15% until the end of September. In early October, at the end of the growing season, the isolation frequency of this fungus increased slightly. On the other hand, the fungus Lb was first isolated in late June and the isolation frequency increased rapidly and kept a high incidence (over 80%) until the end of the growing season (Fig. 3B). Thus, the isolation profiles of *Discula* sp. and Lb appeared to be different. In addition to these two dominant fungal species, *Phomopsis* sp. was isolated at a low frequency (3 to 13%) from early June to the end of the experiment, although there was a small peak in late July (Fig. 3C).

From the old (1- to 5-year-old) twigs, only one fungal species, *Phomopsis* sp., was isolated frequently (Fig. 4). From early May 1995 to late April 1996, it was isolated from more than 85% of the segments.

Discula sp., which was one of the largest components of the fungal assemblage in the leaves, and *Phomopsis* sp., was isolated frequently in the newly developing current-year twigs (Fig. 5). *Phomopsis* sp. was isolated frequently throughout the growing season, but the isolation frequency of *Discula* sp. gradually decreased, as it did in the leaves (Figs. 5A, C).

In petioles, the fungi frequently isolated were *Discula* sp. and Lb, which were also isolated from the leaves, and *Phomopsis* sp., which was frequently isolated from in the twigs (Fig. 6). The patterns of variation in the isolation frequency of *Discula* sp. and Lb were similar in the petioles and the leaves, although isolation frequency of a given species slightly differed among organs (Figs. 6A, B).

The results of the present study also showed an organ specific distribution of fungal species in a given host species. These three fungal species were considered to be the dominant endophytes of the Japanese beech.

Distribution of the endophytes at different sites

Two fungal taxa, *Discula* sp. and an unidentified sterile fungus, Lb were also frequently isolated from the beech leaves in all five sites surveyed (Figs. 2, 7). These two fungi accounted for the great majority of the isolates while other fungi were infrequently or rarely isolated as reported for conifers by Carroll and Carroll (1978). At most sites, *Discula* sp. first appeared in May, had a peak isolation frequency in June and then gradually decreased. On the contrary, the fungus Lb was first isolated in late June to July and the isolation frequency increased until the end of the vegetation period at all sites. The results on major endophyte composition and on temporal pattern of isolation frequency were consistent with the results at Tazawako in 1995. These results strongly indicate that infections of beech leaves by these two fungi are ubiquitous in the Tohoku district and these two fungi are generally associated with leaves of the Japanese beech at different sites.

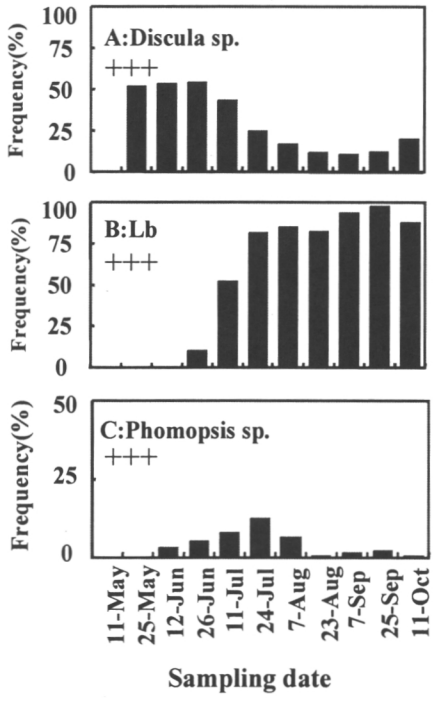


Figure 3. Isolation frequency of endophytic fungi over the growing season from healthy beech leaves. (A) and (B) isolation frequency of two frequent endophyte, *Discula* sp. and sterile Lb. (C) less frequent endophyte *Phomopsis* sp.
+++, significant difference at 0.001 level with sampling date.

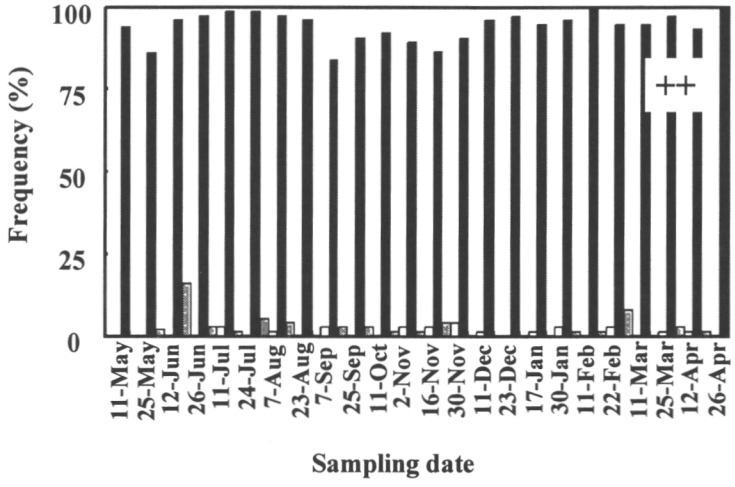


Figure 4. Isolation frequency of the endophytic fungi *Phomopsis* sp. (solid bar), *Discula* sp. (shaded bar) and a sterile Lb (open bar), over one year from healthy twigs.
++, significant difference at 0.01 level with sampling date for *Phomopsis* sp.

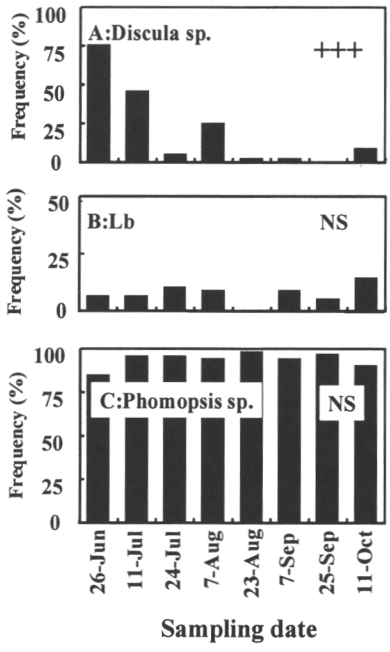


Figure 5. Isolation frequency of endophytic fungi *Discula* sp. (A), sterile Lb. (B) and *Phomopsis* sp. (C), over the growing season from healthy current-year twigs. +++ and NS, significant difference at 0.001 level and no significant difference with sampling date, respectively.

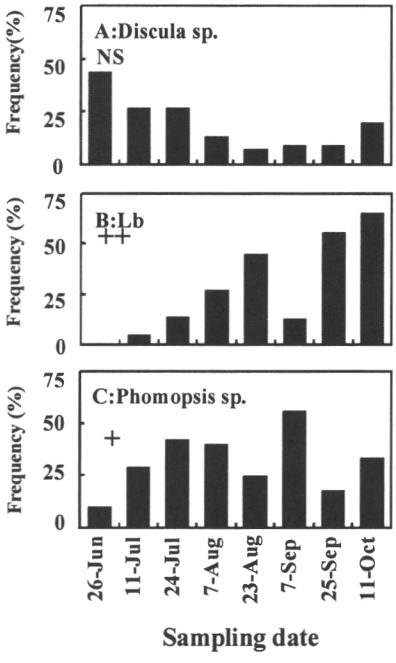


Figure 6. Isolation frequency of endophytic fungi *Discula* sp. (A), sterile Lb. (B) and *Phomopsis* sp. (C), over the growing season from petioles. ++, + and NS, significant difference at 0.01 and 0.05 levels and no significant difference with sampling date, respectively.

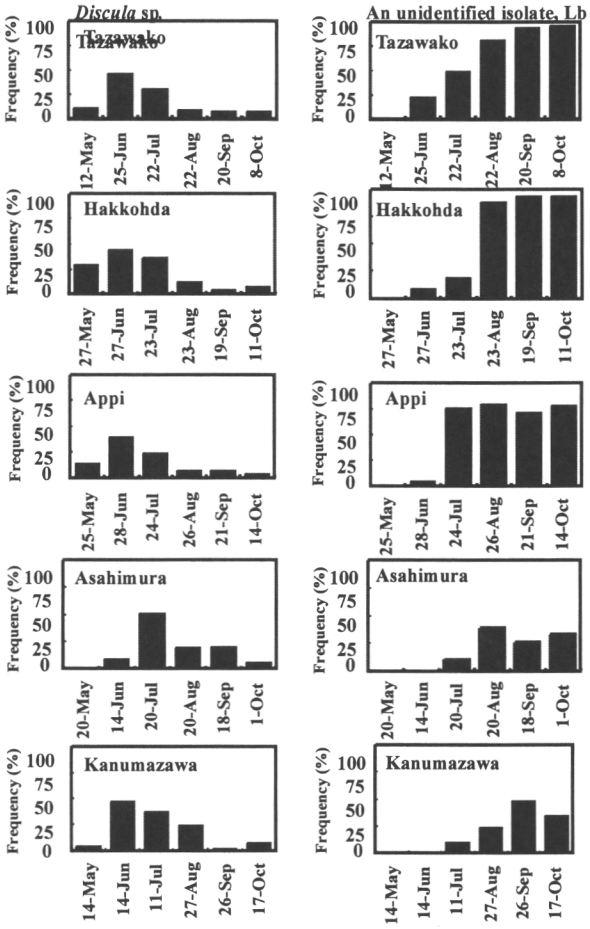


Figure 7. Isolation frequencies (percentage of total 150 segments) of the two main endophyte, *Discula* sp. and the sterile Lb., in beech leaves collected at different experimental sites from May to October.

Spore dispersal of *Discula* sp.

No spore dispersal of *Discula* sp. was detected in the presence of a snow cover. Dispersal of spores was detected for a very short time in late May with the peak of spore dispersal occurring just after the disappearance of snow covering the forest floor (Fig. 8). This suggests the hypothesis that *Discula* sp. overwinters on fallen leaves under the snow and sporulates just after snow melt. It is most likely that dispersed spores play an important role in infecting beech leaves, as suggested by Johnson and Whitney (1992) and Toti et al. (1993) for endophytes of black spruce and European beech, respectively, because current leaves of beech were fully expanded at this time. The observation that no infected young rolled leaflets within winter buds were observed also supports this idea.

Further studies should be made to clarify the ecology and host-endophyte interaction for a better understanding of the role of endophytes in forest ecosystem.

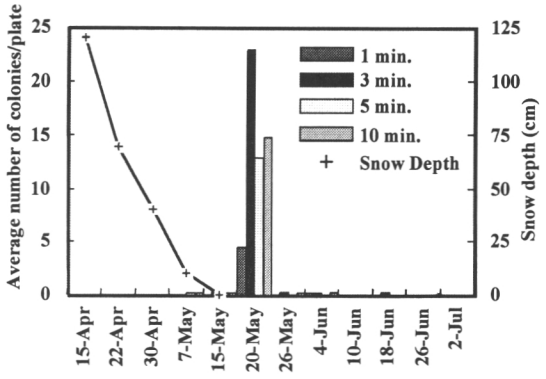


Figure 8. Detection of air-borne spores of *Discula* sp. at Tazawako beech forest.

Acknowledgements

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Effect of increased carbon dioxide and ozone on leaf spot pathogens of birch

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Abstract

We studied how increased carbon dioxide and ozone affect birch – leaf spot pathogens – interaction. 9-year-old ozone sensitive (clone 80) and ozone tolerant (clone 4) silver birch (*Betula pendula*) clones were fumigated with elevated (2 x ambient) concentrations of CO_2, ozone and combined CO_2+O_3 in open-top chambers. In Experiment 1 we monitored the effect of treatments on the development of spots in naturally infected leaves during August-September in 2000 by photographing and image analyzing the leaf spots. In Experiment 2 we studied indirect effects of the gases on disease development by exposing clone plantlets to fumigations for 5 weeks, after which we inoculated the plantlets with *Marssonina betulae* –leaf spot fungus and incubated them in a growth chamber. In Exp. 1 both ozone and CO_2 increased the diseased leaf area (DLA) of clone 4. In clone 4 the increasing effect of fumigations was more than twice higher than in clone 80, though ozone also increased the DLA of the latter. The disease caused by *M. betulae* increased with increasing leaf age. Ozone enhanced this leaf age effect. In Exp. 2 all fumigation treatments increased the DLA of clone 4 in young and fully expanded leaves, and ozone and CO_2+O_3 increased it in old leaves. In clone 80 the DLA decreased in all young and fully expanded fumigated leaves, but in old leaves ozone increased the DLA even more than in clone 4. In most cases CO_2 fumigated together with ozone decreased the effect of ozone.

Keywords: ozone, carbon dioxide, *Betula pendula*, *Marssonina betulae*, leaf disease

Introduction

The effect of ozone and CO_2 on pathogens varies between fungus and host species. The effect may be direct or indirect through altered leaf physiology and structure. Ozone has induced, decreased or had no effect on the germination of fungus spores, sporulation and on the growth of mycelium depending on fungus species (Hibben and Stotzky 1969, Treshow et al. 1969, Beare et al. 1999). On mycelium ozone has caused abnormal characters (Hibben and Stotzky 1969). It has also increased the number of germtubes produced (Beare et al. 1999). Ozone may raise host resistance to pathogens through activation of phenylpropanoid pathways and lignin formation (Booker and Miller 1998), but on the other hand plant injuries caused by ozone may favoure some necrotrophs (Manning and Tiedemann 1995). Ozone also accelerates leaf senescence which may have different kind of effects on biotrophs and necrotrophs. Majority of the studied both necrotrophic and biotrophic fungi have gained benefit

from increased CO_2 (Manning and Tiedemann 1995) through changes in leaf nutrient and water content. There are, however, only a few studies about the effects of ozone and CO_2 on the diseases of deciduous trees. Very little is known especially about the effects of combined CO_2+O_3 fumigations on diseases. The aim of this work was to study how increased carbon dioxide, ozone and combined gases affect birch – leaf spot pathogens – interaction.

Materials and methods

Plant materials used in tests were twenty 9-year-old (year 2000) ozone sensitive (clone 80) and twenty ozone tolerant (clone 4) silver birches (*Betula pendula*) growing at an experimental site at the Suonenjoki Research Station, and 160 micropropagated clonal plantlets.

Fungus material which was used in an inoculation experiment (Exp. 2) was *Marssonina betulae* (Lib.) Sacc. –leaf spot fungus (pure culture provided by Prof. Timo Kurkela; the origin of the isolate was Suonenjoki). Naturally occurring leaf spot fungi in the leaves of experimental trees were utilized for the screening of disease development in trees (Exp.1).

Experimental trees were fumigated with elevated (2 x ambient) concentrations of CO_2, ozone and combined CO_2+O_3 in open-top chambers at the experimental site during May-September 1999 and 2000. There were two kinds of controls: an 'outside control' without a chamber around the tree and a 'chamber control' with a tree enclosed in the chamber but without any gas treatment. Each of the five treatments had four replicate trees of both clones.

Experiment 1 - Monitoring

We monitored the effect of the gas treatments on the development of leaf spots in naturally infected leaves during August-September in 2000. Five leaves with spots in each experimental tree were chosen (20 leaves/treatment), and the test leaves which were left attached to the trees, were photographed with a digital camera three times (7.8., 24.8. and 5.9.2000). The development of spots (amount of spots, % of leaf area) was analysed from the photographs by using a ColAn™-image analyses computer program.

Experiment 2 – Inoculation experiment

To study the indirect effect of the gases on the disease development we exposed 160 clone plantlets to the five different treatments at the experimental site during June-July 2000. Totally 144 plantlets were placed in the chambers (two plantlets of clone 4 and two plantlets of clone 80 in each chamber) and as outdoor controls were left 16 plantlets (eight plantlets of clone 4 and eight plantlets of clone 80). After 5 weeks we moved the plantlets into a greenhouse and inoculated them with *Marssonina betulae*. Of the 160 exposed plantlets 140 plantlets were used in the inoculations. Three healthy

leaves in each plantlet were sprayed with *M. betulae* spore suspension (120 000 spores/ml; about 0.8 ml for each leaf) on the abaxial side of a leaf, and one leaf was sprayed with water only to control the effect of inoculation treatment and the amount of natural infection. After spraying the leaves were enclosed in a plastic bag for 5 days to keep the moisture content high for ensuring the infection. Inoculated plantlets were then incubated for 4 weeks in a growth chamber. At the end of the experiment, the test leaves were detached and photographed with a digital camera. The amount of spots (% of leaf area) was analysed by using a ColAn- program.

Results

Experiment 1

In the monitorings some leaves fell down before last monitoring. Those leaves were usually the most spotted ones. The amount of diseased leaf area (DLA) of fallen leaves is not included in the DLA of monitoring 3. The higher is the number of fallen leaves the higher should also the DLA be, however.

In clone 4 the DLA increased most in ozone and CO_2 fumigated leaves (Table 1). The number of fallen leaves was also high in both treatments (7 and 8, respectively). Also several CO_2+O_3 fumigated leaves fell down before last monitoring. The DLA, when the small number of fallen leaves is also taken into account, was the smallest in both controls.

In clone 80 the highest amount of disease was in ozone fumigated leaves, when fallen leaves are also included. Next highest number of fallen leaves and DLA

Table 1. Diseased leaf area, %, in the naturally infected leaves of CO_2, ozone and CO_2+O_3 fumigated clone 4 and clone 80 silver birch trees in three different monitorings (n=20/ fumigation treatment). Column 'fallen leaves' indicates the number of before monitoring 3 fallen leaves, which are not included in the diseased leaf area of monitoring 3.

		Diseased leaf area, %				
	Fumigation treatments	Chamber control	CO2	O3	CO2+O3	Outside control
Clone 4	Monitoring 1	0.76	1.2	1.07	0.85	0.26
	stdev	*0.93*	*1.16*	*1.14*	*0.95*	*0.21*
	Monitoring 2	3.09	5.53	4.62	4	1.15
	stdev	*5.19*	*5.9*	*4.85*	*5.36*	*1.91*
	Monitoring 3	5.01	6.15	7.31	4.86	2.99
	stdev	*5.87*	*9.49*	*4.79*	*7.47*	*4.33*
	Fallen leaves	3	8	7	8	2
Clone 80	Monitoring 1	0.53	0.69	0.41	0.53	0.27
	stdev	*0.91*	*1.54*	*0.52*	*0.63*	*0.2*
	Monitoring 2	2.3	1.5	1.48	1.61	0.86
	stdev	*2.91*	*2.03*	*1.5*	*2.3*	*0.69*
	Monitoring 3	3.01	2.95	2.97	4.01	2.86
	stdev	*2.59*	*4.72*	*2.97*	*5.1*	*1.75*
	Fallen leaves	5	3	7	0	4

was in chamber control leaves. The amount of disease was smaller and quite similar in CO_2+O_3, CO_2 and outside control leaves.

Experiment 2

In the control leaves of the inoculation experiment the amount of disease was small (about 0.1% of leaf area) in all other treatments except outside control, where the amount of disease in the control leaves was as high as in *Marssonina* -inoculated leaves.

The severity of disease increased with an increasing leaf age in each treatment and in both clones. The results were therefore interpreted in three different classes of leaf ages: Age1 – young, expanding leaves, leaves numbers 1 – 4 from the top of the plantlet; Age2 – adult, fully expanded leaves, numbers 5 – 7 from the top of the plantlet; and Age3 – old leaves, numbers 8 – 11.

In clone 4, in Age1 leaves the highest DLA (1.4%) was in CO_2 treated leaves (Fig. 1). In the other treatments the DLA was 0.1 – 0.6%. In Age2 leaves the amount of disease was the highest (1.2 %) in ozone treated leaves and the DLA was slightly higher than in Age1 leaves in all other treatments except in CO_2 treated and in outside control leaves. In Age3 leaves the DLA was twice as high than in younger leaves in outside control, and 4 – 9 times higher than in Age2 leaves in the other treatments. In ozone treated leaves the amount of disease was highest, 4.8% and in CO_2+O_3 treated leaves next highest, 4.4%, when in chamber control it was 3.0%.

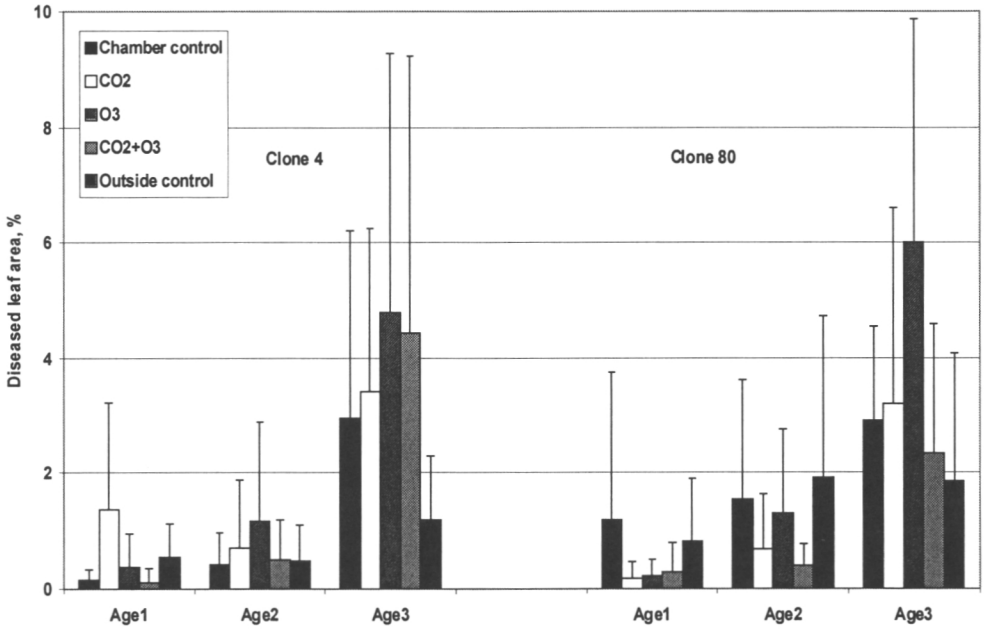


Figure 1. Diseased leaf area (DLA), %, caused by *Marssonina betulae,* in the different leaf age classes of silver birch clonal plantlets 4 and 80 exposed for 5 weeks with CO_2, ozone or CO_2+O_3 before the inoculation. Bars indicate STDEV.

In clone 80, in Age1 and Age2 leaves the DLA was highest in chamber control and outside control leaves (0.8 – 1.9%). In Age3 leaves the DLA was twice as high in ozone treated leaves (6.0%) than in chamber control (2.9%). In CO_2 treated leaves the amount of disease was nearly the same than in chamber control, and in combined gases treatment and outside control slightly lower.

Discussion

In the monitorings the amount of disease was in both clones almost the same in chamber control, but in outside control it was slightly lower in clone 4. In the inoculation experiment the DLA caused by *M. betulae* was smaller in clone 4 than in clone 80 in both controls in young and fully expanded leaves, and in outside control in the old leaves. In the earlier studies made with the same birch clones and *Pyrenopeziza betulicola* Fuckel –leaf spot fungus, but without any gas treatments, clone 4 has been more resistant to *P. betulicola* than clone 80 (unpublished data).

According to the results of monitorings clone 4 was more affected by the fumigation treatments than clone 80. The DLA and/or the number of fallen leaves in the fumigation treatments was more than twice higher in clone 4 than in the other clone. In the inoculation experiment all fumigation treatments increased the DLA in clone 4 in the young and fully expanded leaves, and ozone and combined gases treatment increased it in the old leaves. In clone 80 the DLA was lower in all young and fully expanded fumigated leaves than in chamber control leaves, but in old leaves the ozone treatment increased the DLA in clone 80 even more than in clone 4.

In the fumigated plantlets ozone increased the DLA caused by *M. betulae* in clone 4 in fully expanded and old leaves, and in clone 80 in old leaves. Also the amount of natural infection and the development of leaf spots was increased in ozone fumigations in both clones. When the clone plantlets were fumigated with ozone combined to CO_2, the DLA was lower than in ozone alone fumigated leaves in the fully expanded and old leaves of clone 80, and in the young and fully expanded leaves of clone 4. In the monitorings the DLA was lower in both clones when ozone and CO_2 were fumigated together than alone. Thus CO_2 seemed to reduce the disease increasing effect of ozone.

The results of Exp. 1 and Exp. 2 were quite similar. In the monitorings the direct and indirect effects of the fumigations were not possible to separate. The effects were similar, however, to the indirect effect in the inoculation experiment. The increasing effect of leaf age on leaf spot disease caused by *M. betulae* was clear. Leaf age effect varies between host and fungus species. In *Populus* spp. fully expanded leaves, which were at the state of sink-source transition, were most susceptible to biotrophic fungi (Coleman 1986). Young leaves may be more resistant to fungi, because they contain more some harmful chemical compounds than older leaves, like phenols (Coleman 1986, Cline and Neely 1984). On the other hand, young leaves may be more susceptible to fungi, because the leaf structure of soft expanding leaves is not yet as resistant as that of the mature leaves (Spiers and Hopcroft 1984). In our results ozone fumigation increased leaf age effect. Pre-fumigation of *Populus trichocarpa* x *Populus balsamifera* with ozone has also increased significantly the

size of lesions caused by *Marssonina tremulae* on old leaves, but has decreased it significantly on young leaves (Beare et al. 1999). The strong leaf age effect was apparent also in the absence of O_3, with larger lesions occurring on older leaves. This effect was enhanced by the ozone.

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Occurrence, host range and impact of leaf pathogen fungi on forest trees in Hungary

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Abstract

The results of a recent inventory of leaf and shoot diseases of forest trees in Hungary are presented. During the last few years field investigations were executed in the main forest regions of the country, leaves with symptoms were collected, the pathogens identified and occasionally isolated in pure culture. All species of forest trees and shrubs presenting leaf symptoms were surveyed. About 150 species of leaf pathogenic fungi were identified on more than one hundred tree and shrub species. Occurrence of several leaf pathogens has been recorded for the first time in Hungary: *Asteromella tiliae, Cristulariella depraedans, Dothistroma septospora, Drepanopeziza sphaeroides, Glomerella miyabeana, Hadrotrichum dryophylum, Meria laricis, Microsphaera vanbruntiana, Oidium carpini, Phaeocryptopus gauemannii, Phloeospora associata, Phyllosticta concentrica, Phyllosticta globulosa, Phyllosticta hypoglossi, Phyllosticta minima, Septogloeum carthusianum, Thedgonia ligustrina, Tubakia dryina, Uromyces laburni. A. tataricum* and *A. saccharinum* are first reported as hosts of *Cristulariella depraedans* and *Quercus pubescens* as host of *Tubakia dryina* in Europe.

Keywords: leaf pathogen fungi, forest trees

Introduction

The rate of forestation in Hungary amounts to about 19%. The majority of the forests lies under 350 m altitude in 9 main forest regions in continental, subatlantic and submediterranean climate conditions. As species composition the hardwoods are dominating (85.5%), represented primarily by oaks, black locust, beech and hybrid poplars. Among the conifers mainly Scotch and Austrian pines have a considerable forest surface.

The leaf diseases of hardwoods caused by fungi do not occur generally in mass of economic importance in stands, although epidemics of certain species as *Microsphaera alphitoides* Griffon et Maubl. on oaks, *Melampsora* rusts and *Marssonina* leaf disease of hybrid poplars are to be observed regularly. On conifers *Sphaeropsis* blight and *Dothistroma* red band disease are the economically important fungal leaf diseases. Although a few species cause sensible damages for the forestry, the whole spectrum of the leaf fungi of forest trees is of interest in phytopathological aspect.

Material and method

Selected forest compartments representing all forest types and tree species were surveyed annually for occurrence of leaf disease symptoms in each mean forest region of the country. The frequency of the symptoms was estimated and pathological material collected. The field investigations were carried out at the start and middle to late growing season to reach both the early and the late symptoms of foliage diseases.

The collected material was examined for identification of causal pathogens. The identification was done on fresh material in case of presence of fungal fructifications. For stimulation of the sporulation wet chamber method was used for a few days after surface sterilisation of the plant material with 2 g/l active chlorine containing NaOCl solution. In case of lack of fructifications the pathogens were isolated from symptomatic tissues on PDA and identified on the cultural characters and occasionally sporulation in the pure culture.

Results and discussions

The identified pathogens, caused symptoms and importance of the diseases are discussed in order of main wood species for the forestry in Hungary.

Deciduous trees

Oaks are the most important forest trees in Hungary as occupied territory and growing stock as well. The main species are the sessile (*Quercus robur*), pedunculate (*Q. petraea*) and Turkey (*Q. cerris*) oaks covering together about 33% of the forest surface. The downy oak (*Q. pubescens*) and the introduced red oak (*Q. rubra*) have a low rate of 1-2%. The powdery mildew caused by *Microsphaera alphitoides* is the most frequent foliage disease of oaks. It is ubiquitous especially on sessile oak, not rare on pedunculate and downy oaks. *Mycrosphaera hypophylla* Nevodovskij also was found rarely on *Q. robur*. *Apiognomonia quercina* (Kleb.) Höhn. (anamorphe *Discula quercina* /Westend./Arx) was found on sessile, pedunculate and downy oaks. As endophyte species its symptoms are not common in forest stands. Sometimes occur together with powdery mildew on sessile oak or frequent in association with foliar galls on other oaks. *Septoria quercicola* (Desm.) Sacc. was epidemical on pedunculate oak in 1999 in some regions of SW Hungary. It causes small, 1-3 mm, round brown foliar spots. *Microstroma album* (Desm.) Sacc. was found on pedunculate and Turkey oaks. Its characteristic white colonies appear on the lower surface of the leaves. On the upper surface confluent yellow, later necrotic points can be observed. The disease was epidemical in some places but without considerable effect on the healthy status of the trees. *Tubakia dryina* (Sacc.) Sutton caused symptoms on pedunculate, downy and Turkey oaks. On *Q. petraea* the round, 5-15 mm sized, brown spots appeared around the suck points of *Phylloxera* aphids. On *Q. pubescens* the disease was epidemical in some places. and the round, concentric zoned brown spots extended finally on large leaf surfaces. The pycnidia were observed in concen

tric circles epi- and hypophyllous. On Turkey oak small, 2-4 mm, round brown spots could be observed without fungal fructifications even after wet chamber incubation. The causal agent was identified after the isolation of the fungus, based on the cultural characters. In culture the fungus produces abundantly pycnidia with characteristic microconidia. *T. dryina* is associated with leaf spots of different oak species in Europe and Northern America. In Europe it is known primarily on *Q. petraea* (Butin 1996), than on *Q. rubra* and *Q. cerris* (Belisario 1993). It is frequently isolated as an endophyte of *Q. petraea* and *Q. robur* (Halmschlager pers. Com.). Its occurrence on downy oak has not been reported yet, so this is the first report of this pathogen on *Q. pubescens*. Further identified foliar pathogens *Hadrotrychum dryophylum* Sacc. on pedunculate, *Phloeospora associata* Bubák on downy, and *Phyllosticta globulosa* Thüm. on sessile oak proved to be rare, each were found at one occasion only. These three species as well as *Tubakia dryina* have been first recorded in Hungary (Szabó 2000).

Beech (*Fagus sylvatica*) has an amount of 6.3% in the forest surface. The anthracnose disease caused by *Apiognomonia errabunda* (Rob.) Höhn. (anamorphe *Discula umbrinella* /Berk et Br./Sutton) is not rare in Hungary. In some years it is epidemical in stands. In the nurseries it is common also. Frequent is associated with galls (*Mikiola fagi*) or other insect damages (*Rhynchaenus fagi*). Other foliage fungi of beech appeared rarely, for example the powdery mildew *Phyllactinia guttata* (Wallr ex Fr.) Lév. late summer, without considerable importance.

Black locust (*Robinia pseudoacacia*), is one of the most important forest trees, having a rate of nearly 21%. *Phloeospora robiniae* (Lib.)Höhn. is the most frequent foliage pathogen of black locust (Szabó 1993). Generally spread, it causes brown, necrotic spots and early defoliation, especially of the upper crown. *Microsphaera pseudacaciae* (Marczenko) U. Braun occurs mostly on young plants in nurseries and on sprouts, without importance in stands. The sporadically found *Ectostroma robiniae* (uncertain taxon) causes small, 1-3 mm, round black stromata without fungal fructifications. The isolation essay also was unsuccessfully.

Hornbeam (*Carpinus betulus*) is wide-spread admixed forest tree species. Its rate amounts nearly 6%. *Asteroma carpini* (Lib.)Sutton is the most common foliar fungus of hornbeam. Its symptoms appear on senescent leaves late summer. The other, less frequent observed leaf pathogen is *Monostichella robergei* (Desm.)Höhn. This one is more pathogen, causes extended, grey-light brown leaf necrosis. Its occurrence is not general, but local appears epidemically, frequent in association with foliar damages caused by drought. *Oidium carpini* Foitzik, the recently described powdery mildew of hornbeam (U. Braun 1995) was found in 1999 for the first time in Hungary.

The rate of ash species (*Fraxinus excelsior, F. ornus, F. pennsilvanica*) is about 2%. The appearance of their leaf diseases was not frequent in the years of investigations. The followings are worthy of mention: powdery mildew *Phyllactinia fraxini* (DC.)Homma, than *Phomopsis pterophila* (Nits.)Died. and *Phoma macrostoma* Mont. on *F. excelsior*, *Phyllosticta orni* Bubák on *F. ornus*. The *Phloeospora* state of *Mycosphaerella fraxini* Niessl. was found for the first time in Hungary on *F. ornus*.

Maples (*Acer campestre, A. platanoides, A. pseudoplatanus, A. tataricum, A. negundo*) occur in all type of forest having a rate of about 1% of the forest surface.

The white leaf spot of maples caused by *Cristulariella depraedans* (Cooke)Höhn. was found first in Hungary in 1992 on *Acer saccharinum* (Szabó 1994), then in 1998-99 on *A. campestre, A. pseudoplatanus* and *A. tataricum*. The pathogen is known in Europe preponderantly on *A. pseudoplatanus* (Butin 1981), but has also been reported on *A. platanoides* and mentioned on *A. campestre* among the 21 different woody and herbaceous hosts (Lang 2000). Its occurrence on *A. tataricum* and *A. saccharinum* in Europe is first reported in this paper. Tar spot disease caused by *Rhytisma acerinum* (Pers.)Fr. is generally spread, especially on *A. pseudoplatanus* but it is not rare also on *A. campestre* and *A. platanoides*. The powdery mildews of maples, *Sawadaea tulasney* (Fuck.)Homma and *S. bicornis* (Wallr. ex Fr.)Homma were found frequent on maples. The first is frequent on *A. campestre, A. negundo, A. platanoides, A. pseudoplatanus* and *A. tataricum*, the second on *A. campestre, A. platanoides* and *A. pseudoplatanus*. *Phyllosticta minima* (Berk et Curt.)Underw. et Earle is a foliar pathogen recorded newly in Hungary (Szabó 1997a, 1997b). It is a true *Phyllosticta* with typical conidia bearing gelatinous appendage and spermatia of *Leptodothiorella* type (Van der Aa 1973). In Germany was found rarely on *A. pseudoplatanus* (Wulf 1994). In Hungary occurs on *A. pseudoplatanus, A. campestre* and *A. negundo*. *Phyllosticta aceris* (Lib.)Sacc. and *P. negundinis* Sacc. et Speg. were found on *Acer campestre* and *A. negundo* respecivelly. *Mycosphaerella latebrosa* (Cooke)Schroet. (anamorphe *Phloeospora aceris* /Lib./Sacc) causes small, point shaped leaf spots, premature yellowing and falling of the leaves. It was found on *A. campestre* and *A. pseudoplatanus*. *Diplodina acerina* (Pass.)Sutton was found on *A. pseudoplatanus* and *A. tataricum*, *Didymosporina aceris* (Lib.)Höhn. was epidemical on *A. campestre* in 1995, *Discula campestris* (Pass.)Arx occurs sporadically on *A. campestre*.

The elm species (*Ulmus glabra, U. laevis, U. minor, U. procera, U. pumila* var. *arborea*) have a low rate of less than 1%. *Mycosphaerella ulmi* Kleb. (anamorphe *Phloeospora ulmi* /Fr. ex Kunze/Wallr.) is generally spread on all elm species. The pink mass of conidia spread from hypophyllous acervuli is characteristic. *Platychora ulmi* (Schleich. et Duval)Petr. is wide-spread biotrophic pathogen on *U. minor* and *U. pumila* var. *arborea*. Its black stromata are remarkable on the upper surface of the leaves.

Hybrid poplars, especially *Populus* x *euramericana* are important cultivated trees in Hungary, their rate amounts to 6,6%. The autochthonous poplar species (*Populus alba, P.* x *canescens, P. tremula, P. nigra,*) cover nearly 3% of the forest area.On hybrid poplars *Melampsora larici-populina* Kleb. is wide spread. *M. allii-populina* Kleb. was found less frequent in stands, sporadically on *P. nigra* in flood sites. On white poplars *M. pinitorqua* Rostr. and some other *Melampsora* species occur, but the confirmation of their identification carried out on the uredospore morpholpgy only is necessary. The most frequent occurring *Drepanopeziza* species on hybrid poplars is *D. punctiformis* Gremmen (anamorphe *Marssonina brunnea* / Ellis et Everh./Magn.). *D. populi-albae* (Kleb.)Nannf. (anamorphe *Marssonina castagnei* /Desm. et Mont./Magn.) is also not rare on white and grey poplars in lowland forests. *D. populorum* (Desm.)Höhn. (anamorphe *M. populi* /Lib./Magn.) was found only sporadically on *P. nigra*. *Venturia macularis* (Fr.) E.Müller et Arx

(anamorphe *Pollaccia radiosa* /Lib./Bald. et Cif.) is frequent on white and grey poplars as on aspen causing not only leaf disease, but also top and bark necrosis of shoots. Its impact is of considerable importance in nurseries. *Mycosphaerella populi* (Auersw.)Schröt. (anamorphe *Septoria populi* Desm.) causes small 2-3 mm sized, dark limited spots. It is frequent especially on *Populus nigra* 'Italica'. *Asteroma frondicola* (Fr. ex Ficinus et Schubert) Morelet is frequent in flood plain causing extended, roundish, grey leaf spots with concentrically arranged, epiphyllous acervuli on *P. alba* and *P. x canescens*.

More then ten *Salix* species occur naturally in Hungary. The investigations covered mostly the tree shaped willows, *Salix. alba, S. fragilis* (about 1% of the forest surface) and *S. caprea*. *Melampsora* rusts occur frequently on willows (*M. allii-salicisalbae* Kleb., *M. caprearum* Thüm., *M. euonymi-caprearum* Kleb. *M. galanthi-fragilis* Kleb. etc.) Their identification on uredospores morphology only needs confirmation. *Drepanopeziza salicis* (Tul. et C.Tul.)Höhn (anamorphe *Monostichella salicis* /Westend./Arx) is frequent, especially on *S. fragilis*, causing round dark brown, confluent epiphyllous spots. *Drepanopeziza sphaeroides* (Pers.)Höhn (anamorphe *Marssonin salicicola* /Bres./Magn.) occurs on weeping willow (*S. alba* var. *vitellina pendula*). It causes spotting and premature yellowing of leaves, top necrosis and bark crusting of shoots. *Glomerella miyabeana* (Fuckel)Arx (anamorphe *Colletotrichum gloeosporioides* Penz.) was found on aspen and white willow. These two latest pathogens were identified for the first time in Hungary in 1991 (Szabó 1992). *Venturia* species (*V. chlorospora*/Ces./Aderhold) were not found frequent either in willow stands or in riparian associations. *Phyllosticta salicicola* Thüm. causes sporadically brown spots on *S. alba* cultivars.

Common alder (*Alnus glutinosa*) is cultivated on ca 2% of the forest surface. It occurs also in riparian associations. Leaf blister caused by *Taphrina tosquinetii* (Wesend.)Tul. is not rare on young trees and on sprouts. *T. sadebeckii* Johanson was found on riparian alder of different age. Further leaf pathogens as *Gnomoniella tubiformis* (Tode)Sacc. (anamorphe *Asteroma alneum* /Pers. ex Fr./Sutton) and *Discula* sp. were found sporadically.

Limes (*Tilia cordata, T. platyphyllos, T. tomentosa*) amount less than 1% of the forest surface. *Cercospora microsora* Sacc. is a frequent leaf fungus of *T. cordata* causing small, 1-2 mm, dark spots, premature yellowing and falling of the leaves. It occurs also on *T. tomentosa* but more rarely and the symptoms are less intensive. *Apiognomonia tiliae* (Rehm)Höhn (anamorphe *Discula* sp.) appears early after budding, causing leaf- and shoot anthracnose. It is frequent on *T. cordata*, sporadic on *T. tomentosa*. At the end of the growing season the conspicuous symptoms of leaf blotch caused by *Asteromella tiliae* (Rud.)Butin et Kehr appear frequently, especially on *T. platyphyllos*, but also on the two other linden. Formerly the symptom was connected to *Asteroma tiliae* Rud.. The pathogen was recently correct described and its connection to *Didymosphaeria petrakiana* Sacc. demonstrated (Butin et Kehr 1995). Its occurrence in Hungary has been recently recorded (Szabó 1997b).

On silver birch (*Betula verrucosa*) the powdery mildew *Phyllactinia guttata* (Wallr. ex Fr.)Lév. is frequent late summer, autumn as loose, hypophyllous texture. *Discula betulina* (Westend.)Arx causes brown spots and premature leaf fall. The grey, round spots of *Venturia ditricha* (Fr.)Karsten (anamorphe *Fusicladium betulae*

Aderhold) appear late summer. Birch rust (*Melampsoridium betulinum* /Pers./Kleb.) is epidemical in some years, as it was in 1999. *Kabatiella apocrypta* (Ellis et Everh.)Arx proved to be a general spread ubiquitous leaf pathogen. It was found in leaf necrosis on many different forest trees (*Acer, Alnus, Betula, Cerasus, Fagus, Populus, Salix* etc.).

The shrub layer of the deciduous forests is rich in species. More then 50 leaf pathogen fungi were identified on about 30 shrub species. Only a few fungi from among the first recorded ones in Hungary are enumerated there: *Microsphaera vanbruntiana* Gerard on *Sambucus racemosa*, *Phyllosticta hypoglossi* (Mont.)Allesch. on *Ruscus aculeatus, Septogloeum carthusianum* Sacc. on *Euonymus europaeus, Thedgonia ligustrina* (Boerema)Sutton on *Ligustrum vulgare*, *Uromyces laburni* (DC.)Fuckel on *Laburnum anagyroides*.

Conifers

Pinus sylvestris and *P. nigra* are planted on poor sites and in lowland forestations. Their rate amounts to about 13%. Several pathogens cause important leaf- and shoot diseases of these tree species. In nurseries and young forestations *Lophodermium seditiosum* Minter, Staley et Millar causes needle cast of seedlings and young trees. *L. pinastri* (Schrad.)Chev. is also present as week pathogen of aged needles. *Sphaeropsis sapinea* (Fr.)Dyco et Sutton causes shoot blight especially on *P. nigra*, but it is present also on *P. sylvestris*. This disease appeared in mass during 1980s causes serious damages especially in association with other factors as drought, poor nutrition and some week pathogens as *Cenangium ferruginosum* Fr.. *Mycosphaerella pini* E.Rostrup (anamorphe *Dothistroma septospora* /Dorog./Morelet)) was first identified in 1991 (Szabó 1997c). Late 1990s the epidemic and damages caused by this fungus have been generalized in young forests of *P. nigra* (Koltay 2001). Other identified needle pathogens of pines are *Sclerophoma pithyophila* (Corda)Höhn, *Lophodermella conjuncta* Darker, *Coleosporium* species and some week pathogens in old needles as *Cyclaneusma niveum* (Pers.)DiCosmo, Peredo et Minter and *Cytospora pinastri* Fr..

The leaf pathogen of larch (*Larix decidua*) is *Mycosphaerella laricina* (Hartig)Neger. It causes intensive leaf fall of young trees some years. In the nurseries *Meria laricis* Vuill. has been identified first time in Hungary (Szabó 1999).

The spruce (*Picea abies*) needles are sporadically attacked by *Lirula macrospora* (Hartig)Darker. The shoots and young needles of spruce and larch sometimes are infected by *Botrytis cinerea* Pers. and *Sclerophoma pythiophila*.

Douglas fir (*Pseudotsuga menziesii*) is affected by *Phaeocryptopus gauemannii* (Rhode)Petrak and *Rhabdocline pseudotsugae* Sydow. *P. gauemannii* was newly recorded in Hungary. In the last years it seems to become generalized in Western Hungary.

On the needles of different conifers in arboretums and Christmas tree plantations some further pathogens were identified as *Chrysomyxa abietis* (Wallr.)Unger and *Rhizosphaera kalkhoffii* Bubák on blue spruce, *Lirula nervisequia* (DC)Darker and *Cytospora friesii* Sacc. on fir. The old needles of *Taxus baccata* were infected by

Phyllosticta concentrica Sacc.. This fungus has been reported recently in Hungary (Szabó 1997a).

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Flammulina velutipes (curt). Fr. Sing. population structure

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Abstract

The first three stages of life cycle of wood-eating Basidiomycetes: germination of basidiospores, haploid homokaryotic mycelium and fertile mycelium, in which the nuclei are associated as specialized heterokaryon – dikaryon are very important in the plan of research of infection biology of fungi, protective mechanisms of plant and of the increases it resistance to diseases. The study of population structure of separate species fungi enables to understand mechanisms of its development in wood. With this purpose we conduct a research of the genetic status of fruit bodies of fungus *Flammulina velutipes*, which developed on same plant - host.

The distribution of the factors of compatibility at the fruit bodies of *F. velutipes* assumes initial development of one dikaryon and availability of numerous spore infection. The comparison of the genetic status of fruit bodies assumes distribution of infection with spores, development homokaryotic mycelium and limited distribution of clones.
Rather high factor of outbreeding is marked.

The researches of joint cultivation dikaryons, monokaryons, both di- and monokaryons in pure culture have allowed to trace features of vegetative compatibility at this species.

Bacterial canker of *Maackia amurensis* var. *buergeri* – Occurrence and pathological anatomy

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Abstract

A new disease of *Maackia amurensis* var. *buergeri* was recently found on the northern island of Hokkaido, Japan. Affected trees were heavily damaged and had cankers on both trunks and branches. After natural infection, a series of swellings on the bark surface developed longitudinally. These swellings burst and coalesced to become long cankers with exposed woods. The causal pathogen was isolated and characterized as *Pseudomonas syringae* on the basis of laboratory tests. Pathogenicity of the bacterium was confirmed by inoculation into the host. Hence, we propose that the disease be designated "bacterial canker of *M. amurensis*". Anatomical observations to elucidate the process of the canker formation were carried out. First, abnormalities appeared near the cambial zone. Xylem formation was suppressed, hyperplasia of irregular ray parenchyma cells occurred, then the swellings appeared. The irregular cells increased in number; after that, the surface of the swollen bark burst open. The ruptured swellings coalesced to form long irregular cankers.

Keywords: Bacterial canker, *Maackia amurensis* var. *buergeri*, *Pseudomonas syringae*, anatomy, canker formation

Introduction

Maackia amurensis Rupr. et Maxim var. *buergeri* Schn. (hereafter referred to as *M. amurensis*) is a leguminous deciduous tree. In Japan, it is mainly found on the islands of Hokkaido, Honshu and Shikoku. The wood of *M. amurensis* exhibits a beautiful combination of yellowish white sapwood and dark brown heartwood and is very durable. Recently, a major outbreak of a new canker disease of *M. amurensis* was reported in two plantations at Kawayu in Teshikaga city (eastern Hokkaido) and Higashiyama in Furano city (central Hokkaido). Some trees were also affected in natural forests in Kimobetsu city (southern Hokkaido), Chitose and Sapporo city (central Hokkaido), in the garden of "Yotei seinen no mori" Forest Park in Makkari city (south Hokkaido) and in roadside trees in Sapporo and Tokoro (eastern Hokkaido).

The bacterium was isolated from the affected tissues and its pathogenicity to *M. amurensis* was confirmed. The bacterium was characterized as *Pseudomonas syringae* by its bacteriological characteristics.

This report deals with the description of the symptoms, the isolation of the pathogen and its characterization and pathogenicity tests, then proposes the name of this disease as "bacterial canker of *M. amurensis*" caused by *P. syringae* (Sakamoto et al. 2000). There is also an anatomical study of the disease development (Sakamoto 1999).

Symptoms

Symptoms were observed regularly from 1993 to 1997 in the Higashiyama stand. Cankers were observed on trunks and branches alike. On trunks, they grew longitudinally (Fig. 1). The initial symptom developed from late spring to early summer as a series of longitudinal swellings (approximately 3 × 1.5 cm) on the surface of the bark, which were interspersed with slightly swollen bark (hereafter referred to as SSB). These swellings gradually burst open and coalesced to form longitudinal cracks (Figs. 2a, b). These long cankers on the trunks sometimes spread horizontally and girdled the trunks. The tissues of the affected inner bark were observed to crack and break off easily. Finally, brownish black, dead inner bark tissues tore and fell off, exposing the xylem tissues (Fig. 3).



Figure 1. Serious canker of *M. amurensis* (photographed in Higashiyama).

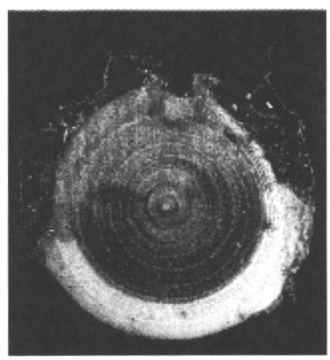


Figure 3. Transverse view of the canker with exposed wood.



Figure 2. Development of natural symptoms in Higashiyama stand. Arrows indicate the same site.
a. Photographed at 10 August 1993.
b. Photographed at 6 July 1995.

Material and methods

Isolation of the pathogen

Small pieces of inner bark tissue (approximately 5×5×5 mm) were excised from swollen, water-soaked areas in the swellings and from the margins of cankers. Each piece was macerated in 5 ml of sterile peptone water (1%). The resulting suspensions were streaked on plates of nutrient agar (NA: Eiken E-MC01) and incubated at 20°C. After 2-3 days, single colonies were re-streaked on fresh NA plates to ensure purity.

Characterization of the pathogen

The pathogen was characterized based on their colony morphology, cell morphology and bacteriological characteristics (Sakamoto et al. 2000).

Pathogenicity

Pathogenicity tests were performed on 7-year-old seedlings of *M. amurensis* in 1994. Holes were bored into the xylem of the trunks of seedlings with a cork borer (5 mm in diameter), then filled with a mass of bacterial cells from NA plates and sealed with vinyl tape. The vinyl tape was removed 2-3 weeks after inoculation.

Anatomy

Eighteen affected and five trees were selected. Samples of the SSB, the swellings, the margins of cankers and the healthy tissues (approximately 1.5×1.5×1.5 cm, including phloem and xylem) were fixed with FAA (formalin: acetic acid: 50% ethanol = 5:5:90). After washing under running water for 3-4 hours, they were dehydrated in ethanol series and embedded in 10% celloidin (Toyokuni Chemical Co.). Transverse, tangential and radial sections of 20-25μm in thickness were cut on a sliding microtome. They were stained with safranin O (1% solution in 50% ethanol) and fast green (0.1% solution in 95% ethanol), and were then examined under a light microscope.

Results

Isolation of the pathogen

The same bacterium was regularly isolated from all the affected freshly tissues. The colonies were 1-2 mm in diameter on NA after 3 days at 20°C, with an entire margin, and were circular, convex, smooth and glistening.

Characterization of the pathogen

The current isolates were Gram-negative, non-sporing, straight rods, motile with one to three flagella, produced a white to cream glistening growth, metabolized glucose oxidatively, did not grow at 40°C, nor did they accumulate poly-ß-hydroxy butyrate granules. Other results are shown in Table 1.

Pathogenicity

The inoculated inner bark tissues began to proliferate vigorously in the vicinity of the inoculation points after approximately two weeks. The bark became cracked and the symptoms continued to proliferate until the end of September 1994. At the end of April 1995, the symptoms started to develop again. Several swelling (Fig. 4a) and noticeable cankers (Fig. 4b) were observed.

Table 1. Characteristics of the current isolates and reference isolates.

Characteristics	Isolated strains [a)]	*P. syringae* pv. *syringae* [a)]	*P. syringae* pv. *myricae* [a)]	*P. syringae* pv. *tremae* [a)]	*P. syringae* pv. *actinidiae* [b)]
Fluorescent pigment on King's B medium	-	+	-	-	-
Hydrolysis of Aescrin	-	+	-	-	-
Starch	-	-	-	-	-
Tween 80	+	+	+	+	+
Levan production	V	+	+	-	+
Activity of Tyrosinase	-	-	+	+	-
Urease	-	+	-	-	-
Lecithinase	-	-	-	-	-
Reaction in purple milk	-*)	KD	-*)	-*)	Kw
Gluconate oxidation	-	-	-	-	-
H2S production	-	-	-	-	-
Indole test	-	-	-	-	-
Liquefaction of Pectin	-	-	-	-	·
Gelatin	-	+	-	-	-
Reducing substances from sucrose	+	+	+	+	+
V.P.-M.R. test	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-
Utilization of Glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
D-Galactose	+	+	+	+	+
Fructose	+	+	+	+	+
D-Ribose	+	+	+	+	+
D-Arabinose	-	-	-	-	-
Lactose	-	-	-	-	-
Maltose	-	-	-	-	-
L-Arginine	+	+	+	+	+
Gluconate	+	+	+	+	+
Citrate	+	+	+	+	+
L-Malate	+	+	+	+	+
L-Tyrosine	-	-	-	-	+
Formate	-	-	-	-	-
Propionate	-	-	-	-	-
L-Valine	-	-	-	-	-

a = Author's data, b = Data from Takikawa et al. (1989).
+ = positive, - = negative, K = alkaline reaction, D = digestion, w= weak reaction
* = No visible change was observed, V= Variable results among the isolates.

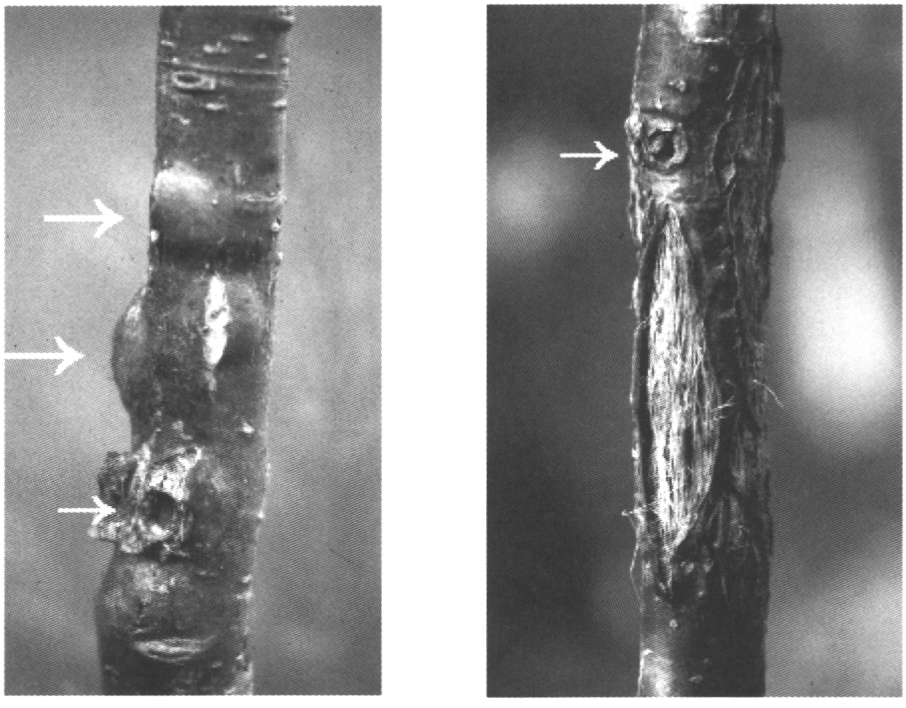


Figure 4. Symptoms of bacterial canker of *M. amurensis* on inoculated seedling.
a. Swellings (big arrows) which developed after artificial inoculation (date of inoculation: 23 June 1994; date of photography: 22 June 1995). Small arrow indicates the inoculation site.
b. Canker which formed after artificial inoculation (date of inoculation: 15 July 1994; date of photography: 31 August 1995). Arrow indicates the inoculation site.

Anatomy

In the tissues of the SSB far from the swelling, the cambial zone was clearly observed. The most remarkable difference between healthy tissues and SSB was a suppressed lignification of the xylem tissues in inner areas of the cambial zone. In these areas, vessel formation was also suppressed (Figs. 5, 6).

In the SSB near swelling, the cambial zone became fuzzy or disappeared (Fig. 7).

In the swellings, hyperplasia of irregular-shaped parenchyma cells was commonly observed. Masses of bacteria were observed in intercellular spaces of the irregular ray cells. The irregular-shaped parenchyma cells increased in number with the development of the disease until the cambial zone had completely disappeared. In the phloem tissues, the bacteria degraded the parenchyma cells, and then the bacterial lesions (the areas where the mixture of degraded parenchyma cells and bacteria was observed) became more and more numerous (Fig. 8). These areas correspond to the stained spots in the macroscopic transverse views of the swellings (Fig. 9). The irregular-shaped parenchyma cells increased in number with degradation, the bacterial lesions got bigger and bigger, then the phloem tissues got thicker and thicker.

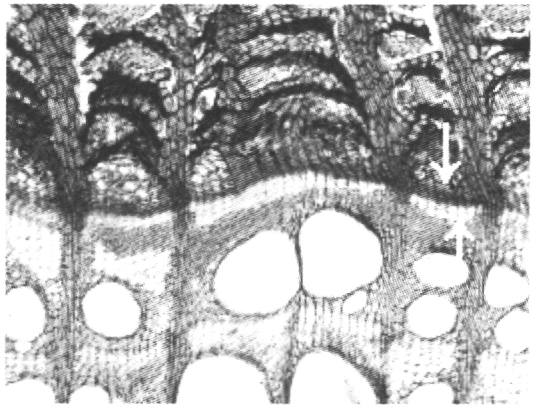


Figure 5. Transverse view of the healthy tissue. Arrows indicate the cambial zone.

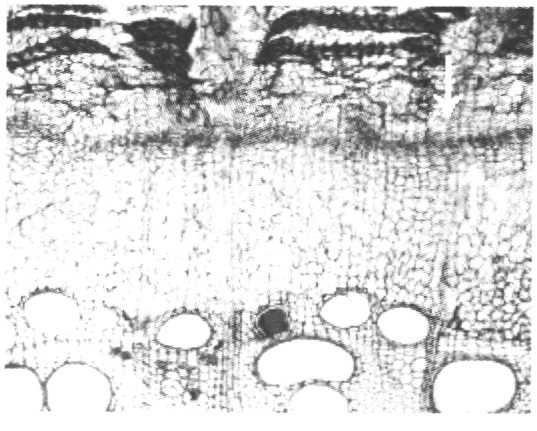


Figure 6. Transverse view of the SSB far from the swelling. Arrows indicate the cambial zone.

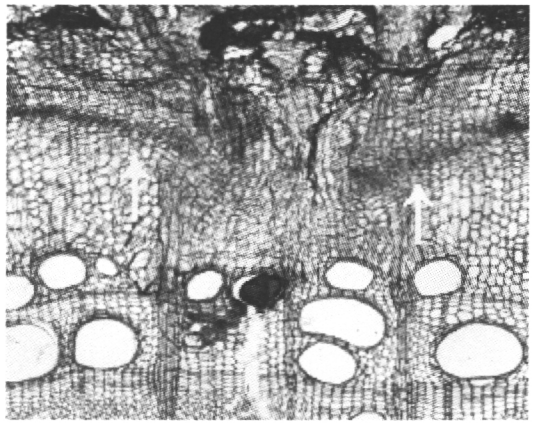


Figure 7. Transverse view of the SSB near the swelling. Arrows indicate the cambial zone.

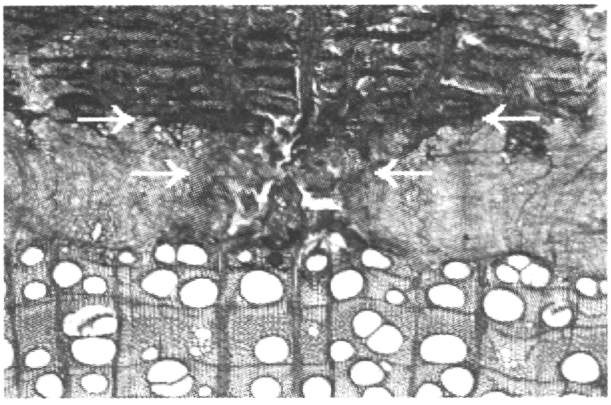


Figure 8. Transverse view of the swelling. Arrows indicate the bacterial lesions.

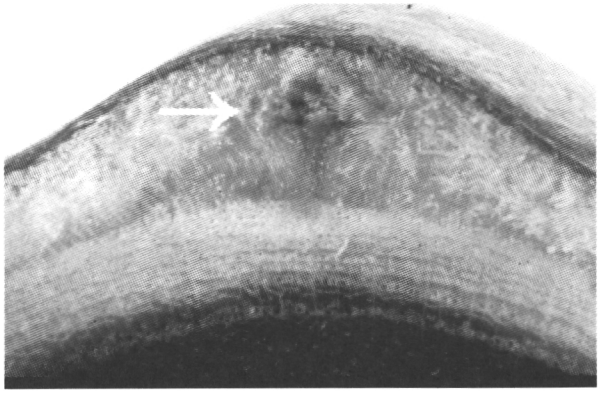


Figure 9. Transverse view of the swelling. Arrow indicates the stained spot.

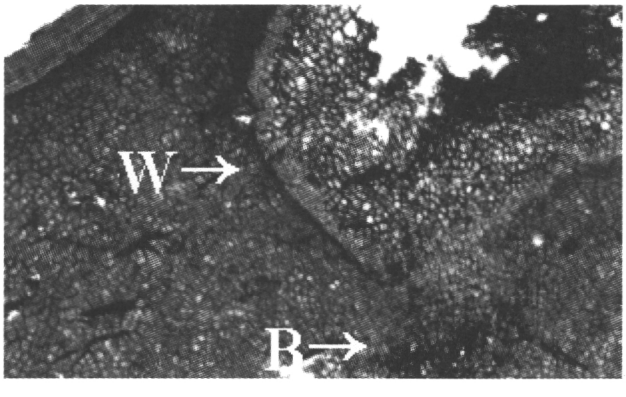


Figure 10. Transverse view of the ruptured swellings. W indicates the wound periderm and B indicates bacterial lesion.

In the tissues of ruptured swellings, wound periderms were formed to protect the living cells in the cortex and phloem. However, bacterial lesions expanded to the cortex and phloem with the development of the disease, and coalesced with the wound periderm (Fig. 10). The coalesced tissues were observed to crack and break off easily.

Discussion

Based on the bacteriological characteristics mentioned above, the current isolates should be included in the genus *Pseudomonas* (Palleroni 1984). Other characteristics of the current isolates were very similar to the characteristics of *P. syringae* pathovars that were pathogenic to woody plants, such as *P. syringae* pv. *syringae* (Tables 1, Young 1991), *P. s.* pv. *myricae* (Tables 1, Ogimi and Higuchi 1981), *P. s.* pv. *dendropanacis* (Ogimi et al. 1988a), *P. s.* pv. *tremae* (Tables 1, Ogimi et al. 1988b), *P. s.* pv. *actinidiae* (Tables 1, Takikawa et al. 1989) and *P. s.* pv. *daphniphylli* (Ogimi et al. 1990). Therefore, the isolates were classified as a member of *P. syringae* (Sakamoto et al. 2000). Bacterial canker of *M. amurensis* has not been previously reported in the world. Hence, the authors proposes the common name "bacterial canker of *M. amurensis*" caused by *P. syringae* (Sakamoto et al. 2000).

The results of the current research clarified the anatomical characteristics of the development of the disease as follows. The first anatomical abnormalities of the disease appeared near the cambial zone as the suppression of normal xylem formation. Then, the hyperplasia began to form irregular-shaped parenchyma cells, and caused several swellings and SSB on the bark. The irregular cells increased in number with degradation by the bacteria, and the surface of swollen bark burst. The cracks of the swellings coalesced to form long irregular cankers.

This study revealed the pathogens and mechanisms of disease development of the canker disease. It presents many important suggestions for pathological research of other bacterial cankers and galls.

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Watermark disease of willows in Japan – Occurrence and pathological anatomy

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Abstract

The watermark disease of willows (*Salix* spp.) was identified in Japan for the first time, in the northern island of Hokkaido. During spring and summer, the leaves and branches suddenly wilted, and the affected trees sometimes died. When affected branches or trunks were cut, a distinct, watery zone stained reddish brown or brownish black (watermark) was observed in the sapwood. The pathogen was isolated from the watermark and identified as *Erwinia salicis*. Inoculation tests confirmed its pathogenicity to willows. The characteristics of the watermark were investigated. In the watermark, some of the ray parenchyma cells caused necrosis. Dye-injection tests revealed that water conduction did not take place in the watermark. Therefore, the non-conductive watermark in sapwood may be analogous to discolored wood, and its formation and expansion in the affected sapwood may be the reason for the wilting symptoms. Soft X-ray photography and cryo-scanning electron microscopy revealed that the watermark had a high level of moisture (wetwood). Osmotic potentials ($\phi\pi$) of the watermark were substantially lower than those in healthy sapwood. ^{13}C-NMR spectroscopy revealed that *E. salicis* produced levan in the watermark, which is suspected to be a causal agent of low $\phi\pi$. Vessel-ray parenchyma and interfibre pit membranes were often damaged and absent. They could serve as effective pathways for the accumulation of water in the watermark.

Keywords: Watermark disease, *Erwinia salicis*, willows (*Salix* spp.), discolored wood, bacterial wetwood

Introduction

Watermark disease is one of the most serious diseases on willows (*Salix* spp.) and has been reported in the U.K (Day 1924, Dowson 1937), the Netherlands (Lindeyer 1931) and Belgium (Rijckaert et al. 1984). The causal bacterium was first named as *Bacterium salicis* by Day (1924), but the name was later changed to *Erwinia salicis* (Day 1924) Chester 1939 (Chester 1939).

In August 1993, a serious shoot blight, wilt and dieback was found on willows in natural forests around the mountainous area of Mount Taisetsu in central Hokkaido.

Currently, this disease is observed on three local *Salix* species: *S. bakko* Kimura, *S. sachalinensis* Fr. Schm. and *S. kinuyanagi* Kimura in the natural forests of the mountainous area of Mt. Taisetsu (Kamikawa, Kamisihoro, Rubeshibe and Oketo) and in the natural forests of the upper part of the Nissho-Pass (Hidaka).

The development of the wilting symptoms in the leaves and shoots of this disease suggests a progressive inhibition in its ability to conduct water.

The watermark (affected wood tissues) had an abnormally high level of moisture compared to the surrounding regions (Sakamoto and Sano 2000). This kind of wood tissue is called wetwood (Panshin and de Zeeuw 1980). Several kinds of bacteria in the xylem tissues are suspected to be associated with wetwood in many cases (e.g., Hartley et al. 1961, Ward and Pong 1980, Ward and Zeikus 1980), and the term "bacterial wetwood" has been applied to such kinds of wetwoods (Murdoch and Campana 1983).

This report deals with symptoms of the disease, isolation and characterization of the pathogen and establishment of its pathogenicity (Sakamoto et al. 1999). Wood anatomy, water conductivity and physiological and chemical properties of the watermark are also studied (Sakamoto and Sano 2000, Sakamoto and Kato, submitted).

Symptoms

Symptoms were observed regularly from 1993 to 1998 in the mountainous area near Mt. Taisetsu. During spring and summer, the leaves on some branches suddenly wilted and turned reddish brown, but remained on the trees for a time. On some trees almost all the branches were affected. Some branches and trunks died and then became leafless. Sometimes recovery shoots developed on the affected branches but they often became affected later. Seriously affected trees died within 4 to 5 years (Fig. 1). The internal symptom is called a 'watermark'. It was found in the sapwood of affected branches and trunks as a distinct, watery zone stained reddish brown or



Figure 1. Dieback of *S. sachalinensis* (photographed in Kamikawa).

brownish black. It forms a circumferential stain (Fig. 2), sometimes covering almost the entire transverse section in seriously affected trees. Bacteria tended to ooze from watermark on cutting. On exposure to air, the watermark quickly turned dark brown or black and a dark brownish-black liquid (mixture of bacterial ooze and metabolic substances in watermark) was exuded.

Material and methods

Isolation of the pathogen

Small pieces of the watermark (approximately 5×5×5 mm) were macerated in 5 ml of sterile peptone water (1%). The resulting suspensions were streaked on plates of nutrient agar (NA: Eiken E-MC01), and incubated at 20°C. After 4-6 days, single colonies were re-streaked on fresh NA plates to ensure purity.

Identification of the pathogen

The pathogen was identified based on their colony morphology, cell morphology and bacteriological characteristics (Sakamoto et al. 1999).

Pathogenicity

Pathogenicity tests were performed on 2-year-old seedlings from trees of *S. sachalinensis* and *S. kinuyanagi* in 1998. One-year-old branches were pricked to the xylem with a needle through a drop of bacterial suspension (approximately 10^9 cfu/mL).

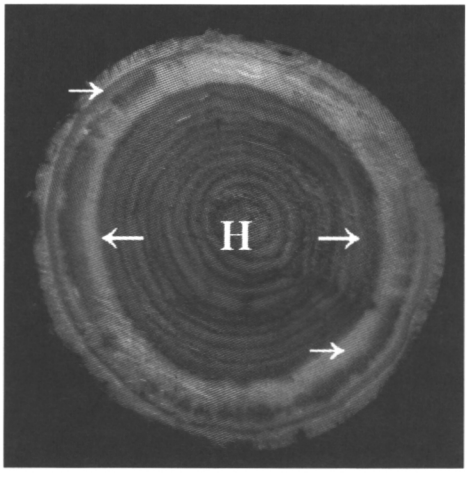


Figure 2. Circumferential watermark stain (arrows) in *S. bakko* (collected in Kamikawa). H indicates heartwood.

Light microscopy

Six affected and three healthy trees were selected. Samples of the watermark and the healthy sapwood (approximately 1.5×1.5×1.5cm) were fixed with FAA (formalin: acetic acid: 50% ethanol = 5: 5: 90) for one week, and then washed under running water for 3-4 hours. Twenty to twenty five μm thick transverse, tangential and radial sections were cut on a sliding microtome. They were stained with safranin O (1% solution in 50% ethanol) and fast green (0.1% solution in 95% ethanol), and were then examined under a light microscope. Some unstained sections were also observed.

Dye-injection test

Three affected and one healthy tree (for control) were selected. Funnel-shaped plastic collars were attached to the trunks, and then a safranin O solution (0.2% aqueous solution) was poured in the collars. Four to six holes (8.5 mm in diameter, average depth of 59.1 mm) were bored with an electric drill under the surface of the solution in the collars. About four hours later, the trees were cut down and divided into 1 m in long sections. The distribution of the dye was examined to detect the zone of xylem, which remained conductive.

Soft X-ray photography

Cylindrical specimens were excised from the trunks of one affected and one healthy tree. The preparation of samples for soft X-ray photography was made according to the methods developed by Sano et al. (1995). Soft X-ray has low penetrability; it cannot penetrate the zone which has a high level of moisture in the green wood disk. Hence, the zones with high level of moisture shown as dark (black) zone in the photograph.

Cryo-scanning electron microscopy

Cylindrical specimens were excised from the trunks of one affected and one healthy tree, which were used for soft X-ray photography. The collection of samples was made according to the method developed by Utsumi et al. (1996), and preparations of samples were made according to the procedures described by Sano et al. (1995). The samples were kept frozen and were never melted during the course of this study. The transverse surfaces of the samples were examined under the cryo-SEM (JSM 840A equipped with CRU-40) to observe the distribution of ice (water in natural condition) in the tissues.

Scanning electron microscopy

Four affected and two healthy trees were selected, then wood blocks (approximately

5×5×5 mm) were taken from the watermark and healthy sapwood. They were freeze-dried according to the methods described by Sano and Fukuzawa (1994). After drying, the blocks were split radially or tangentially with razor blades, and two halves of each specimens were coated with gold using an ion sputter (FC-1100; JEOL), and observed with a SEM (JSM-5600LV; JEOL) at 10 or 15kV.

Osmotic potential

Four affected and two healthy trees, which were selected for scanning electron microscopy, were used. Osmotic potentials ($\phi\pi$) of the liquid exuded from the watermark and the small disks (approximately 5 mm in diameter, 2 mm in thickness) of unaffected sapwood (from healthy trees) were measured by a dew-point microvoltmeter (HR-33T; WESCOR INC., USA) equipped with a sample chamber (C-52-SF; WESCOR INC., USA).

Polysaccharide in watermark

1) Isolation of extracellular polysaccharide (EPS) from bacterial culture
The isolate of *E. salicis* was incubated on the high-sucrose medium developed by Dye (1968) at 25°C for 2 days. The crude EPS slime on the plate medium was collected and purified.

2) Isolation of polysaccharide from watermark
Four affected and two healthy trees, which were selected for scanning electron microscopy, were used. The wood samples (10g in fresh weight) were excised from the watermark and healthy sapwood. The water extracts of milled samples were collected, purified and measured their dried weight.

3) ^{13}C-NMR spectroscopy
Samples of the EPS fraction from bacterial culture, the polysaccharide fraction from watermark and the healthy sapwood were dissolved in deuterium oxide and subjected to ^{13}C-NMR spectroscopy using ALPHA-500 (JEOL) operated at 125 MHz. Commercial levan (ß-2,6-D-fructofuranan) (WAKO Pure Chemical Industries, Ltd.) was served as the reference standard.

Results

Isolation of the pathogen

The same bacterium was regularly isolated from all the freshly affected tissues. The colonies were 1-2 mm in diameter on NA after 4 days at 20°C, with an entire margin, and were circular, convex, smooth and glistening.

Identification of the pathogen

The current isolates were Gram-negative, nonsporing, straight rods, motile with peritrichous flagella, produced a transparent to white growth, metabolized glucose fermentatively, and showed negative reactions in the potato soft rot, oxidase activity, and nitrate reduction tests. Other results are shown in Table 1.

Pathogenicity

The reactions were observed approximately 2-3 weeks after inoculation. Initially a few leaves became light brown and curled, but about 3-4 days later, other leaves also turned brown. Then whole branches wilted, the affected leaves fell off, and the branches died within a month. The watermark stain was observed in transverse sections of affected branches.

Table 1. Some characteristics of the current isolates and *Erwinia salicis*.

	Current isolates[a]	*E. salicis*[b]
Characteristics		
O/F test	F	F
Potato soft rot test	-	-
Oxidase activity	-	-
Catalase activity	+	+
Nitrate reduction	-	-
Reaction in purple milk	-	-
Growth factor requirements	-	-
Growth at 36°C	-	-
Levan (Mucoid growth)	+	+
H2S from Cystein	+	+
Casein hydrolysis	-	-
Aesculin hydrolysis	+	+
Gelatin liquefaction	-	-
Acetoin	+	+
Methyl Red test	-	-
Indole test	-	-
Acid production from		
Glucose	+	+
Fructose	+	+
Lactose	-	-
Maltose	-	-
Utilization of		
Acetate	+	+
Fumarate	+	+
Benzoate	-	-
Oxalate	-	-

a = The author's data, b = Data from Dye (1968).
+ = 80-100% isolates positive, - = 0-20% isolates positive.

Light microscopy

In the unstained tissues of the watermark, contents of the affected ray parenchyma cells were yellow to brown, and some cells lost their contents.

In the stained tissues of the watermark, not all the vessels were clogged with tyloses and masses of bacteria, and some of the ray parenchyma cells caused plasmolysis and necrosis (Fig. 3). Masses of bacteria formed in the vessels irrespective of the presence of tyloses (Fig. 4). Tyloses were also found in the tissues without the watermark, but the masses of bacteria were only found in the watermark. The ray parenchyma cells adjoining the vessels clogged with bacteria were affected first. Then, the parenchyma cells in the watermark gradually died as the disease progressed. Sometimes, the bacteria were seen in dead ray parenchyma cells in contact with the vessels clogged with the bacteria. In the healthy tissues, no tyloses, no masses of bacteria in vessels and no necrosis of ray parenchyma cells were observed.

Dye-injection test

In the healthy tree, all the sapwood had conductivity (but only late wood in old sapwood). However, no conductivity was observed in the watermark. In seriously affected trees (the entire transverse section was almost completely covered by the watermark), only part of sapwood in the outer layers was conductive.

Soft X-ray photography

When comparing the normal black and white photograph and the soft X-ray photograph of the affected green wood disks, the watermark was observed to be darker than the surrounding sapwood on the soft X-ray photograph taken of the green (frozen) wood (Figs. 5a, b). This indicated that there was a high accumulation of moisture. There were no black coloured areas (a high level of moisture) in the soft X-ray photograph of the dried disk from the affected wood.

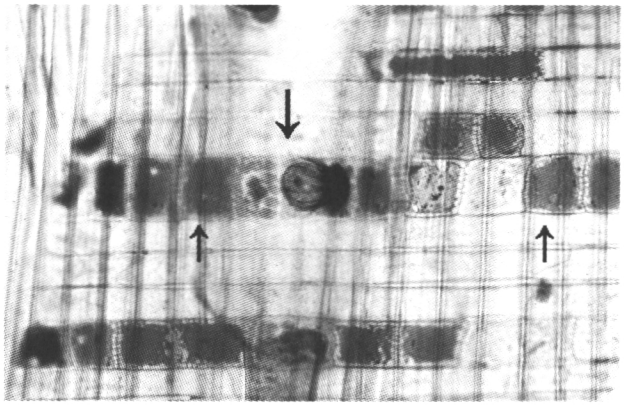


Figure 3. Radial view of the ray parenchyma cells in the watermark. Big arrow indicates plasmolysised cell and small arrows indicate necrotic cells.

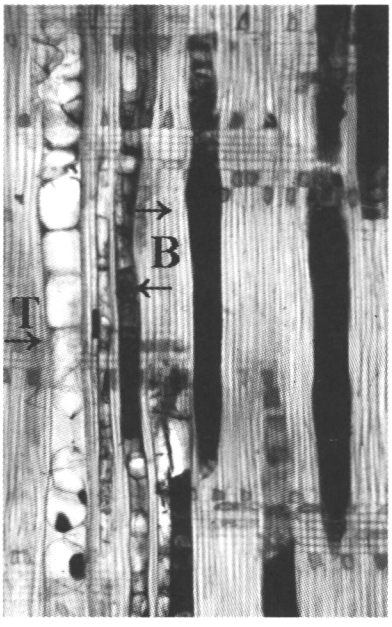


Figure 4. Radial view of the watermark. T indicates tyloses and B indicates masses of bacteria.

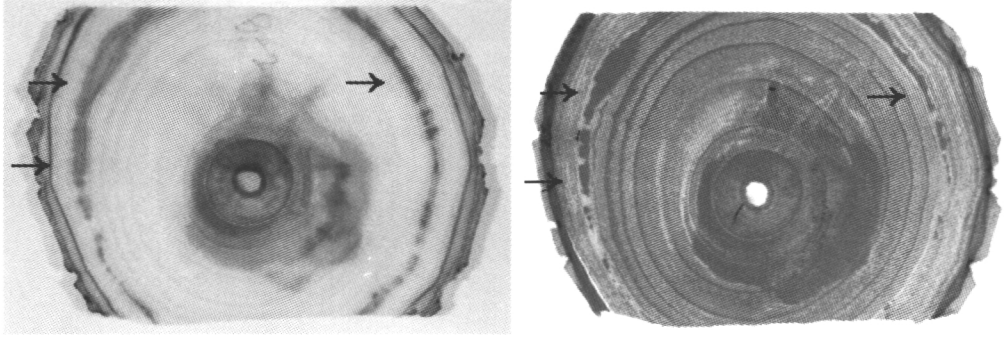


Figure 5. Soft X-ray photography.
a. A normal photograph of the wood disk from the affected tree in green state. Arrows indicate the watermark.
b. A soft X-ray photograph of the disk in **a** in green state. Note that the dark zone (arrows) corresponds with the watermark in **a**.

Cryo-scanning electron microscopy

The cryo-scanning electron microscopy revealed that almost all the cells in the watermark were filled with ice irrespective of the type of cell (Fig. 6). However, ice was seldom present in the lumina of wood fibers in the sapwood of healthy samples.

Scanning electron microscopy

In the watermark, vessel-ray parenchyma pit membranes were heavily incrusted, damaged, or often absent (Fig. 7). Figure 8 shows a complementary pair of interfibre surfaces in which the pit membranes are absent.

In contrast to the watermark, appreciable incrustations were rarely observed in tissues of healthy sapwood. Also, vessel-ray parenchyma and interfibre pit membranes typically remained intact.

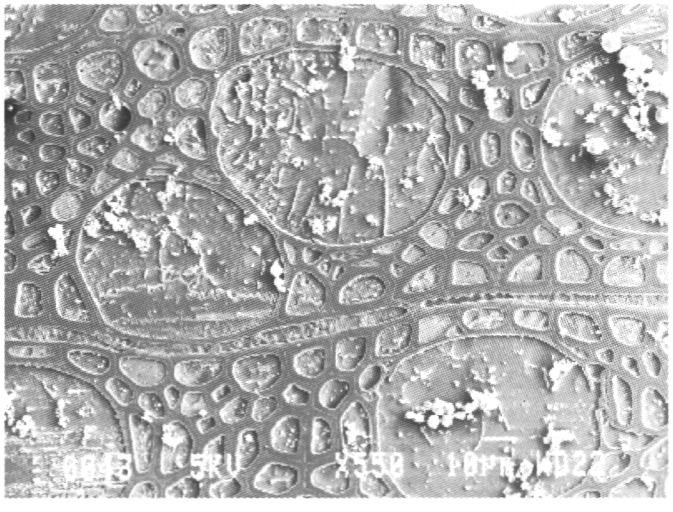


Figure 6. A cryo-SEM photograph of the watermark. Note that almost all the cells were filled with ice (water in natural conditions).

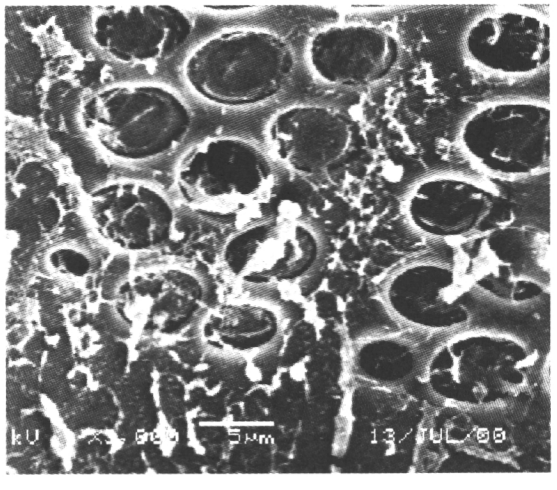


Figure 7. SEM photographs of the vessel-wall surfaces between vessels and ray parenchyma cells. Pit membranes are often heavily incrusted or absent.

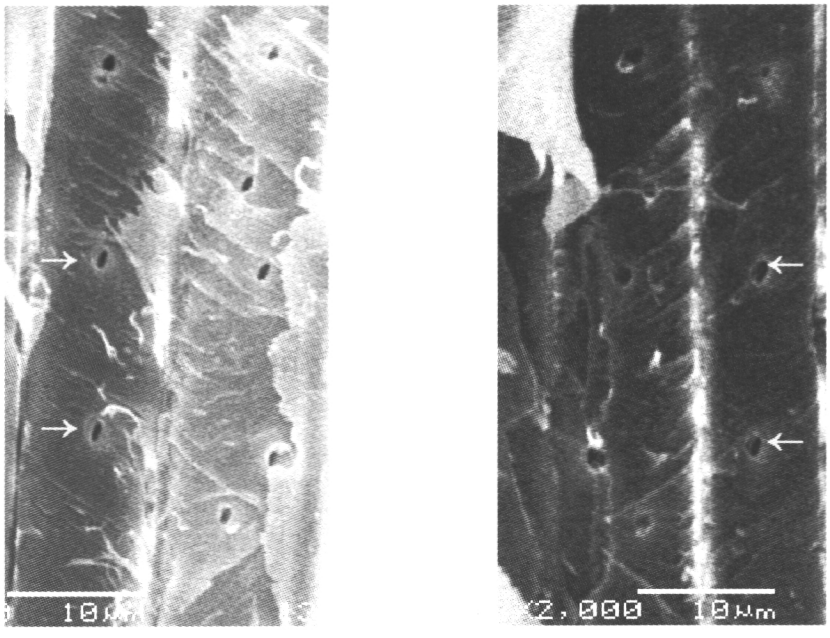


Figure 8a, b. SEM photographs of complementary pair of surfaces between wood fibers. Pit membranes are often absent on both faces of pit pairs (arrows).

Osmotic potential

Substantial decrease in the mean values of $\phi\pi$ was observed in the watermark (-0.34 MPa) compared to the values of the unaffected sapwood (-0.04 MPa).

Polysaccharide in watermark

The mean values of the dried weight of the polysaccharide fraction from watermark and healthy sapwood were 40.0 mg and 31.2 mg, respectively.

The signals in the ^{13}C-NMR spectrum of the commercial levan appeared in the range of 60-110 ppm, and these signals completely agreed with the signals in the spectrum of the EPS fraction (arrowheads in Fig. 9). Thus, the EPS fraction was identified as levan. The spectrum of the polysaccharide fraction of the watermark showed the same signals as the EPS fraction and commercial levan, while the spectrum of healthy sapwood did not (Fig. 9). This fact obviously indicated that the watermark contained levan; on the contrary, the healthy sapwood did not.

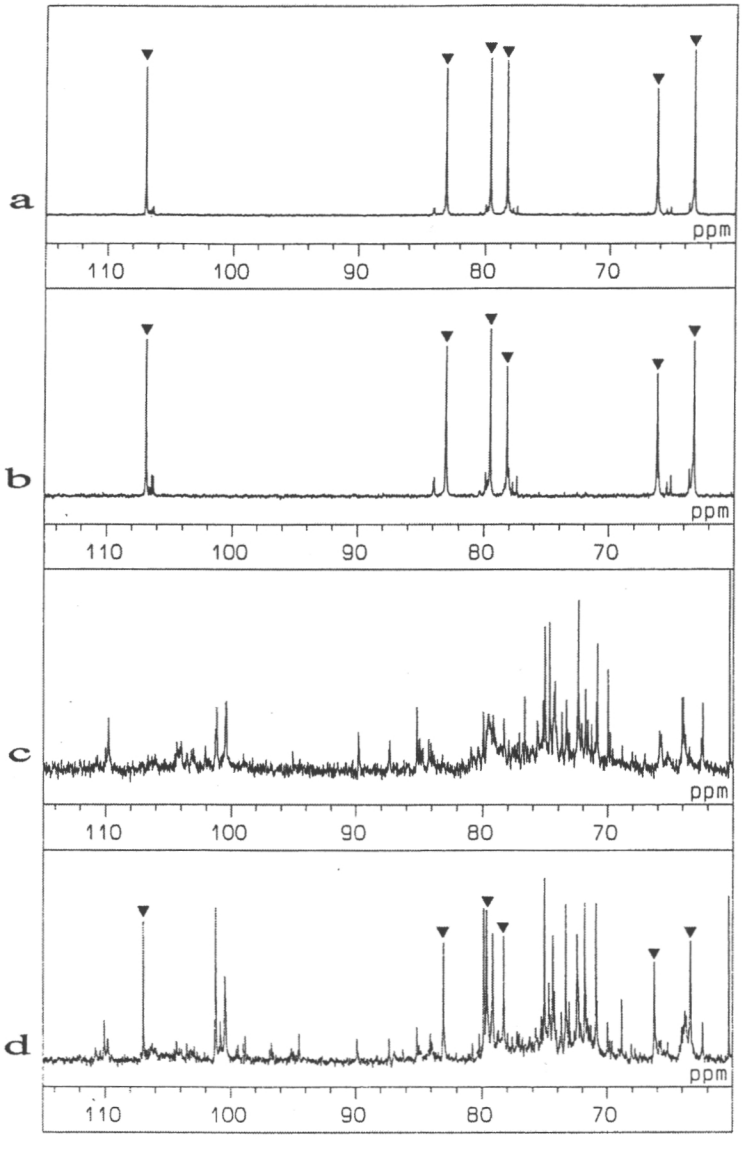


Figure 9. ^{13}C-NMR spectroscopy.
a. Commercial levan.
b. EPS from the bacterial culture.
c. Polysaccharide from healthy sapwood.
d. Polysaccharide from the watermark. Arrowheads indicate the signals of levan.

Discussion

Based on the bacteriological characteristics mentioned above, the current isolates should be included in the *Erwinia amylovora* group (Bradbury 1970). Other characteristics of the current isolates corresponded to those of *E. salicis* (Table 1), and their pathogenicity to *Salix* spp. was confirmed. Therefore, the current isolates were identified as *E. salicis*. The external and internal symptoms corresponded to the symptoms of watermark disease of willows as previously reported in Europe (Day 1924, Dowson 1937, Smith et al. 1986, Strouts and Winter 1994). Our report (Sakamoto et al. 1999) is the first description confirming the presence of the watermark disease in willows, and its pathogen, *E. salicis*, in Japan. Although *E. salicis* was reported to be present in Japan in one textbook (Bradbury 1986), no evidence to support this statement was provided.

The ability to conduct water was not observed in the watermark. The contents of the affected ray parenchyma cells in the watermark were yellow to brown. This suggests that there was an accumulation and oxidation of phenolic compounds. A direct detection test of the phenolic compounds in the watermark was not performed in this study; however, Wong and Preece (1978) reported an increased level of phenolic compounds in infected willow wood. The present anatomical study showed that the parenchyma cells in the watermark caused plasmolysis and necrosis with the development of the disease. These facts suggest that, in this respect, the watermark in sapwood may be analogous to wound wood 'discolored wood'. Discolored wood is the tissue that forms as a kind of defense reaction in sapwood, with similar processes to heartwood formation. The wood is believed to become discolored wood as follows. When xylem tissues in healthy sapwood are attacked by microorganisms or are mechanically injured, the accumulation level of secondary substances and antimicrobiological substances, such as phenolic compounds, increases. Then, the parenchyma cells gradually die. The color of these tissues will turn brown to black brown with oxidation and polymerization of the secondary substances, then lose the ability to conduct water as in normal heartwood (Shigo and Hillis 1973, Bauch 1984, Shigo 1984). Therefore, the non-conductive watermark formation and its expansion in the affected sapwood as the disease progresses may be the reason for the wilting symptoms.

This report revealed that masses of *E. salicis* were only found in watermark and not in surrounding or healthy sapwood. Except for levan, ^{13}C-NMR spectroscopy revealed that both watermark and healthy sapwood contained the same kinds of polysaccharides (not identified in this study). These facts indicated that the increment of dried weight of the polysaccharide fraction of the watermark was due to levan, which was produced by *E. salicis*. The low $\phi\pi$ in the watermark can be attributed in large part to levan, which acts as the osmotically active material. Furthermore, as shown in Figure 4, masses of *E. salicis* were often found in the lumina of tyloses. This fact indicated that the tyloses were often collapsed and did not block the vessel lumina from water invasion. This study also revealed that the vessel-ray parenchyma and interfibre pit membranes were often damaged. They could also serve as effective pathways for the accumulation of water in the watermark. These data support the concept that levan production decreases $\phi\pi$, leading to water accu-

mulation through those pathways.

Studying the more precise mechanism of water inhibition and water accumulation in the watermark might also provide relevant information to elucidate the mechanisms of wilt diseases and bacterial wetwood in other tree species.

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Responses of juvenile poplars to inoculation with *Septoria musiva* can predict their long-term canker disease resistance

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Abstract

Septoria musiva causes cankers that severely limit production of hybrid poplar in eastern North America. A field trial in 1998 and a greenhouse trial in 1999 were conducted to determine whether a short-term assay of response to stem infection by *S. musiva* was predictive of long-term field performance (LTFP). Fresh leaf scars of 27 clones (field) and 15 clones (greenhouse), representing a range in long-term performance as reflected by canker disease, were inoculated with five mm MEA plugs colonized with *S. musiva*. Results were measured four months after inoculation in the field trial, and three months after inoculation in the greenhouse trial. Incidence, canker length, and percent of stem circumference affected (girdle) were recorded for each cankered tree. Responses were compared with ratings of canker damage from longer-term field studies by logistic regression. Analysis showed that the field assay predicted LTFP better than the greenhouse assay. In general, girdle was the best predictor for LTFP.

Neofabraea populi in Scandinavia

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Abstract

The ascomycete *Neofabraea populi* was identified from two damaged hybrid aspen stands in Southern Finland. Genetic markers from 24 single-ascospore isolates, two isolates from wood tissue and three Norwegian reference isolates of *N. populi* were compared. No observable genetic variation was found with 32 random amplified microsatellite markers. The identity of five RAMS loci between samples was assured by successful PCR amplification with sequence specific primers. The results indicate that the genetic variation within *N. populi* is extremely low, suggesting prescence of widely spread clone. This might be due to introduction of small founder population of the fungus to Europe. It is also likely that the fungus is homothallic.

Keywords: *Neofabraea populi*, hybrid aspen, genetic variation, introduced species

Introduction

Neofabraea populi Thompson causes cankers on *Populus grandidentata, P. tacamahaca* and *P. tremuloides* and also hybrid aspen (*Populus tremula* x *P. tremuloides*) (Thompson 1939, Roll-Hansen and Roll-Hansen 1969). Originally *N. populi* was described in Northern America (Thompson 1939). The disease had also been noted on several hybrids poplars in Japan since late 1940's, although it was identified in the end of 1960's (Ito et al. 1969). The affected species included *P. alba*, *P. sieboldii*, *P. tremula* var. *davidiana*, *P. alba* × *P. sieboldii, P. tremula* var. *davidiana* × *P. canescens*, *P. sieboldii* × *P. canescens*, *P. nigra*, *P. deltoides* var. *monilifera*, *P nigra* × *P. deltoides* var. *monilifera*, *P. nigra* × *P .maximoviczii*, *P. maximoviczii* and *P. simonii* (Ito et al. 1969).

The first damages caused by the fungus in Europe were noted few years after hybrid aspen plantations were established in Norway. Thus it was assumed that the fungus had been accidentally introduced from North America (Roll-Hansen and Roll-Hansen 1969). There are only few documented observations of *N. populi* in Finland (Semb and Hirvonen-Semb 1968), but it is thought to occur commonly with planted hybrid aspen. Recently, the importance of hybrid aspen as raw material for pulp production has increased. The wood is planned to be grown by short-rotation forestry on reforested agricultural land. Since conditions for spread of *N. populi* are optimal in dense coppice stands, the fungus is a potential threat for production of hybrid aspen.

The aim of this study was to identify the causal agent of canker disease observed in hybrid aspen stands in Finland. In the disease, 2nd generation (root suckers) of trees are seriously damaged with cankers and dead bark. Both morphological characters and DNA markers were used.

Material and methods

Fungal isolates

Hybrid aspen stands in Fiskars (South-West Finland) and Pornainen (Southern Finland) with dense 2nd generation of trees, were visited in October 2000. Serious canker damages and dead stems of aspen suckers were observed within both stands. A total of 25 cankers of young hybrid aspen with cankers were cut apart. In Fiskars, fresh cankers with apothecia were frequent and easy to cut from living stems. In Pornainen, no apothecia bearing cankers on living trees were found, because the stand was almost destroyed by the disease and most trees had died during pervious years.

In the laboratory, slice of the apothecia bearing bark was cut from each stem, and placed under lid of a petri dish containing water agar. Released single ascospores were identified from the water agar by microscope and isolated with modified pasteur pipette. The single-ascospore isolates were further cultured on 1,5% malt agar. A total of 24 isolates were isolated from this stand.

Although apothecia were not found on canker samples collected from Pornainen, it was possible to isolate *N. populi* directly from the wood material. Pieces of wood were cut from the barrier zone of a canker, surface-sterilized by rinsing in 4% NaOCl, 75% ethanol and finally sterilized water (10 sec each) and incubated on 1,5% malt agar. The morphology of the isolates was compared to single ascospore isolates. In addition to number of saprophytic fungi, also two isolates of *N. populi* were found.

Three isolates were obtained from Halvor Solheim, Norwegian Forestry Research Institute. They were isolated during the *N. populi* epidemy in 1960's.

Microscopy

Ascospores were identified on the water agar plates under a low power microscope. For microscopic purposes, blocks of water agar carrying spores were cut from the culture, placed on a microscope slide and covered with a coverslip. The slides were placed on a warm plate at 50°C to dry and reduce agar to a thin layer before the spores were photographed. One hundred ascospores were measured from the microscope slides.

DNA markers

For DNA isolation the fungal cultures were grown on malt agar covered with cello-

phane membrane. Nuclear DNA was isolated as described in Vainio et al. (1998). A total of 36 RAMS (Random Amplified MicroSatellite; Hantula et al. 1996) markers were PCR amplified by using anchored di- and trinucleotide repeat primers and analysed on 1% agarose gels. RAMS primers used were DBV$(CAT)_5$ DDB$(CCA)_5$, DBD$(CT)_5$C, HBH$(GAG)_5$ and VDD$(TCC)_5$, where B= C/G/T, D=A/G/T, H=A/C/T and V=A/C/G), respectively. Primers for amplification of five sequence characterized amplified regions (SCAR) were designed; individual RAMS markers were cloned, sequenced and new primers were designed based on the sequence information.

Results

Microscopy

Based on the morphology of ascospores, the aspen canker pathogen in Finland was similar to *N. populi* (Fig. 1). The size of the ascospores was 15-25 × 5-10 µm. The ascospores were oblong-ellipsoid, slightly curved, contents granular, hyaline.It was also noted that the morphology of the colonies *in vitro* was highly similar with reference isolates with practically no variation.

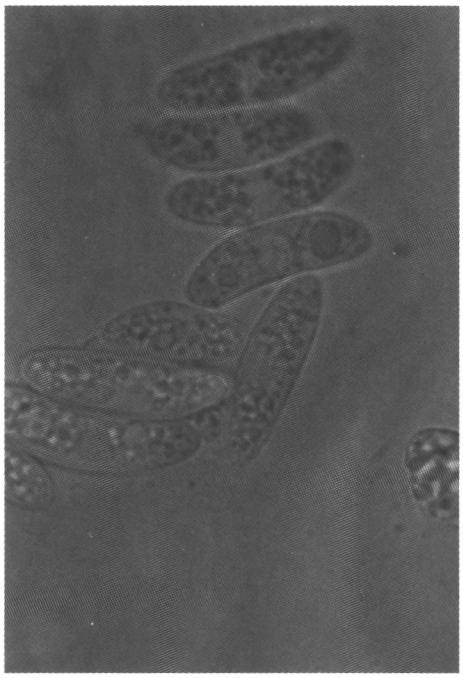


Figure 1. Spores of *N. populi*. Size of the spores is 15-25 × 5-10 µm.

DNA markers

Surprisingly, all the Scandinavian isolates were identical according to the used markers. No variation was observed within 32 RAMS markers amplified from ascospore isolates, canker isolates or reference isolates (Figs. 2, 3). With DBV$(CAT)_5$ primer it was possible to score seven reproducible markers, with DDB$(CCA)_5$ six markers, with DBD$(CT)_5$C eight markers, with HBH$(GAG)_5$ six markers and with VDD$(TCC)_5$ five markers, respectively. Accordingly, the SCAR markers were successfully amplified from all isolates (Fig. 4).

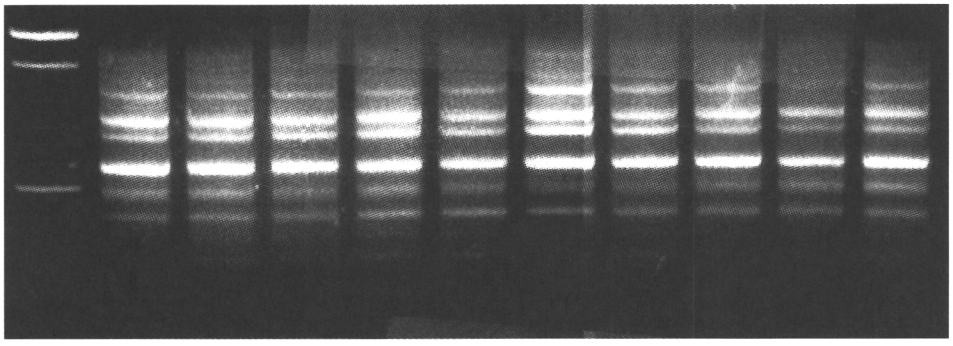


Figure 2. RAMS fingerprints amplified from *N. populi* isolates (Fiskars) with DBV$(CAT)_5$ primer. Note the similarity between lanes. Leftmost lane is 100 bp molecular weight ladder, where the lower intense band is 600 bp.

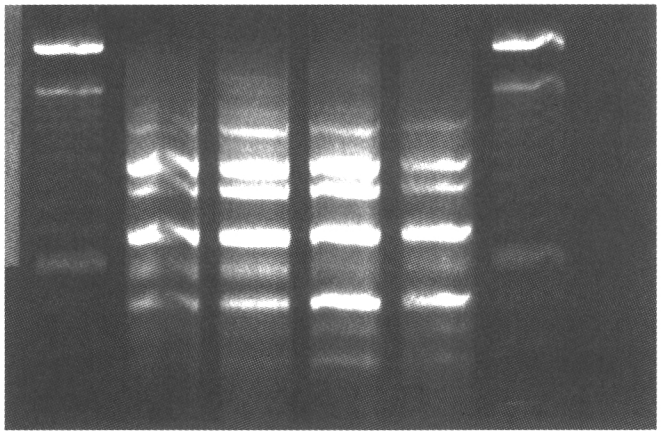


Figure 3. Similar RAMS fingerprints amplified with DBV$(CAT)_5$ primer. Lanes from left: A 100 bp molecular weight ladder, where the lower intense band is 600 bp, three Norwegian isolates, a isolate from Fiskars and a molecular weight ladder (right).



Figure 4. Pairs of SCAR markers amplified from one Norwegian (left band in each pair) and one Finnish isolate (right band in each pair). Distal lanes are molecular weight ladders.

Discussion

Ascospore morphology

The ascomycete *N. populi* was identified from two damaged hybrid aspen stands in Southern Finland. The size of the ascospores measured in this study (15-25 × 5-10 µm) was close to earlier observations; 16-22 × 5-6,5 µm (Thompson (1939), 14-22 × 4-6 µm (Ito et al. 1969), 13,2-20,8 × 4-6,5 µm (Roll-Hansen 1969). Also the slightly curved, ellipsoid shape and hyaline colour of the spores were observed both in this study and previous reports.

DNA markers

No genetic variation was observed with the 32 RAMS markers, although theoretically it would be possible to identify 2^{32}=4 294 967 296 clones of the fungus by scoring the presence or absence of the markers. Although it is known that no marker system has ultimate resolution of genotypes, it can be considrered highly probable that the fungal isolates studied were actually clonal. The same markers amplified from isolates from 1960's and 2000's suggest also exclusive clonality of the fungus in Scandinavia, since no evidence of mating with different genotypes during last four decades was found.

As most of the isolates were isolated from single ascospores, the observations also support the homothallism of *N. populi*, since mating is usually required to produce apothecia. Homothallism was also hypothesized by Thompson (1939), who observed that the fungus produced apothecia *in vitro*. However, no apothecia or similar structures have been observed within cultures in this study. The extremely low genetic variation was also in correspondece with the previous theory of introduction of the fungus to Europe, and suggested that the founder population had been only few isolates, perhaps one isolate. However, more isolates from Scandinavia and North America are needed, to obtain more information about the population structure of the fungus within Scandinavia and also the possible introduction of the fungus from North America to Europe.

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Effect of fertilisation on the resistance of spruce seedlings to shoot blight caused by *Sirococcus conigenus*

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Abstract

The effect of fertilisation with different Mg-fertilisers on the susceptibility of four-year-old *Picea abies* seedlings to *Sirococcus* shoot blight was examined in an artificial inoculation experiment. Prior to inoculation the nutritional and the physiological status of the seedlings was assessed by measurements of chlorophyll fluorescence and the contents of proteins, low molecular weight carbohydrates and starch in needles.

Four weeks after inoculation shoot dieback was highest on seedlings fertilised with a neutral Mg-salt (Bittersalz), whereas no significant differences were found between unfertilised seedlings and seedlings fertilised with an alkaline fertiliser derived from magnesite and dolomite and amended with organic compounds (Magnosol). The Bittersalz treatment was also characterised by reduced chlorophyll fluorescence in the last year's needles and by higher contents of glucose and pinitol in the current year needles.

The reisolation revealed striking differences between the infection rate of shoots and the expression of shoot blight symptoms, indicating that the infection of current year shoots by *Sirococcus conigenus* is not necessarily associated with dieback or development of symptoms.

Keywords: *Sirococcus conigenus*, *Picea abies*, carbohydrates, proteins, chlorophyll fluorescence

Introduction

The mitosporic fungus *Sirococcus conigenus* (DC.) P. Cannon & Minter causes shoot blight and seedling mortality on many conifer hosts in the northern temperate zone (Sutherland 1987, Farr et al. 1989). It has been implicated in shoot dieback of pine, spruce and hemlock from the seedling stage (Funk 1972, Illingworth 1973, Redfern 1973, Smith 1973, Magasi et al. 1975, Sutherland et al. 1981, Motta et al. 1996) to mature size (O'Brien 1973, Neumüller 1994, Maresi and Capretti 1995). In Central Europe the disease mainly affects Norway spruce (*Picea abies* [L.] Karst.).

In Austria, *Sirococcus* shoot blight has caused severe damage in some loca-

tions since the early 1980s. Symptoms of crown thinning, dieback of twigs and branches as well as top dying of mature Norway spruce have been observed especially in secondary spruce forests on poor and acidified soils additionally impoverished by litter raking and lack of admixed tree species (Halmschlager et al. 2000a, 2000b, Anglberger and Halmschlager 2000, Jandl et al. 2000). Preliminary results from field experiments indicated that imbalances in tree nutrition, particularly a deficiency of magnesium and calcium, increased the susceptibility of Norway spruce to *Sirococcus* shoot blight (Anglberger and Halmschlager 2000, Jandl et al. 2000). To verify these results under controlled conditions, the effect of fertilisation on the resistance of spruce seedlings to *Sirococcus* shoot blight was tested in an inoculation experiment.

Material and methods

Three-year-old potted spruce seedlings from a single clone, grown on homogenised and autoclaved mineral soil obtained from a Norway spruce stand severely affected by the fungus, were fertilised in spring 1998 with "Magnosol®" (an alkaline fertiliser derived from magnesite and dolomite and amended with organic compounds) and "Bittersalz®" (a neutral Mg-salt), respectively. A third collective of seedlings remained unfertilised. After one year, 20 seedlings of each treatment were inoculated during flushing by spraying with a conidial spore suspension of *S. conigenus*. Inoculations were carried out twice with a nine day interval using conidial suspensions of two different *S. conigenus* isolates (8,1 x 10^5 and 1,1 x 10^6 conidia/ml). Ten seedlings from each fertiliser treatment served as control and were sprayed with sterile deionised water only.

Following inoculation, seedlings were transferred to growth chambers and placed in polyethylene bags to maintain a high humidity (near 95% relative humidity). According to Wall and Magasi (1976) the seedlings were incubated under the following day/night conditions: 16 h/8 h; 21°C/16°C; 2000 lx/0 lx. Eighteen days after inoculation seedlings were removed from the growth chambers and exposed to open air. Evaluation of shoot blight symptoms was carried out 29 days after inoculation. Seedlings were examined for the presence or absence of shoot blight symptoms and seedlings with at least one severely damaged shoot were classified as damaged. In order to estimate the rate of fungal infection reisolations were carried out 37 days after inoculation. One shoot, apparently healthy, from each of the 90 seedlings used in the inoculation experiment and 40 shoots from damaged seedlings were cut, surface sterilised and plated onto malt extract agar (MEA 2%).

The nutritional and the physiological status of the host prior to inoculation was assessed on seedlings from each treatment which were not used for the inoculation. Proteins, low molecular weight carbohydrates and starch were analysed from current year needles (7 replicates per treatment) and chlorophyll fluorescence was measured in current and last year's needles. For the chemical analyses the needles

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were frozen in liquid nitrogen, lyophilised, ground to = 2 mm and stored at – 30°C. For carbohydrate analyses aliquots of the frozen powder were pulverised in a sample mill (MM2, Retsch) and extracted with hot distilled water (4% w/v). 1 ml of the water extracts was purified using a coupled cation and anion exchange resin (Dowex 50W x 8, 50-100 mesh, H^+-form, and Dowex 1x8, 50-100 mesh, COO^- form, Fluka), with arabinose added as an internal standard. The neutral fraction (low molecular weight carbohydrates) was analysed by HPLC (Hewlett Packard, Aminex HPX-87 P, 300 x 7,8 mm, Biorad, refractive index detector) (Meister 1998).

Starch was degraded to glucose (Winter et al. 1996) which was measured photometrically following Boehringer-Mannheim (1995). Proteins were analysed according to Peterson (1977).

Chlorophyll fluorescence (F_v/F_m) of dark adapted current and last year's needles was measured with a MINI-PAM Photosynthesis Yield Analyser (Walz, Effeltrich, Germany). Reflectance of photosynthetically active radiation in the range of 430 - 680 nm was measured with an SD2000 spectrometer (OceanOptics, Dunedin, Florida) on current year needles. Datt (1999) showed that the chlorophyll content correlates with relative reflectance $[(r_{850}-r_{710})/(r_{850}-r_{680})]$, which was used to obtain a relative chlorophyll content of the spruce needles.

Results and discussion

Although development of symptoms was very low in general (only 0-12% of the shoots from a seedling were damaged by *Sirococcus* shoot blight), number of damaged seedlings differed significantly between treatments (x^2=6.172; p=0.046, Table 1). Number of damaged seedlings was highest in the Bittersalz treatment, whereas no significant differences occurred between the unfertilised seedlings and the Magnosol treatment. Thus seedlings fertilised with Bittersalz appeared more susceptible to shoot dieback. Control seedlings remained free of symptoms.

Table 1. Number of damaged spruce seedlings, 29 days after inoculation with a spore suspension of *S. conigenus*, related to fertiliser treatments (x^2=6.172; p=0.046).

	Treatment		
	Magnosol	Bittersalz	Not fertilised
Not damaged	10	3	9
Damaged	10	17	11

Interestingly, reisolation from inoculated current year shoots indicated a generally high rate of infection (Table 2). Although highest from needles of wilted shoots, the fungus also was frequently reisolated from needles and stems of inoculated shoots showing no visible symptoms. These results suggest that development of symptoms is related to the chemistry and physiology of the current year shoots as affected by the treatment with different fertilisers. However, it remains unclear which factor actually results in expression of symptoms. Because length of current year shoots

did not differ at the date of inoculation, differences in disease incidence cannot be explained by varying susceptibility of the seedlings due to different stages of shoot development.

Table 2. Reisolation rate (%) of *S. conigenus* from stems and needles of diseased and healthy looking inoculated current year shoots as well as from shoots of the control variant (n = number of shoots used for reisolation).

	Inoculated		Control	
	Damaged	No symptoms	Damaged	No symptoms
	n = 40	n = 60	n = 0	n = 30
Stems	67.5 %	28.3 %	-	0,0 %
Needles	88.6 %	73.3 %	-	3.3 %

In contrast to the other treatments seedlings fertilised with Bittersalz showed needle yellowing, especially on older needles, and reduced chlorophyll fluorescence in the last year's needles prior to inoculation, indicating impaired photosynthesis (Table 3). Reduced photosynthetic performance therefore perhaps promoted development of disease symptoms. The fact that the fertiliser had no effect on the chlorophyll fluorescence of the current year needles suggests that plants from all treatments translocated nutrients to the young needles. The reflectance from needles of Bittersalz-fertilised plants also indicated lower chlorophyll content.

The carbohydrate contents in current year needles related to treatments are given in Table 4. Values of the carbohydrate content did not much differ from those obtained by Dermutz (1992) and Schafellner et al. (1996) who investigated the carbohydrate contents of *Picea abies* growing in young plantations. Furthermore, the contents of glucose and pinitol in the current year needles of the Bittersalz treatment were significantly higher than in the Magnosol fertilised and the unfertilised seedlings, indicating higher contents of soluble carbohydrates. No differences in carbohydrate and protein contents occurred between the Magnosol treatment and the unfertilised seedlings.

The results of this study do not agree with the hypothesis of Funk (1972), who claimed *Sirococcus* shoot blight as a "low sugar disease", according to Horsfall and Dimond (1957). Seedlings fertilised with Bittersalz were most severely affected by *Sirococcus* shoot blight in spite of the high carbohydrate contents prior to inoculation. Since no additional data are available on the dynamics of carbohydrate contents after inoculation and during incubation of the seedlings in the growth chambers, an explanation of the relatively strong symptoms in the Bittersalz treatment is difficult.

Whatever the biochemical mechanism is, this study showed that the expression of symptoms was affected by variations in needle chemistry and physiology resulting from treatments with different fertilisers. Furthermore, fertilisation with magnesium sulphate (Bittersalz) was not appropriate to reduce susceptibility of seedlings, grown on nutrient poor and acidified soils, to *Sirococcus* shoot blight.

Table 3. Relative estimate of total chlorophyll content and dark fluorescence yield (Fv/Fm) of current and last year's needles due to fertiliser treatment.

Treatment	Control	Bittersalz	Magnosol	Significance (ANOVA)
Total chlorophyll (relative values)	0.492 (±0.013)	0.512 (±0.004)	0.540 (±0.012)	0.0163
F_v/F_m 1-year-old needles	0.767 (±0.007)	0.751 (±0.008)	0.774 (±0.005)	0.041
F_v/F_m current year needles	0.769 (±0.006)	0.769 (±0.005)	0.770 (±0.005)	0.993

Table 4. Concentrations (mg/g dry weight) of carbohydrates and proteins in current year needles prior to inoculation, related to fertiliser treatments (means followed by different letters are significantly separated at $p<0.05$ by ANOVA).

	Sucrose	Glucose	Pinitol	Fructose	Myo inositol	Starch	Protein
Magnosol	$6,64^a$	$22,09^a$	$30,41^{ab}$	$24,47^a$	$3,93^a$	$9,1^a$	$27,72^a$
Bittersalz	$6,09^a$	$28,19^b$	$34,42^b$	$24,35^a$	$4,42^a$	$10,5^a$	$26,78^a$
unfertil.	$6,10^a$	$22,42^a$	$28,45^a$	$22,85^a$	$4,48^a$	$12,2^a$	$26,29^a$

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Nutritional status of mature Norway spruce related to infection by *Sirococcus* shoot blight

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Abstract

An inventory on the nutritional status of 72 mature Norway spruce trees (*Picea abies*) revealed significant differences in element contents of the current year and 3-year-old needles between healthy and *Sirococcus*-diseased trees. Contents of P, Ca and Mg were significantly lower in the current year and 3-year-old needles of diseased trees, and supply of Ca and Mg was insufficient, when compared with threshold values. Diseased trees further showed higher N/Mg and N/Ca-ratios compared with healthy trees, indicating imbalanced nutritional status.

Keywords: *Sirococcus conigenus*, shoot blight, Norway spruce, *Picea abies*, nutrition

Introduction

The mitosporic fungus *Sirococcus conigenus* (DC.) P. Cannon & Minter is constantly associated with shoot blight and branch dieback in secondary spruce forests on poor soils in Upper Austria, leading to top and branch mortality of Norway spruce. Symptoms have been observed since the early eighties (Neumüller 1992, 1994, Halmschlager et al. 1998, 2000a, 2000b), however, differences in disease severity often occur within a single stand, probably due to different nutritional status of individual trees (Anglberger and Halmschlager 2000, Jandl et al. 2000). To verify this hypothesis, the nutritional status of healthy and diseased mature Norway spruce trees was assessed on a homogenous site.

Material and methods

Investigations were carried out in a 90-year-old pure Norway spruce stand in the Kobernausser Wald (Fig. 1). Half of the 72 sample trees showed severe crown thinning due to shoot, twig and branch dieback, caused by *S. conigenus* whereas the other trees were apparently healthy and vigorous. In December 2000 one branch at

Site	Kobernausser Wald
Elevation (m)	600
Aspect	W
Bedrock	Tertiary gravels
Soil type	Dystric Cambisol
Humus type	Moderhumus



Figure 1. Location and site characteristics of the experimental site.

the 7th whorl was cut from each sample tree. Current year needles and 3-year-old needles were individually analyzed after oven drying and acidic extraction with HNO_3-$HClO_4$ for K, Ca, Mg, P and S using inductively coupled plasma emission spectrometry (ICP-OES). N-content was determined by semi-micro-Kjeldahl method. Only living needles were used as analytic samples and current year needles used for analyses were taken from shoots expressing no symptoms of *Sirococcus* shoot blight.

Differences between needle element contents of severely affected and healthy trees were tested by Student's t-test using SPSS statistical software, release SPSS 8.0, SPSS Inc. (Kähler 1998).

Results and discussion

Table 1 shows the macronutrient contents in the current year and 3-year-old needles of *P. abies* related to infection. Results obtained from needle analysis revealed significant lower contents of P, Ca and Mg in the current year and 3-year-old needles of diseased trees. Furthermore the nutrient contents of needles from trees severely affected by *S. conigenus* reflected insufficient Mg and Ca supply and enhanced N/Mg and N/Ca-ratios, when compared to threshold values given by Hüttl (1986) and Stefan (1995), respectively (Tables 1 and 2). The content of P in the current year needles of diseased trees was just at the limit to deficient supply (Table 1). Contents of N, K and S showed no relationship to tree health status, in both current year and 3-year-old needles.

Increased host susceptibility due to unbalanced nutrition has already been demonstrated from other fungi, causing shoot blight and canker in conifers. Ylimartimo (1990/1991) reports about increased disease severity on Scots pine seedlings with latent infections by *Gremmeniella abietina* (Lagerb.) Morelet after increasing N-availability. Increased susceptibility of Scots pine to *G. abietina* related to nutritional imbalances also has been demonstrated by Barklund (1993). As well the out-

Table 1. Means of macronutrient contents (mg/g dry weight) in the current year and 3-year-old needles of *Picea abies* related to infection by *S. conigenus*. Means followed by different letters are significantly separated at $p<0.05$ by t-test.

Health status	N(mg/g)	K(mg/g)	P(mg/g)	Ca(mg/g)	Mg(mg/g)	S(mg/g)
Current year needles						
Severely affected	15,44a	4,40a	1,17a	0,75a	0,57a	0,85a
Healthy	15,73a	4,61a	1,34b	1,31b	0,80b	0,92a
3 year old needles						
Severely affected	14,33a	4,26a	0,82a	0,99a	0,33a	0,90a
Healthy	15,22a	4,31a	0,91b	1,78b	0,40b	0,93a
Deficient supply[1]	< 13, 0	< 4,0 - 4,5	< 1,1 - 1,2	< 1,0 - 2,0	< 0,7 - 0,8	-

[1] Threshold values for current year needles from Hüttl (1986)

Table 2. N/Ca and N/Mg ratios in current year needles of *Picea abies* related to infection by *S. conigenus*. Means followed by different letters are significantly separated at $p<0.01$ by t-test.

Health status	N/Ca	N/Mg
Severely affected	24,50a	29,41a
Healthy	13,97b	22,34b
Ratios for balanced nutrition [1]	2,0 - 7,0	8,0 - 14,0

[1] Threshold values from Stefan (1995)

break of this disease has been associated with application of nitrogen fertilizer (Donaubauer 1972, Kallio et al. 1985). However, excessive foliar N contents seem to have only an indirect effect on host susceptibility through increasing nutrient imbalances as has been demonstrated by Ylimartimo (1991) in a laboratory experiment. After all, increased imbalances expressed by high N/K and N/Mg ratios reduced resistance of Scots pine seedlings against *G. abietina* infections (Ylimartimo 1991).

Similar results were obtained by Roelofs et al. (1985) who reported lower foliar K and Mg levels in *Pinus nigra* var. *maritima* trees infected by *G. abietina* and/or *Sphaeropsis sapinea* (Fr.) Dyko & Sutton, compared to the non infected trees, whereas availability of N was increased in the damaged trees. Increased disease incidence stimulated by high N content in host tissues was also reported for *S. sapinea* by Van Dijk et al. (1992) and Stanosz and Trobaugh (1996).

Results of the present study also correspond well with the findings of Anglberger

and Halmschlager (2000) in an earlier study, comparing the severity of *Sirococcus* shoot blight on fertilized and unfertilised plots in degraded Norway spruce stands on poor podzolic soils over tertiary gravels and old silicate bedrock, respectively. In their study the application of magnesium rich carbonate fertilizers harmonized tree nutrition and decreased severity of *Sirococcus* shoot blight at the investigated sites, whereas the application of fertilizers with relatively high N-contents and only small portions of Mg did not achieve similar results.

Results of the present study therefore indicated that expression of disease symptoms obviously was related to acute nutritional imbalances or that fungal infection itself is affecting needle nutrient contents, respectively. Further investigations at this site will indicate, whether the improvement of the nutritional status of single trees by application of appropriate fertilizers will modify the resistance of trees against infection and promote recovery.

Acknowledgements

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Growth disturbances in spruce forest on burn-beaten areas

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Abstract

Growth disturbances of spruce forest appear in burn beaten areas. These areas have by turns been agricultural land and after several years cultivation have been grown forest and they have often also been pastured areas by cows and sheep. Today most of these areas are forest. The pioneer tree has often been alder and later to, those areas has been planted spruce and birch. In these forests appear growth disturbances, which typical feature is the dieback of leader shoot, and the bush like appearance of these trees. The leader bud of shoots does not develop normally during growing period and that's why new or several leader shoots grow from abbatial buds. In these areas there is deficiency of boron (B), but the content of main nutrients P, K, Ca and N are in normal level. Fungal injuries develop in these shoots later and seem to be secondary. Primary infections cause *Thekopsora areolata* which causes cankers and also kills the new shoots. These injuries are heaviest near the bird cherries, which is the other host plant of this fungus. The other most common fungi are *Sclerophoma pythiophila* and *Sirococcus strobilinus*.

Detection, identification and quantification of latent needlecast pathogens and endophytes in symptomless conifer foliage by PCR and Dot-blot assays

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Abstract

We are developing sensitive and reliable detection methods to determine whether pathogens are present in symptomless foliage. Sequences of the ITS-rDNA are obtained from fungi in foliage of pine, spruce, juniper and Douglas fir. Oligonucleotide probes are developed based on differences among the aligned sequences. Direct primer PCR amplifications are used to determine the specificity of the probes. Probes that are proven to be species-specific are further studied. The optimal conditions for the use of each probe are determined experimentally in both PCR reactions and dot-blot assays. The probes are highly reliable in detecting infections in symptomless needles of *Pseudotsugae menziesii* and sometimes are specific to the subspecies level in identification. Probes readily detected the pathogens and endophytes in needles taken from the beginning of the infection period in May, again in November, and in May of the following year. Measurements of the density of PCR products correspond to the progress of the disease. Also, the density of PCR products correspond to the relative susceptibility of each tree as visually rated in the field, in blind studies.

Introduction

Detection of plant pathogens directly from infected tissues by Polymerase Chain Reaction amplification (PCR) is new and is increasingly being developed for disease diagnosis (Hemlin). The use of molecular probes has particular value in detection and identification of fungi that cause needlecasts of conifers. Needlecast pathogens cause latent infections, grow slowly in living foliage tissues, and are particularly difficult to culture. Most of the fungi that cause needlecasts grow slowly in culture relative to other fungi. Endophytic fungi such as *Hormonema dematioides* are numerous in conifer foliage and usually they will overgrow the pathogenic fungi on agar medium. For example, following the culturing of approximately 10,000 green needles of *Pinus sylvestris* over a six year period for the purposes of quantifying *Cyclaneusma minus* infections, at least 95% of needles yielded *H. dematioides* cultures in our studies in Michigan. Some needlecast fungi form colonies in culture that do not exceed 1 cm in diameter after many months such as *Dothistroma pini, C.*

minus and *Scirrhia acicola*, still others are not culturable such as many species of *Rhabdocline*.

Needlecast pathogens have a latency period of 9-months to 24-months following infection of foliage. It is known that these pathogens can be disseminated particularly into Christmas tree farms by sale of symptomless infected nursery stock. To prevent new introductions of the pathogens from infected areas and to stop the spread of infection where pathogens are locally absent, programs of inspection of nursery stock exist to provide certification that seedlings are healthy prior to sale. Additionally, quarantine programs exist to prevent introduction of exotic pathogens such as the European strain of *Gremmeniella abietina*. Inspection programs rely primarily on visual inspections and therefore identification of pathogens is usually done on seedlings showing symptoms. Unfortunately, symptoms are often not observed at the time of inspection that is usually in early spring. Identifiable fruiting bodies are usually absent, and isolation and cultivation is very slow and seldom successful. Development of effective quarantine and certification programs may depend on development of new technologies.

PCR is an extremely sensitive technique that should allow detection of a fungal pathogen without the need for isolation on agar media. We were uncertain whether PCR could be successful in detection of the minute amount of fungal cells that some needlecast pathogens form in green needles because species such as *Rhabdocline* may be confined to a single epidermal cell in the early months following infection (Stone 1987). Hyphae of other pathogens have not been detectable in needles prior to disease symptoms (Peterson and Walla 1978; Jewell 1983, 1990). Additionally, conifer foliage has been found to have many chemicals that interfere with PCR amplification and the chemicals have been difficult to remove during DNA extraction and purification (Bahnweg et al. 1998). Identification of the pathogen by PCR can be complex, as many of the pathogens are closely related (share nearly identical ITS sequence) to non-pathogenic endophytes, for example the pathogenic species of *Kabatina* and *Rhizosphaera* are related to endophytic *H. dematioides*. Also, several related species or subspecies in a genus like *Rhabdocline* may have different levels of infection and cause different levels of damage on one host within a local region. Furthermore, needles on one host plant may be infected by more than one pathogen.

The strategy that has usually been used in PCR detection of fungi is to amplify extracts of genomic DNA with universal primers. Universal primers are designed from conserved coding regions of the ribosomal DNA subunit (rDNA). However, the conserved regions used are those flanking variable non-coding regions of rDNA like the internal transcribed spacers (ITS) because the sequences obtained are usually variable enough to differentiate species or subspecies of fungi. Based on sequence differences new primers are designed that are specific to the target species. We adapted this experimental approach and designed species-specific and subspecies-specific primers for use in amplifying the ITS-rDNA of the target fungus in PCR tests. We also used these specific primers as ^{32}P-isotope labeled oligonucleotide probes in Dot-Blot assays of DNA extracts. Most challenging, we evaluated the use of these methods to quantify the level of infection in asymptomatic needles over the time course of disease development.

Our objectives are to develop PCR or Dot-Blot detection methods and test the

reliability and sensitivity of each method in detection of species and subspecies of needlecast fungi directly from infected needles, especially asymptomatic needles. Additionally, we wish to use the detection to quantify increasing levels of infection during disease development and to evaluate host susceptibility. Michigan has a large and diverse conifer nursery industry producing over 9 million conifer seedlings a year plus it has a diverse Christmas tree industry harvesting 4 million trees a year. Therefore, our purpose is to develop detection methods for all the relevant needlecast pathogens we encounter in the host plants. We outline and summarize some successful results of the research here, but the finer details of the methodologies and primer sequences will be published elsewhere.

The pathogenic and related non-pathogenic endophytic fungi that we have studied are listed below by host in Table 1. Included in Table 1 are the abbreviations of each species. The abbreviations are used in labeling the lanes of electrophoretic gels or Dot-blot membranes (Species code) to indicate that the DNA of that organism is used in the test corresponding to the gel lane. Also, we list the size of the DNA sequence in base pairs (bp) that we obtained by PCR amplification using the universal primers ITS1 and ITS4 (White et al 1990). The fungal cultures and fruiting bodies are obtained from Michigan plants except *C. niveus*, *K. thujae, R. kobayashi, R. macrospora, R. oudemansi* obtained from CBS, *R. weirii* ssp. *weirii*, *R. weirii* ssp. *obovata*, and *R. pseudotsugae* ssp. *pseudotsugae* obtained from Jeffery Stone, and *L. macrospora* obtained from James Walla.

In Table 2 we have listed the names of the primer/probes used as species-specific primers in direct PCR amplifications and as probes in Dot-blot assays. The effective temperatures for annealing during PCR reactions (Tm) and for DNA-DNA hybridization (Th) are also listed with the correlated size of the oligonucleotide in base pairs. Primer/probes with –1 (e.g., LS-1) read the non-coding strand in the same direction as the universal primer ITS1. Similarly, those that read the coding strand have –4 designating the same direction as ITS4.

Figure 1 demonstrates the effectiveness of using pairs of designed primers (LS-1 and LS-4, or LP-1 and LP-4) in PCR amplifications of various DNA minipreps from needle inhabiting fungi including endophytes and pathogens. It is possible with careful design to select primers for closely related species in a genus such as *Lophodermium* that only amplify one of the species. The primers LS-1 and LS-4 strongly amplify *L. seditiosum* while not amplifying *L. pinastri, L. conigenum, L. juniperi* or *Lophodermium* sp (undescribed species). Likewise, primers LP-1 and LP-4 amplify *L. pinastri* and do not amplify *L. seditiosum* or other species of *Lophodermium* (Figure 1).

Figure 2 demonstrates the use of a ^{32}P-isotope labeled oligonucleotide probe in species-specific detection of the endophyte *H. dematioides*. The probe is highly specific when used at the optimum hybridization temperature. Note the faint ghosts of reactions with two *Rhizosphaera,* two *Kabatina* and *Aureobasidium.* These ghost reactions are due to the similarity of the DNA sequence among these closely related black yeast.

In the past two years we have concentrated our efforts with probes to investigate the needle pathogens of *Pseudotsuga menziesii* (Douglas fir). Michigan suffered several severe epidemics of *Rhabdocline* needlecast in the Christmas tree in-

Table 1.

Host & Fungus	Source	Species code	Sequence Length (bp)
Pine			
Lophodermium seditiosum	Live culture	LS	569
Lophodermium pinastri	Live culture	LP	560
Cyclaneusma minus	Live culture	CM	548
Cyclaneusma niveus	Live culture	CN	805
Dothistroma pini	Live culture	DP	625
Hormonema dematioides	Live culture	HD	627
Aureobasidium pullulans	Live culture	AP	623
Gremmeniella abietina	Live culture	SL	559
Sirococcus conigenus	Live culture	SC	641
Scirrhia acicola 1	Live culture	SA1	575
S. acicola 2	Live culture	SA2	575
Lophodermium conigenum	Live culture	LC	ND
Lophodermium sp.	Live culture	LSP	ND
Juniper			
Kabatina juniperi	Live culture	KJ	625
K. thujae	Live culture	KT	628
Sclerophoma pithyophila	Live culture	SP	627
Phomopsis juniperovora	Live culture	PJ	620
Lophodermium juniperi	Live culture	LJ	ND
Spruce			
Rhizosphaera kalkhoffii 1	Live culture	RKA1	626
R. kalkhoffii 2	Live culture	RKA2	628
R. kobayashi	Live culture	RKO	618
R. macrospora	Live culture	RMAC	629
R. oudemansii	Live culture	RO	625
R. pini 1	Live culture	RPI1	629
R. pini 2	Live culture	RPI2	629
Lirula macrospora	fruiting bodies	LIRM	571
Douglas fir			
Rhabdocline parkeri 1	Live culture	RP	575
R. pseudotsugae ssp. epiphylla	fruiting bodies	RPE	ND
R. pseudotsugae ssp. pseudotsugae 1	fruiting bodies	RPP1	573
R. weirii ssp. weirii	fruiting bodies	RWW	ND
R. weirii ssp obovata	fruiting bodies	RWOBV	ND
R. weirii ssp oblonga	fruiting bodies	RWOBL	572
Phaeocryptopus gaeumannii	Live culture	PG	626

ND = not determined

dustry. *Rhabdocline* needlecast is a devastating disease in the Michigan Christmas tree industry because epidemics occur only sporadicallly, often separated by 10 years without disease and therefore the growers are not using control measures. Also, during epidemic years the disease often extends into new regions of the state with no prior experience with the disease. Figure 3 is a composite picture showing the damage to the tree including blisters on the leaves and the water-soaked expanded

apothecia on the 2-3 year old needle. Infection of a plantation of trees of the age for sale usually results in the total loss of the crop. Farmers destroy the trees because recovery to an acceptable appearance to allow sale requires three disease-free years, about one third of the rotation period. Douglar fir is not grown as a timber tree in Michigan. *Rhabdocline* is a complex of species on Douglas fir. *Rhabdocline parkeri* is a ubiquitous endophyte in the coastal seed-sources of Douglas fir but its occurrence was not known in Intermountain seed sources (IM) until this study. *Rhabdocline pseudotsugae* and *R. weirii* cause similarly damaging diseases on Douglas fir. *Rhabdocline pseudotsugae* occurs in two subspecies, *R. pseudotsugae* ssp. *pseudotsugae* and *R. pseudotsugae* ssp. *epiphylla. Rhabdocline weirii* occurs as three subspecies, *R. weirii* ssp. *weirii, R. weirii* ssp. *obovata,* and *R. weirii* ssp. *oblonga.* The two species *R. pseudotsugae* and *R. weirii* have been reported as pathogens in Michigan (O'Brien 1983) but the subspecies present were not known until this study.

Table 2.

Fungus	Probes	Oligo-nucleotide size (bp)	Tm (PCR) °C	Th (Dot-blot) °C
Lophodermium seditiosum	LS-1	18	62	56
	LS-4	22		ND
Lophodermium pinastri	LP-1	19	60	56
	LP-4	20		ND
Cyclaneusma minus	CM-1	20	58	60
	CM-4	22		ND
Cyclaneusma niveus	CN-1	22	60	52
	CN-1	20		ND
Dothistroma pini	DP-1	19	60	57
	DP-4	16		57
Hormonema dematioides	HD-1	20	56	51
	HD-4	19		51
Aureobasidium pullulans	AP-1	18	58	55
	AP-4	18		55
Kabatina juniperi	KJ-1	20	58	ND
	KJ-4	18		ND
Rhizosphaera kalkhoffii 1	RK-1	17	54	55
	RK-4	18		55
Rhabdocline parkeri 1	RP-1	19	59	58
	RP-4	19		60
R. pseudotsugae ssp. *pseudotsugae*	RPP-1	18	56	64
	RPP-4	17	52	55
R. weirii ssp. *weirii*	RWW-1	22	61	71
	RWW-4	22	60	65
R. weirii ssp. *oblonga/obovata*	RWO-1	22	49	55
	RWO-4	20	52	55
Phaeocryptopus gaeumannii	PG-1	20	58	57
	PG-4	19		57

ND = not determined

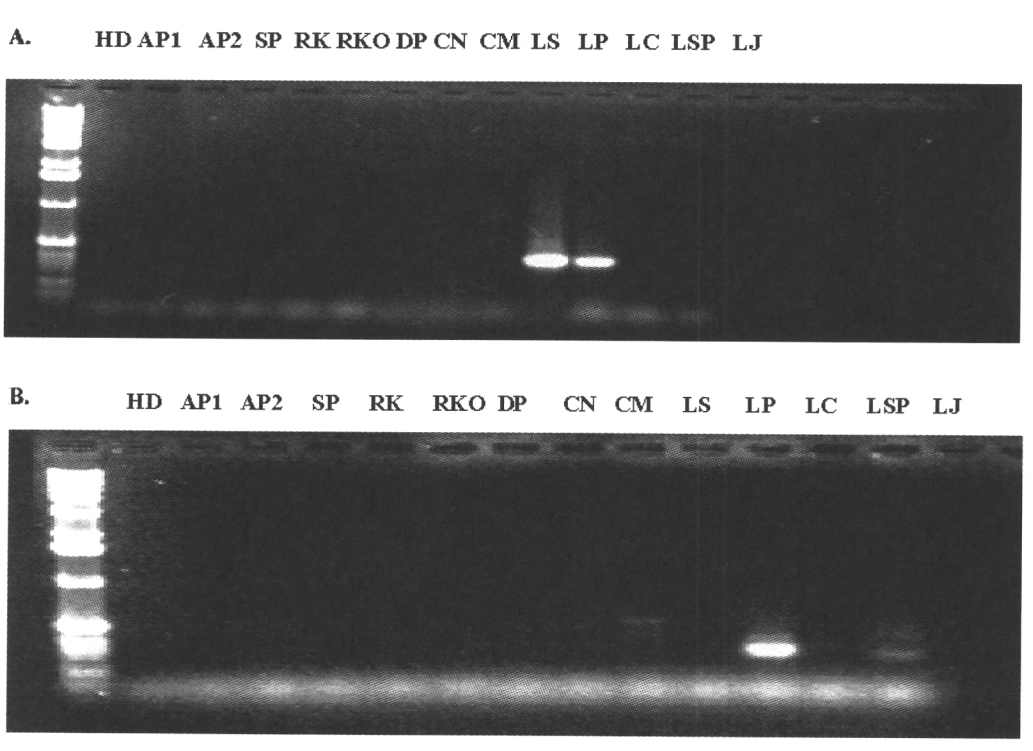


Figure 1. A. *Lophodermium seditiosum* probes LS-1 and LS-4 amplification. B. *Lophodermium pinastri* probes LP-1 and LP-4 amplification.

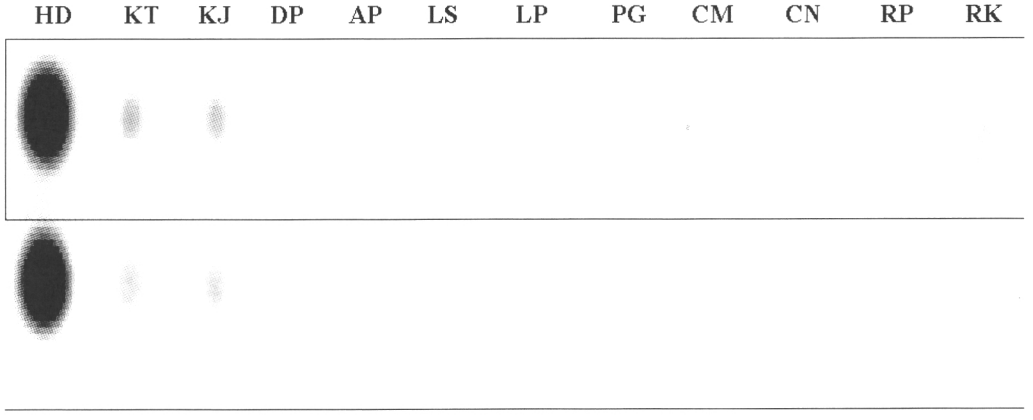


Figure 2. *Hormonema dematioides* probes HD-1 and HD-4 hybridized to PCR products of universal primers.

Michigan is a northern state with cold long winters and the industry relies on the cold-hardy interior mountain seed sources of Douglas fir which are susceptible to *Rhabdocline* needlecasts. The IM seed-sources are from various national forests on isolated mountain peaks in the western United States and Canada. The coastal

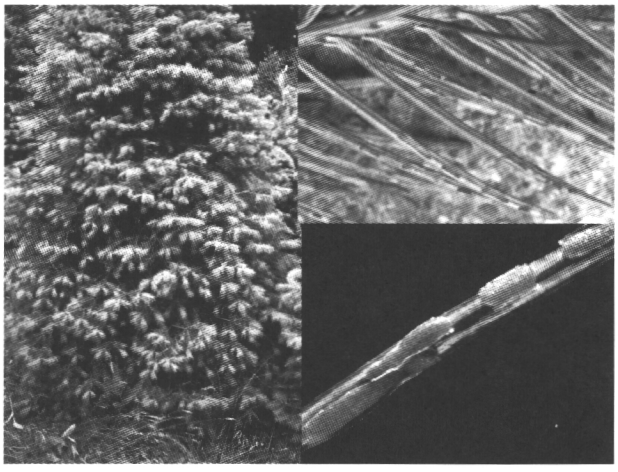


Figure 3. *Rhabdocline* needle casts of Douglas fir (*R. pseudotsugae, R. weirii*)

seed-sources of Douglas fir are generally cold-intolerant and immune to *Rhabdocline* needlecasts. Needlecasts of these latter seed-sources have been studied recently by Jeffery Stone at Oregon State University.

The development of the species-specific probe methodology in the Adams lab in Michigan was combined with the evaluation of a collection of interior mountain seed-sources for disease tolerance in the Chastagner lab in Washington. This cooperative effort allowed the testing of probes for *Rhabdocline* species in seed-sources and individual trees and coorelation of probe detection to visual ratings for disease severity and tree susceptibility. The cooperative effort was conducted as a blind test where the results of probe detection were completed in a lab that did not have the visual ratings data and vice-versa. The Chastagner lab was evaluating disease susceptibility of Douglas fir, in replicated plots, including the following seed-sources: Apache National Forest (N. F.), Carson N. F., Cibola N. F., Clearwater N. F., Coconino N. F., Lincoln N. F. source a, Lincoln N. F. source b, Rio Grande N. F., San Isabel N. F., and Santa Fe N. F. Additionally, individual trees in each seed-source were being rated for susceptibility each year over 3 years. A map of the origins of the IM seed sources is shown in Figure 4.

The remainder of this report will concern the primer/probes for the *Rhabdocline* species. Figure 5 demonstrates the effects of optimizing the temperature of hybridization (Th) in increasing the specificity of the probe for the target fungus. Changing the temperature changes RPP-1 from a specificity at 55 C for all *Rhabdocline* species including *R. parkeri* (RP) an endophyte [anamorph = *Meria parkeri* (MP)], *R. pseudotsugae* and *R. weirii,* to a probe that at 64 C is specific only for the subspecies of *R. pseudotsugae*. This probe has dual value for diagnosis when used at either temperature.

In Figure 6 the primer/probes RPP-1 and RPP-4 when used as a pair specifically amplify *R. pseudotsugae* ssp. *pseudotsugae* and *R. pseudotsugae* ssp. *epiphylla* in direct PCR amplification. Nested amplification did not improve specificity. Figure 7 shows that primer/probes RWW-1 and RWW-4 specifically amplify *R. weirii* ssp. *weirii* and not other subspecies of *R. weirii* and both primer/probes were indi-

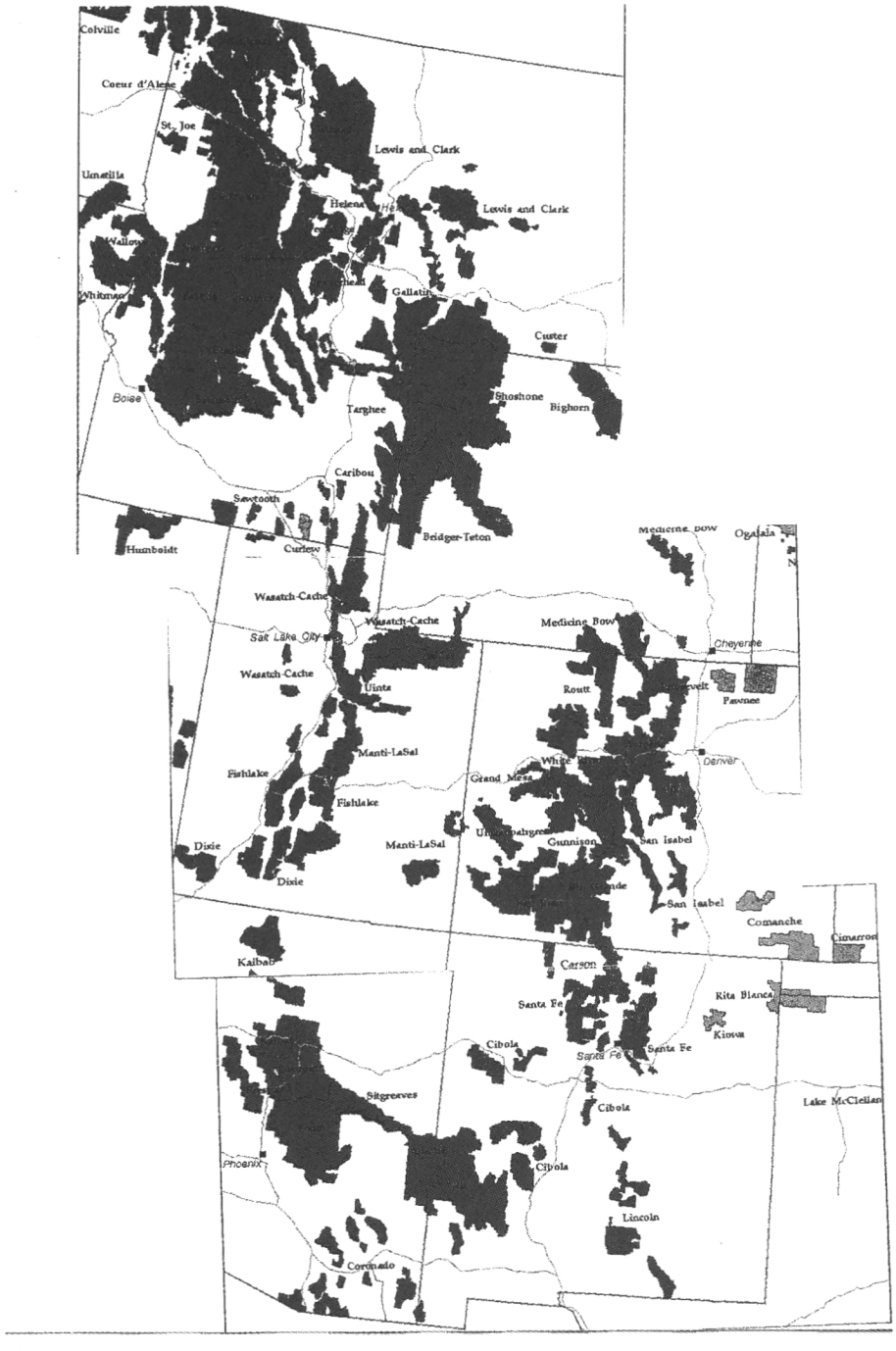


Figure 4.

vidually specific for *R. weirii* ssp. *weirii* only, in Dot-blot assays. Several primer/probes designed for *R. weirii* ssp. *oblonga* and *obovata* were tested also. Due to similarities in ITS sequence primer/probes RWO-1 and RWO-4 were selected as they gave strong reactions to subspecies *oblonga* and *obovata* while not amplifying

R. weirii ssp. *weirii,* Figure 8. *Rhabdocline parkeri* and subspecies of *R. pseudotsugae,* however, were also amplified though less strongly. Because we could not improve the specificity of the primers we believe that *R. weirii* ssp. *oblonga* and ssp. *obovata* might be more closely related to *R. pseudotsugae* than to *R. weirii* ssp. *weirii.*

With the *Rhabdocline* primer/probes we began examining needles from 44 selected trees from different seed-sources that had shown differential responses to needlecast in field trials over 3 years in Washington. Bulked samples of about 200

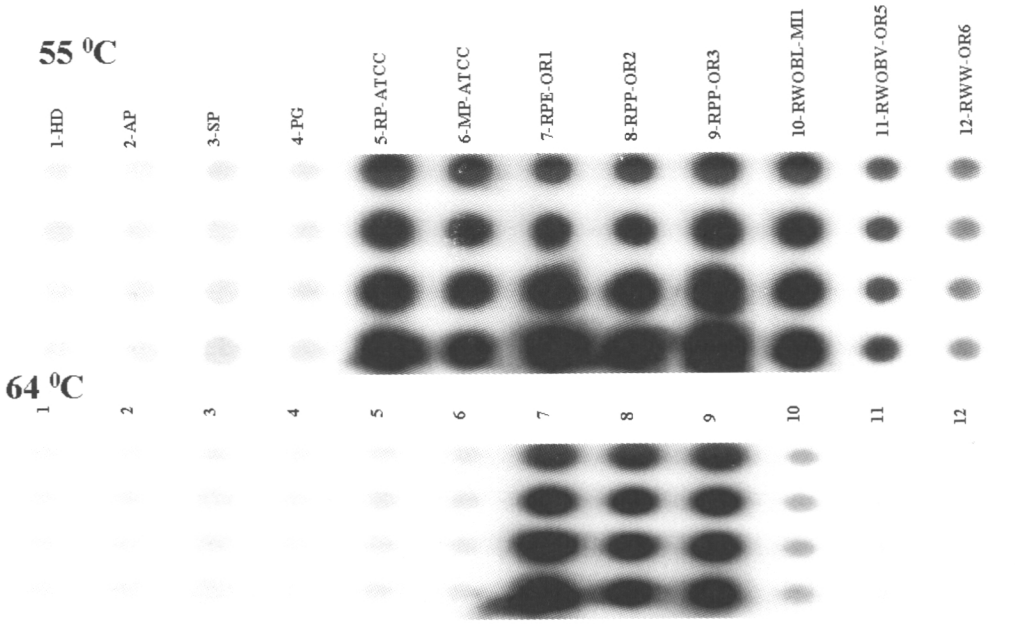


Figure 5. *Rhabdocline pseudotsugae* ssp. *pseudotsugae* probe RPP-1 hybridized to PCR products of universal primers at 55 & 64°C.

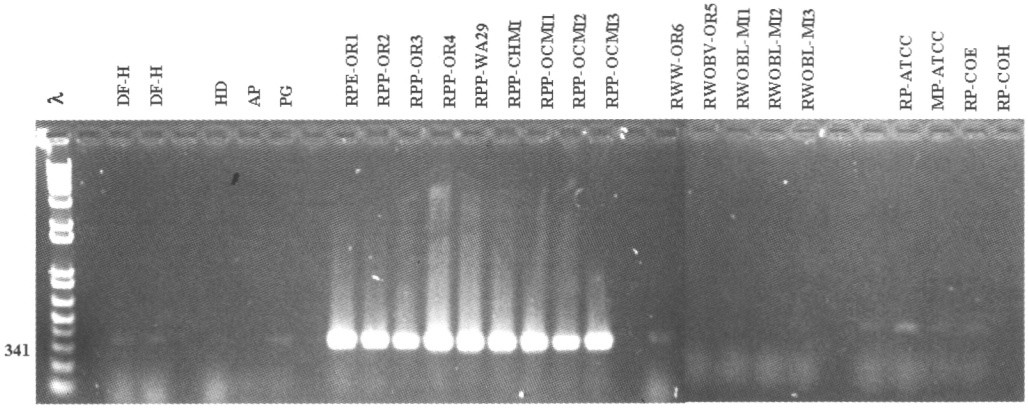


Figure 6. *Rhabdocline pseudotsugae* ssp. *pseudotsugae* specific primers RPP-1 and RPP-4 amplification - Direct PCR.

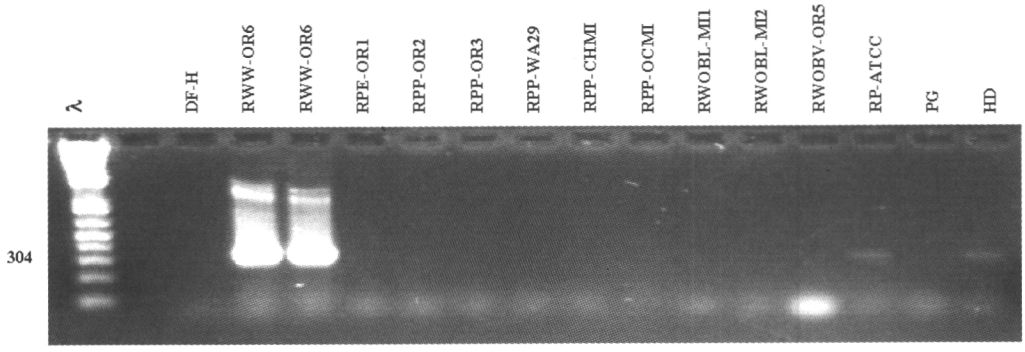


Figure 7. *Rhabdocline weirii* ssp. *weirii* specific primers RWW-1 and RWW-4 amplification - Direct PCR.

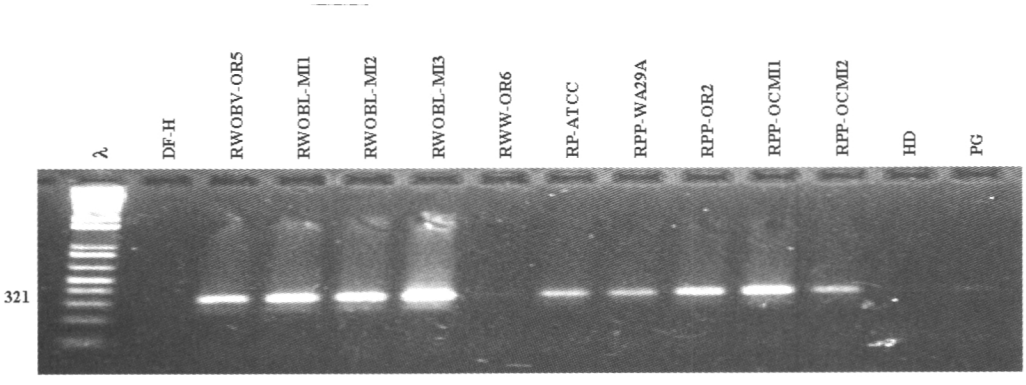


Figure 8. *Rhabdocline weirii* ssp. *oblonga/obovata* specific primers RWO-1 and RWO-4 amplification - Direct PCR.

grams of needles selected from needles that were all formed in the same season of growth were collected and placed into a bag. Three bags of needles were collected from each tree: current year's needles collected in May 2000, current years needles collected in November 2000, and one year old needles collected also in May 2000. Disease severity and tree susceptibility was visually rated for each tree in March 2000, and again in March 2001. The visual rating scale used was 0 (no disease) to 100 (severe disease) as present on greater than 75 percent of the needles on the lower two-thirds of the tree on four sides.

The three lots of needles/tree were tested separately with primer/probes RPP-1 and RPP-4, and also for probes of *Phaeocryptopus gaumannii* and *R. parkeri*. Results for the latter two species are not reported here. Each lot of needles was tested with 4 to 8 replications using two different DNA extraction methods. One method used a random sample of 10 g of needles from a bag, and DNA was extracted from these needles using a common household blender (4 replications/bag). A second method used just 5 needles collected randomly from the bag which were ground in a mortar and pestle in liquid nitrogen for DNA extraction (8 replications/bag).

The PCR amplified products of 4 replications for each of the 44 trees were

loaded onto a single gel in a large format gel apparatus, Figure 9. This provided for a uniform measurement of background fluorescence versus PCR product fluorescence on ultraviolet transillumination of ethidium bromide stained gels. A Bio-Rad gel reader and software system called Quantity One® was used to quantify the intensity of the DNA fluorescence. Each gel lane is separately recorded and the background signal subtracted. The Quantity One® reader reports the amount of DNA in the PCR product as a percentage of pixels (mm^2) fluorescing in a gel lane. This measurement can be converted to nanograms of DNA based on the known concentration of a specific band of the DNA marker ladder in the leftmost lane of each gel.

In Figures 10, 11 and 12 the first row of numbers is the sample (bag) number which is a known tree with a known susceptibility. The second row of numbers is the Quantity One® measurement of the intensity of the PCR product in the lane (concentration of DNA). Below the gels is the visual rating of disease severity given to each individual tree in March. Examine a well that does not contain a fluorescent band then check the Quantity One® reading above and the visual rating below. Agreement is remarkably good and better than we had assumed was possible. Thus, PCR detection of *Rhabdocline* pathogens in Douglas fir needles can be effectively used in quantifying the infection level or susceptibility of a tree. Figure 10 shows results from the 5 needle (mortar and pestle) extraction assay whereas Figure 11 shows results from the bulk 10g (blender) extraction assay. The two gels have good agreement. Figure 12 is of November needles that can be compared with the same year of needles collected in May (Fig. 10) but the visual rating was completed in March 2001 for Figure 12.

Figure 13 is a graph that summarizes results of the gels shown in Figures 10, 11, and 12. The visual rating of each tree is graphed as dark shading and the intensity of the PCR product is superimposed as light shading. The agreement between dark and light peaks can be readily compared and agreement is surprisingly good.

We also discovered that the PCR detection methodology is effective in quantifying the increasing amount of pathogen hyphae in the needles as the infection progressed through disease development. Figure 14 shows disease progress is re-

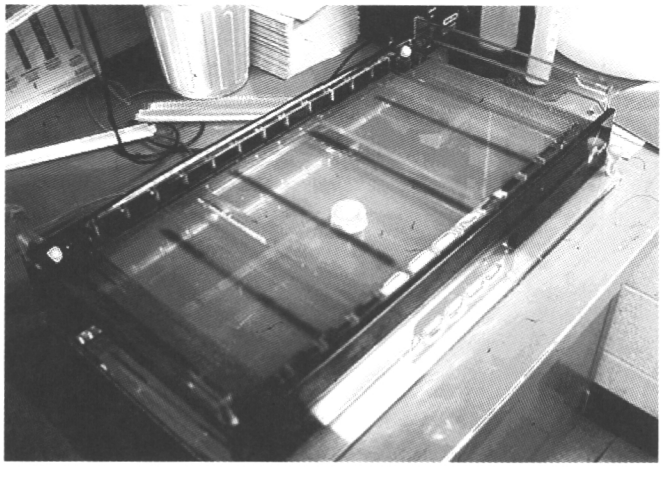


Figure 9.

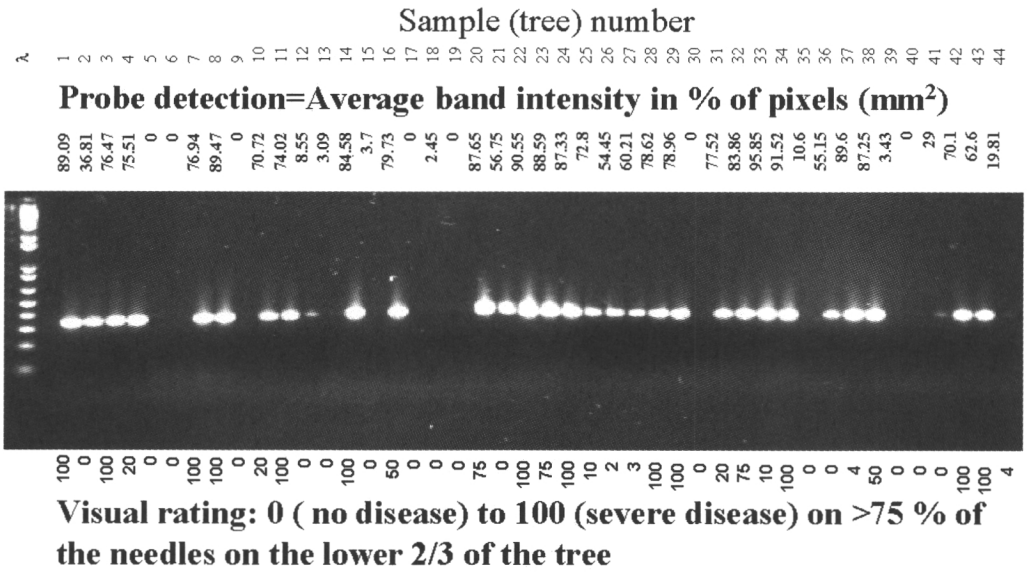


Figure 10. Comparison of probe detection and visual disease ratings of *Rhabdocline* infection of 44 trees. Mini extraction = 5 needles (May 2000 samples; 1 year old needles).

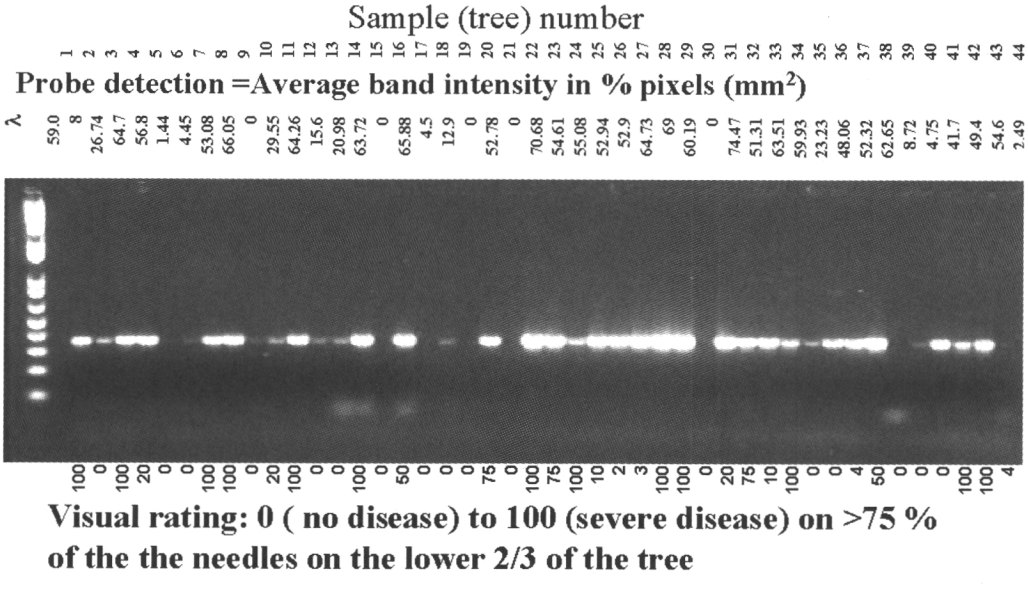


Figure 11. Comparison of probe detection and visual disease ratings of *Rhabdocline* infection of May needles from 44 trees. Bulk extraction = 10 g needles (May 2000 samples; 1 year old needles).

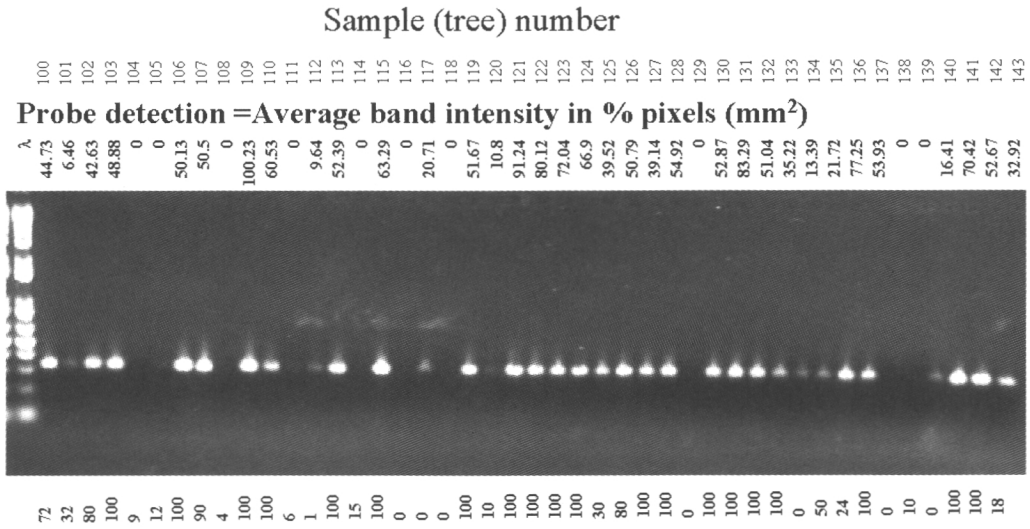


Figure 12. Comparison of probe detection and visual disease ratings of *Rhabdocline* infection of November needles of 44 trees. Mini extractions (November 2000 samples; current year growth needles).

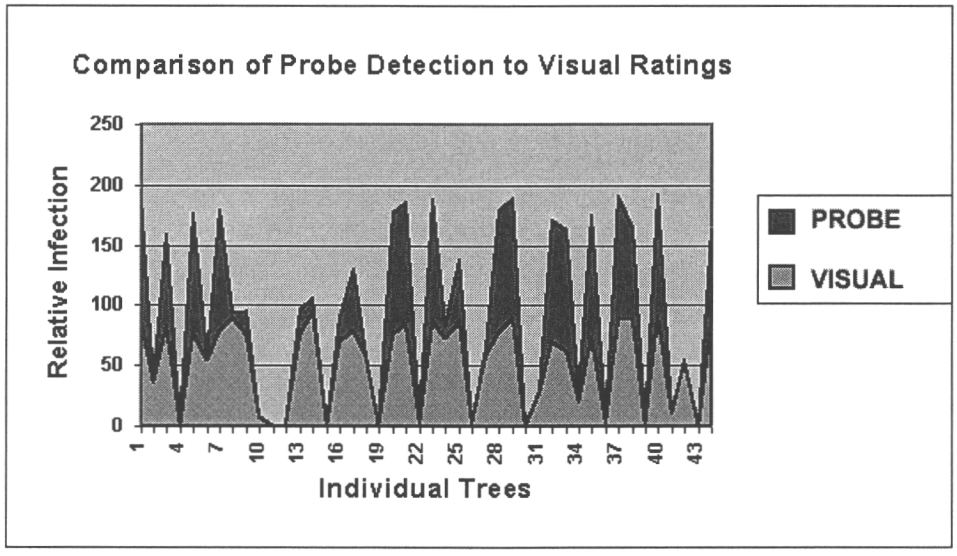


Figure 13.

flected as an increasing intensity of the PCR product amplified from the infected needles. These disease progress curves are separated by IM seed-source in our effort to determine if any of the seed-sources differ in the rate or amount of increasing hyphae of the pathogen in the needles during the year. Such differences might show

a type of disease resistance similar to a "slow-rusting" found in certain cultivars of wheat. Apache N. F. and Santa Fe N. F. sources show relatively higher amounts of pathogen hyphae in needles in early spring and one would expect that symptoms on foliage would be more obvious early in these sources. Additionally, Apache N. F. has greater amounts of pathogen hyphae in infected needles after one year, therefore greater disease severity would be expected at the time the disease symptoms are most evident.

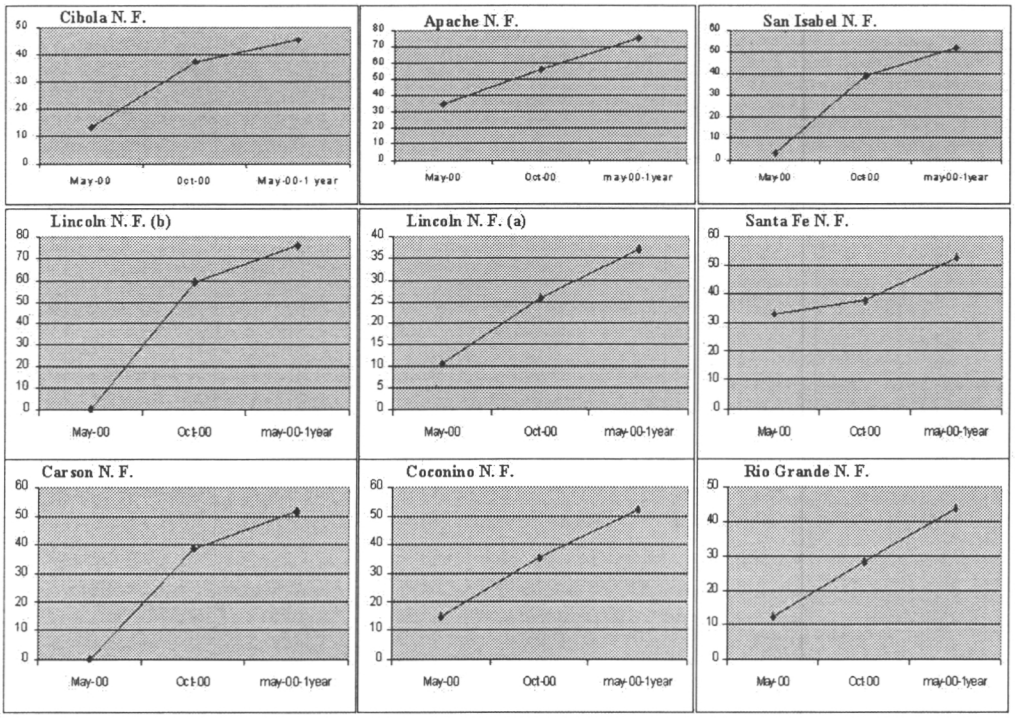


Figure 14. Changes in the amount of *Rhabdocline* mycelium in needles during a season.

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Some interesting fungi found on pine litter of three pine species from Nuevo León, Mexico

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Abstract

Three different types of pine needles of *Pinus pseudostrobus*, *P. cembroides*, and *P. arizonica* var. *stormiae* were sampled during one year. Needles were kept under moist conditions during one month. After this time needles were examined under microscope for fungi. 30 taxa of fungi were identified on this study. Well defined patterns of fungi for each one of the studied pine species were found. *Leptostroma* state of *Lophodermium australe*, *Niesslia exilis*, and *Marasmius androsaceus* fungus were commonly found on attached needles, and on "hanging needles" of *Pinus pseudostrobus*. *Rhizosphaera kalkhoffii*, and *Pestalotiopsis stevensonii* were found on attached, and on hanging needles of *Pinus cembroides*. *Verticicladium trifidium,* and *Discosia strobilina* were found only on needles on the soil. *Coniochaeta lignaria*, *Leptomelanconium pinicola*, and *Pestalotiopsis funerea* were the most common fungi found on needles of *Pinus arizonica* var. *stormiae*.

Keywords: forest pathology, fungal ecology, forest trees

Introduction

The litter plays a prominent role in the forest ecosystems to provide energy, carbon, nitrogen and nutrients. The productivity of the forest is directly related to the degree of degradation of the organic matter (Toutain 1987). For these reasons numerous authors have made investigations on the importance of the fungi and the degrees of decomposition of litter from diverse trees, among them: Hering (1967) studied the decomposition of litter for fungi in oak trees; Rihani et al. (1995) studied the decomposition of litter for fungi and soil fauna in *Fagus sylvatica*; Sankaran (1993), studied the litter decomposition of several tropical trees.

Fungal diversity of pine leaves is very high. Marmolejo and Minter (unpublished data) found in the literature more than 2,600 records of fungi associated with pine needles.

Many of the studies made about fungi on pine litter have been made in Europe and Asia, among this works are to mention Kendrick (1958) who studied the micromycetes of the litter of *Pinus sylvestris;* Hayes (1965) Lists ca. 90 species from unsterilized and surface-sterilized litter of *Pinus sylvestris*; Tubakai and Saito (1969) studied three fungi isolated from fallen *Pinus densiflora* leaves in Japan; Mitchell et al. (1978) studied the colonization of Pinus sylvestris needles by fungi. Finally Meulder

and Meulder (1997) studied the occurrence of microfungi on needles of *Pinus sylvestris* and *Pinus nigra* and found 18 taxa in the area of Antwerp, Belgium.

In Mexico, excepting the works of Heredia (1987, 1994) about fungi associated to three species in a cloud forest and the study of Vidal-Gaona et al. (1997) about fungi associated to litter of *Quercus crassifolia*, there are not antecedents on fungi related to the litter of pines, for what this study represents the first direct contribution within this research line.

The objective of this study was the determination of distribution's patterns of fungi on needles and litter of three pine species in Nuevo León, Mexico.

Material and methods

For this study three different types of pine needles of *Pinus pseudostrobus*, *P. cembroides*, and *P. arizonica* var. *stormiae* were sampled every two weeks during one year. The locations where these samples were collected are described in Table 1.

Table 1. Localities of study.

1.	Bosque Escuela, Iturbide Nuevo León, Pine forest with *Pinus pseudostrobus* Lindl. Elevation: 1630 m a.s.l.
2.	Pablillo, Galeana, Nuevo León, Pine forest with *Pinus arizonica* var. *stormiae* Mart. Elevation: 2000 m a.s.l.
3.	Pablillo, Galeana, Nuevo León, Pine forest with *Pinus cembroides* Zucc. Elevation: 2000 m a.s.l.

The types of sampled pine needles were:
- dead needles, that were still attached to the twigs
- "hanging needles" (already detached needles, that were hanging on the existing vegetation and were not in contact with the pine litter layer)
- needles of the pine litter layer, that were not yet discolored.

Needles were brought to the laboratory, and were kept under moist conditions during one month. After this time, needles were kept in a freezer for one week to prevent acari infestation. After this, samples were examined under microscope for fungi.

Results

30 taxa of fungi were identified on the three type of needles studied. An account of all the species is presented in Table 2.

Table 3 presents the absolute frequence of all the fungi found in this study on the three types of needles on the considered pine species. Ten species were found on *Pinus cembroides*. *Rhizosphaera kalkhoffii*, *Pestalotiopsis stevensoni,* and *Preussia*

sp. were the most common fungi found on attached needles. *Verticicladium trifidum* and *Discosia strobilina* were the most common on detached needles on the soil. *Desmazierella acicola* (Teleomorph from *V. trifidium*) was fruiting from October to December on all the three types of needles.

Fifteen species were found on *Pinus pseudostrobus. Leptostroma durissimum, Niesslia exilis,* and *Marasmius androsaceus* were the most common fungi found on attached needles. *Leptostroma durissimum* and *Discosia strobilina* were the most common on detached needles on the soil.

On *Pinus arizonica* var. *stormie*, 18 taxa were found. Althought, this pine species registered the highest diversity, this pine also presented the lowest absolute frequencies.

Well definied patterns of fungi were observed for the three pine species. Only *Discosia strobilina* and *Preussia* sp. were found on all the three pine species. *Coniochaeta lignaria, Desmazierella acicola, Discosia strobilina, Niesslia exilis, Pestalotiopsis stevensoni,* and *Verticicladium trifidum* are first reported from Mexico.

Table 2. Found Taxa.

Aureobasidium pullullans (de Bary) Arnaud
Chaetomium sp.
Chloridium sp.
Coniochaeta lignaria (Grev.) Cooke
Desmazierella acicola Lib.
Discosia strobilina Libert
Fusarium lateritium Nees ex Fr.
Herpotrichia sp.
Lachnum sp.
Leptomelanconium pinicola (Berk. & M.A. Curtis) R.S. Hunt
Leptostroma durissimum Cooke
Leptostroma sp.
Lophodermium australe Dearn.
Marasmius androsaceus (L. ex Fr.) Fr.
Meloderma desmazieresii (Duby) Darker
Mytilinidium mytilinellum (Fr.) Zogg
Niesslia exilis (Albertini & Schwein.) G. Wint.
Oidiodendron griseum Robak in Melin & Nannf.
Penicillium sp.
Pericona byssoides (Pers.) Fr.
Pestalotiopsis funerea (Desmaz.) Steyaert
Pestalotiopsis stevensoni (Peck) Nag Raj
Phoma sp.
Preussia sp.
Rhinocladiella sp.
Rhizosphaera kalkhoffii Bubák
Scolecobasidium sp.
Septonema sp.
Sphaeropsis sapinea (Fr. ex F.r) Dyko & Sutton
Verticicladium trifidum Preuss.

Table 3. Absolute frequence of the studied fungi. A= attached needles, H= hanging needles, D= detached needels in litter layer.

Pinus	*cembroides*			*pseudostrobus*			*arizonica*		
	A	H	D	A	H	D	A	H	D
Aureobasidium pullullans	5	6	0						
Chaetomium sp.							1	0	1
Chloridium sp.							1	0	1
Coniochaeta lignaria	1	0	1	3	0	0	7	7	4
Desmazierella acicola	3	4	5						
Discosia strobilina	0	1	12	0	0	6	0	0	1
Fusarium lateritium							1	0	0
Herpotrichia sp.				0	0	7			
Lachnum sp.							3	1	0
Leptomelanconium pinicola							6	5	1
Leptostroma durissimum				23	25	18			
Leptostroma sp.							0	0	1
Lophodermium australe				4	0	1			
Marasmius androsaceus.				10	9	1	1	1	0
Meloderma desmazieresii				2	3	3			
Mytilinidium mytilinellum				0	0	3			
Niesslia exilis				12	9	2	1	0	1
Oidiodendron griseum				1	0	0	0	0	1
Penicillium sp.							2	6	4
Pericona byssoides	8	7	3				1	0	0
Pestalotiopsis funerea							3	2	1
Pestalotiopsis stevensoni	15	17	12				1	0	0
Phoma sp.	0	2	0						
Preussia sp.	14	19	6	2	6	2	5	2	0
Rhinocladiella sp.				3	1	0	1	1	0
Rhizosphaera kalkhoffii	18	14	2						
Scolecobasidium sp.				1	0	0			
Septonema sp.							0	0	1
Sphaeropsis sapinea				3	0	0			
Verticicladium trifidum	1	1	20	0	0	1			

Acknowledgements

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Phomopsis occulta as a landscape pathogen of Douglas-fir

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Abstract

Phomopsis occulta was isolated from a mature Douglas-fir (*Pseudotsuga menziesii*) in Madison, Wisconsin in 1999. This fungus had not previously been documented on Douglas-fir in Wisconsin. In addition, any evidence verifying pathogenicity of the fungus on Douglas-fir had not been published. For these reasons, a greenhouse inoculation experiment was conducted using a *P. occulta* isolate from the mature symptomatic Douglas-fir. Four treatments were used, including inoculation of both wounded and nonwounded shoots and wounded and nonwounded controls. Wounded Douglas-fir shoot tips frequently developed shoot blight symptoms and died. The pathogen was reisolated from both symptomatic and asymptomatic shoots that had been wounded and inoculated. These results verify that *P. occulta* is a shoot blight pathogen of Douglas-fir, indicate the potential importance of wounding to subsequent symptom development, and suggest the possibility that *P. occulta* can persist on or in asymptomatic tissue as a latent pathogen.

Some recent findings concerning *Phacidium infestans* in Northern Sweden

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Abstract

Since the 1990's green tree retention has been common in Sweden. This means that a lot of low shelter trees and some high shelter trees are left after harvest. Therefore it is of interest to find out how this environment affects pathogens. In Northern Sweden snow blight, *Phacidium infestans*, is one pathogen that theoretically may be affected by this change in environment on new clear-cuts. I have set up several experiments, which still are active, and now I present only some preliminary results.

Snow blight infection vs clear-cut area

The mycelial growth on 60 cm high Scots pine, *Pinus sylvestris*, seedlings were measured in controlled inoculation experiments (Fig. 1) on 18 well-defined different sized clear-cuts in Swedish Lapland (64°N, 16–18°E). After three winters there was a slightly significant ($p=0.0432$) negative relation between clear-cut size and the infection height (Fig. 2a). The infection width showed a negative, but not significant, trend versus size of clear-cut (Fig. 2b). Snow depth and size of clear-cut showed a curved relation with a minimum at about 15 ha (Fig. 2c). A significant ($p=0.0393$) relation between snow depth and infection height was found, but only a trend ($p>0.05$) concerning the infection width. There was a large variation between years, which means that this experiment must be repeated some more winters before any conclusions can be drawn.

Snow blight infection vs stand residuals

During the winters 1998/99, 99/00, 00/01, totally 45 pair-wise controlled comparisons situated at Svartberget Experimental Station, Vindeln (64°14'N, 19°46'E) have been set up and analysed. The experiment was arranged so that one inoculated 60 cm high Scots pine seedling was placed together with one "secondary seedling" within 30 cm (Fig. 1). This arrangement was repeated both with and without low shelter, consisting of 2 m Birch (*Betula* sp.) saplings. The result varied highly between the years but as an average the mycelial growth within these "inoculation units" was 31 cm wider (horizontally) in the shelter ($p<0.001$). "Secondary seedlings" with shelter

were infected to 40% higher proportion than those standing without shelter ($p<0.001$). There was now significant difference in snow depth.



Figure 1. The "inoculation unit" used by the author in most of the experiment concerning *Phacidium infestans* in Northern Sweden. As inoculumn 2 g of dry infected needles enclosed in a polyester bag with 1 mm masks is used. The pines are approx. 50 cm high detached Scots pine (*Pinus sylvestris*) shoots from an infection free area.

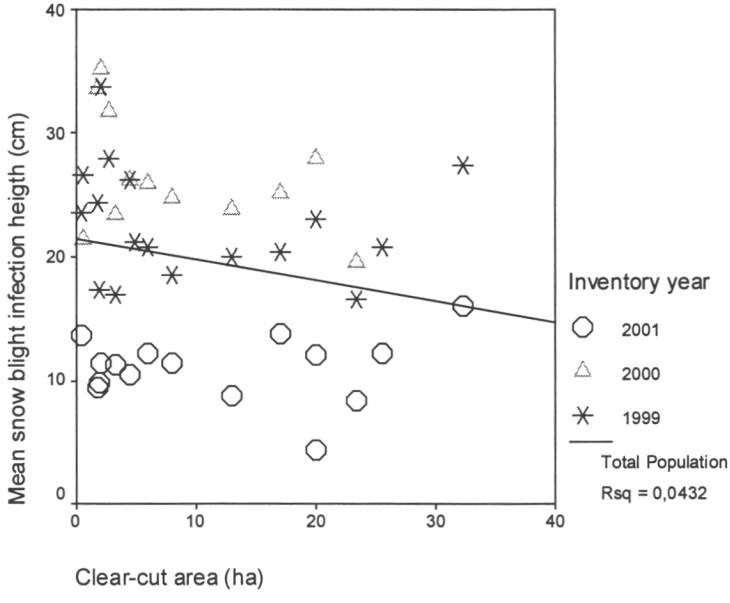


Figure 2a. Mean snow blight infection height versus clear-cut area in Swedish Lapland the winters 1998/99–2000/01.

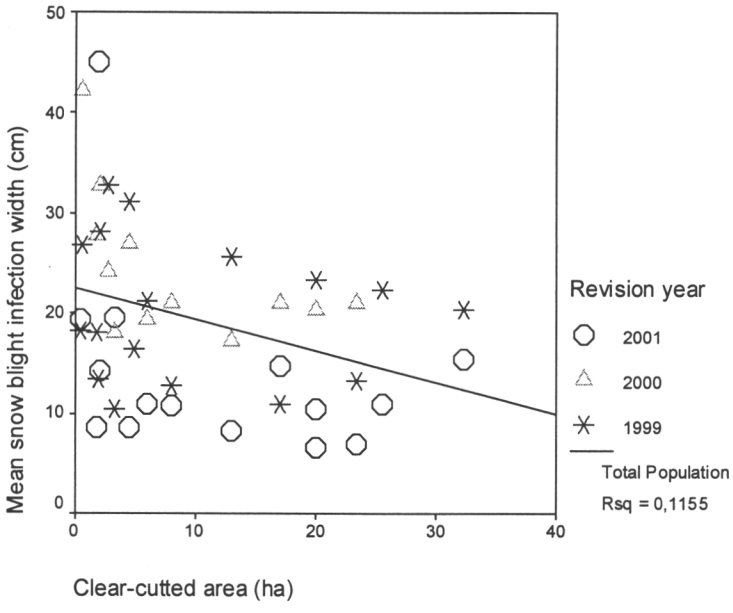


Figure 2b. Mean snow blight infection width versus clear-cut area in Swedish Lapland the winters 1998/99–2000/01.

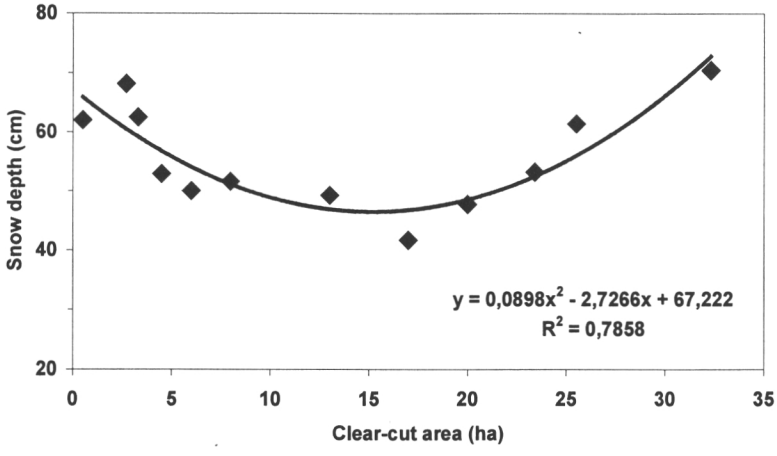


Figure 2c. Mean snow depth 1998/99–2000/01 at the investigated clear-cuts in Swedish Lapland.

Effect of fresh logging slash

In an inoculation experiment near Fredrika in Swedish Lapland, (64°03'N, 18°28'E) the mycelial growth with or without fresh logging slash from Scots pine was investigated. The slash was fresh in the way that the needles still were green and could serve as nutrient source for the pathogen. Already after one winter the infection width was 17 cm ($p<0.001$) wider within the slash than without, and this difference

was at the same level after next winter although the width now was 62 cm within the slash compared to 47 cm without slash ($p<0.001$).

Effect of high shelter trees

At Svartberget Experimental Station, Vindeln (64°14'N, 19°46'E) the mycelial growth of snow blight was tested within a seed tree stand (160 stems/ha) and on an open 1 ha clear-cut during the winter 1998/99–2000/01. In each environment 18 replications of the "inoculation unit" (Fig. 1) were set out each late autumn just before the first snow. In the seed tree stand, the "inoculation units" were placed at three different distances from the seed trees (0.5, 1 and 3 m). The snow depth (mean of 120 measurements during three winters) was 72 cm on the clear-cut compared to 53 cm in the seed tree stand ($p<0.001$). As an average over all three winters, there was significantly higher (28 cm) snow blight infection on the clear-cut area compared to 18 cm in the shelter ($p<0.001$) and significantly ($p<0.001$) higher proportion infected secondary saplings (0.5) on the clear-cut area compared to in the shelter (0.3).

Infection potential through infected needles

As a graduate student project, we have studied the ability for the snow blight fungus to infect through ascospores in a environment with or without low shelter. Preliminary data indicate that ascospore infection occur at least 40 m from the source. In a wind thunnel experiment we have collected ascospores at a distance of 100 m from the source.

Phylogenetic analysis of *Guignardia cryptomeriae* based on rDNA-ITS sequences

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Abstract

In order to infer the taxonomic placement of *G. cryptomeriae*, we determined the sequences of the rDNA-ITS region for isolates of *G. cryptomeriae* and some related fungi. Phylogenetic analysis among these sequences revealed that *G. cryptomeriae* was not distinct from *Botryosphaeria dothidea* and *B. berengeriana*. Phylogenetic analysis among these sequences and *Botryosphaeria* sequences obtained from the DNA databank showed that all isolates of *G. cryptomeriae* were included in a *Botryosphaeria* main clade. These results strongly suggested that *G. cryptomeriae* should be transferred to the genus *Botryosphaeria*.

Introduction

Shoot blight is one of the most important diseases of the Japanese cedar (*Cryptomeria japonica*). The causal agent, *Guignardia cryptomeriae*, was first described in 1950 (Sawada 1950). Some taxonomists assumed that this fungus might be placed in the genus *Botryosphaeria*, but further studies have not been conducted. Consequently, the taxonomy of this fungus remains unclear.

The purpose of this study is to infer the taxonomic placement of *G. cryptomeriae* based on molecular phylogenetic analysis. We determined sequences of the rDNA-ITS region for isolates of *G. cryptomeriae, B. laricina,* which is a causal agent of shoot blight in the Japanese larch (*Larix kaempferi*), an undescribed species of *Botryosphaeria* (tentatively nicknamed the "*Botryosphaeria* large-spore species"), which is a causal agent of shoot blight in the Japanese cypress (*Chamaecyparis obtusa*), and two species of *Botryosphaeria* (*B. dothidea* and *B. berengeriana*), which were isolated from several broadleaved trees. Phylogenetic analyses were made among these sequences and additional sequence data of *Botryosphaeria* spp. taken from the DNA databank.

Material and methods

Fungal strains and DNA isolation

The cultures that were used in this research are listed in Table 1. Total genomic DNA was extracted from mycelium grown in PD broth by the method of Lee and Taylor (Lee and Taylor 1990).

Table 1. Isolates used in this study.

	Isolate	Host
G. cryptomeriae	MA3	Japanese cedar
	MA7	Japanese cedar
	MA12	Japanese cedar
	MA18	Japanese cedar
	MA19	Japanese cedar
	MA8	Japanese cypress
	MA10	Japanese cypress
	MA21	Japanese cypress
	M-T-1	Japanese thuja
B. laricina	GC56	Japanese larch
	GC59	Japanese larch
	GC78	Japanese larch
	GC79	Japanese larch
Botryosphaeria sp.	B-H-1a	Japanese cypress
(~*Botryosphaeria* large-spore species")	B-H-3	Japanese cypress
	B-H-5	Japanese cypress
B. dothidea	M-C-1	Cherry tree
	M-C-6	Cherry tree
	M-C-11	Cherry tree
	B-MU-1	Japanese apricot
	B-JP-1	Japanese pear
B. berengeriana	B-P-1	Peach
	B-P-8	Peach

PCR amplification and sequencing

The nuclear ribosomal ITS1-5.8S-ITS2 region was amplified by polymerase chain reaction (PCR) using conditions and primers (ITS5 and ITS4) described in White et al. (1990). Nucleotide sequences of the PCR products were obtained by an Applied Biosystems 377 DNA sequencer, using the Big Dye Terminator Reaction Kit (Applied Biosystems).

Sequence analysis

Sequences were aligned using the Clustal W (Thompson et al. 1994). The alignment was checked visually, and minor adjustments were made manually. Phylogenetic analysis was performed with PAUP 4.0 (Swofford 2000) for maximum parsimony.

Results

PCR amplification of genomic DNA using the primers ITS5 and ITS4 generated a product of approximately 500 bp for all the isolates. The phylogenetic relationships among *G. cryptomeriae* and *Botryosphaeria* spp. were inferred from heuristic parsimony analysis of the aligned ITS sequences. The analysis showed that tested isolates were divided into four major groups. The first group consisted of 4 isolates of *G. cryptomeriae,* 4 isolates of *B. dothidea,* and all isolates of *B. berengeriana.* The second group consisted of 5 isolates of *G. cryptomeriae* and 1 isolate of *B. dothidea.* The third group consisted of all isolates of *B. laricina.* The fourth group consisted of all isolates of the "*Botryosphaeria* large-spore species".

The sequence data of *G. cryptomeriae* were aligned and analyzed with *Botryosphaeria* spp. sequences obtained from the DNA databank. The results showed that all isolates of *G. cryptomeriae* were included in a *Botryosphaeria* main clade.

Discussion

Although isolates of *G. cryptomeriae* were divided into two groups, both groups contained isolates of *Botryosphaeria*. Furthermore, phylogenetic analysis with *Botryosphaeria* sequences obtained from the DNA databank showed that all isolates of *G. cryptomeriae* were included in a *Botryosphaeria* main clade. These results strongly suggest that *G. cryptomeriae* should be transferred to the genus *Botryosphaeria*.

Acknowledgments

We thank Dr. S. Ito of Mie University and Dr. S. Kanematsu of the National Institute of Fruit Tree Science for providing the fungal isolates.

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Diversity of endophytic fungi of single Norway spruce needles and their role as pioneer decomposers

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Abstract

The diversity of endophytic fungi within single symptomless Norway spruce needles was found to be low at species level as 90% of isolates were identified as *Lophodermium piceae*. The number of individual infections was, however, high. The diversity within detached needles that retained their green color had similar diversity even after a long incubation, but after the loss of colour the needles went through a considerable population change and became inhabited by only one or few individuals belonging to species other than *L. piceae*. The ability of needle endophytes to act as pioneer decomposers was found to be considerable, which, however, does not concern *L. piceae* as this fungus was not found to persist in needles during decomposition.

Keywords: endophytic needle fungi, decomposition, *Lophodermium piceae,* Norway spruce, ITS-RFLP, RAMS

Introduction

In Norway spruce (*Picea abies* (L.) Karst.) the highest diversity of fungi appears to be found in needles as epiphytes and endophytes (Müller and Hallaksela 2000). Sieber (1988) detected one hundred endophytes from Norway spruce needles in Switzerland. The most frequently occurring species is *Lophodermium piceae* (Fuckel) Höhn. inhabiting often more than 50% of the needles (Barklund 1987, Sieber 1988, Solheim 1994, Müller and Hallaksela 1998a). Other less known endophytes occurring in needles of Norway spruce are *Tiarosporella parca* (Berk. & Broome), *Sclerophoma pythiophila* (Corda) Höhn., *Rhizosphaera kalkhoffii* Bubák, *Thysanophora penicillioides* (Roum.) W. B. Kendr. and *Lirula macrospora* (R. Hartig) Darker (Sieber 1988, Solheim 1994, Müller and Hallaksela 1998a).

The significance of *L. piceae* and other Norway spruce needle endophytes to their host has not been solved, but it is clearly not an opportunistic pathogen as it occurs more commonly in young, healthy stands than in stressed, diseased ones (Barklund 1987, Sieber 1988). So far it is not known whether these endophytes are activated from their dormant state within the needle by unusual events like herbivory, attacks by other microbes or by needle senescence. Neither do we know whether these endophytes protect needles against insects or needle pathogens, allow redistri-

bution of nutrients prior to needle cast or are just "waiting" for needle senescence to be the first pioneers during needle decomposition, the last hypothesis of which is tested in a study published recently (Müller et al. 2001). Here we review shortly the results and provide an additional illustration of that study.

Results

Fresh green needles

In the analysis of Norway spruce endophyte diversity Müller et al. (2001) isolated fungi from thin (thickness 0.2 µm) slices of surface sterilized healthy needles. 52% of fresh green needles were infected with endophytic fungi. The fungi were identified to species level by comparing their restriction fragment length polymorphism (RFLP) patterns of PCR-amplified Internal Transcribed Spacer (ITS) sequences to known reference isolates. Using restriction enzymes *Msp*I and *Cfo*I it was possible to identify the majority, i.e. 147 out of 182, isolates obtained from fresh green needles. In single densely infected needles up to eight different ITS-types could be found. The majority of isolated endophytes had similar ITS-patterns as found among the *L. piceae* reference isolates (Müller et al. 2001).

The within species diversity of fungi was analyzed using random amplified microsatellite (RAMS) analysis (Zietkiewicz et al. 1994, Hantula et al. 1996) with two primers (Müller et al. 2001). In this analysis even isolates from adjacent needle slices were almost without exception of different haplotypes (see Fig. 1).

Detached needles

Changes in endophyte communities of detached needles were analyzed from samples incubated on sterilized soil. *L. piceae* remained dominant after needle detachment as long as the needles retained their green color. When the needles became

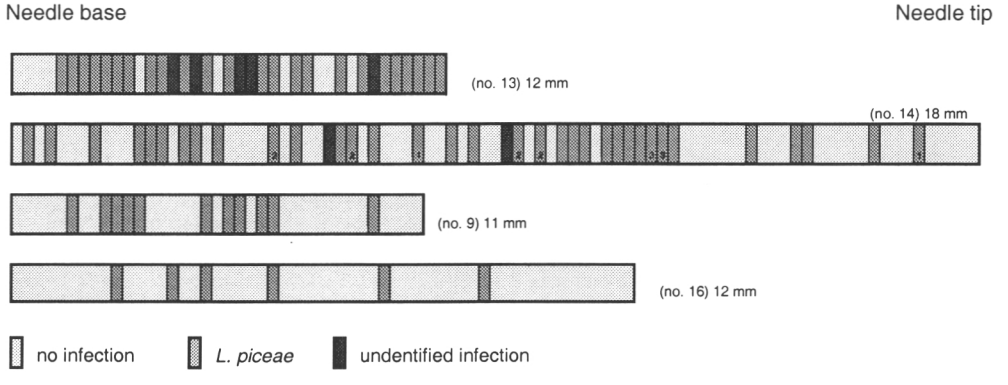


Figure 1. Examples of endophytic infections within single healthy green needles.

brown *L. piceae* made way for *T. parca*, *S. pythiophila* and other (unidentified) endophytes, most of which were not found in fresh green needles (Müller et al. 2001).

In the analysis of Müller et al. (2001) the fungal diversity within needles remained extremely high (i.e. only genetically dissimilar isolates were found) during incubation as long as the needles were green or partly green. After turning brown or black, single fungal haplotypes overgrew the needle and all isolates had the same or only few haplotypes.

Endophytes as primary decomposers

The role of endophytes in primary decomposition was measured as a difference between weight loss of needles incubated on sterilized soil and needles incubated on live soil. After twenty weeks incubation at 15°C a dry weight loss of 13-17% was found in initially green symptomless needles. The dry weight loss of needles on the live soil was somewhat higher after 8 and 20 weeks of incubation but the difference was not statistically significant (Müller et al. 2001).

On average a proportion of 13% of slices of fresh green needles were infected at the start of the incubation experiment. The infected proportion did not change significantly in those needles which remained green during the 20 weeks incubation but in decoloured needles the infected proportion increased considerably (ending from 36% to 93%; Müller et al. 2001).

Conclusions

In the analysis of Norway spruce endophytes (Müller et al. 2001) the two hypotheses suggested by Carroll and Petrini (1983) for endophytes proved true regarding to the dominant Norway spruce needle endophyte *L. piceae*. Norway spruce needles commonly inhabit numerous genetically different endophytic individuals, the majority of which do not contribute significantly to the decomposition of the needles after their abscission. These results are in agreement with earlier hypotheses made by Carroll and Petrini (1983) based on variation in substrate utilization capacity among isolates of single species in single leaves. However, the study produced another as important but highly unexpected result. This was the drastic decrease of fungal genotype diversity in needles during the early phase of the decomposition process. This suggests that the endophytic mycota of plants may in fact be more diverse in terms of the numbers of different genotypes than the saprophytic mycota of the same tissue. It would be important to test whether a similar result would also be obtained from other plant systems.

The results of Müller et al. (2001) confirmed that *L. piceae* is the most frequent endophyte in needles of *P. abies* and that it is a highly diverse species (Müller and Hallaksela 1998a). Further information was obtained from individual level diversity within single needles, which shows that endophytic infections occupy only a small spatial domain of the needle and the needles may thus accommodate a large

number of genetically different fungal individuals. This infection pattern is essentially similar to that of *R. parkeri* in needles of Douglas fir (McCutcheon et al. 1993, Carroll 1995).

As *L. piceae* disappear from decaying needles it clearly has no marked role in the decomposition of spruce needles and is likely to become overgrown by other fungi after needle cast. This is supported by observations that fruiting of *L. piceae* on fallen brown needles is less common than could be expected from the infection frequency of *L. piceae* on green intact needles (Solheim 1994). Thus, it can be concluded that other less frequent endophytes of Norway spruce needles like *S. pythiophila* and *T. parca* seem to be responsible for the initial decomposition process. The ecological function of *L. piceae* in needles of *Abies alba* may be different from that in *P. abies* as microscopic investigations of decomposing fir needles suggest *L. piceae* to be a primary saprophyte (Gourbière et al. 1999).

The result of Müller et al. (2001) on the significance of endophytic needle fungi during the initial needle decomposition is in accordance with earlier results obtained by Mitchell and Millar (1978) on the decomposition of Corsican pine needles. However, the saprophytic ability of *P. abies* needle endophytes varies largely (Müller et al. 2001). This is in good agreement with earlier findings on needle endophytes of *A. alba* (Canavesi 1987).

The overall results of Müller et al (2001) strengthen the hypothesis that *L. piceae* occupies a similar ecological niche in Norway spruce needles as *R. parkeri* in needles of Douglas fir because *L. piceae* (1) occupies small spatial domains in the needles, (2) is genetically highly diverse and (3) seems not to have an essential role during needle decomposition (Carroll 1995, McCutcheon et al. 1993). Taking this in consideration and remembering that *R. parkeri* has been shown to act antagonistically toward gall midges (*Contarinia* spp.) in Douglas fir (*Pseudotsuga menziesii*) needles (Carroll 1986), it would be intriguing to speculate whether *L. piceae* would have a similar role in Norway spruce. Evidence for a mutualistic relationship between *L. piceae* and Norway spruce, however, is not available.

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Black yeast-like fungi of Norway spruce

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Abstract

During two studies on the diversity of endophytic fungi in Norway spruce (*Picea abies*) we obtained 66 dark yeast-like fungal isolates from wood and needles. Some of these isolates could be identified by their morphology as *Exophiala* sp. and *Hortaea werneckii*. Representatives of these genera are known to be human pathogens causing skin mycoses. We characterized ourisolates and several culture collection (CBS) strains of these genera using restriction fragment length polymorphism (RFLP) analysis of their 18S rDNA.

According to their RFLP-patterns the isolates showed to be diverse. None of them are closely related to culture collection strains of *H. Werneckii* or of five *Exophiala* species used for comparison in this study. Black yeast-like fungi could not be cultured from spruce timber available in timber yards.

Needle disease on *Taxus baccata* caused by *Cryptocline taxicola*

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Abstract

The paper reports a conspicuous appearance of *Cryptocline taxicola* at several locations in Germany in the year 2000. The fungus is one of the few parasitic fungi on English Yew and its characteristics and the symptoms of the needle disease are described.

Keywords: *Cryptocline taxicola, Taxus baccata*, English Yew, needle disease

Introduction

In contrast to other European tree species, English Yew *(Taxus baccata)* has a conspicuously low affinity for fungi (Schütt et al. 1994). Not only does this species apparently grow entirely without the presence of mycorrhizal fungi, but it also exhibits little tolerance for substrate-specific saprophytic or parasitic fungi (De Vries et al. 1996). Therefore it is all the more remarkable that a characteristic needle fungus with obvious parasitic potential has occurred more often in recent years. The appearance of a needle disease on English Yew, which was found at several places in Germany and caused several requests for determination especially in the year 2000, seems to be worth a short communication. Information on the disease symptoms and on the causal fungus are presented.

Material and methods

The material for examination was taken from an infected tree in Hamburg, which is described in detail by the portrayal of a botanical garden (Biederstedt 2001). Investigations of the symptoms was carried out macroscopically by hand lens while the examination of the fungal structures required histological preparation. After embedding needle pieces in glycol-methacrylate (GMA, Kulzer method) needle cross sections of 8 µm were prepared and stained with a 1% aqueous solution of thionine. For cultivation of the fungus malt-extract-agar (MEA) containing 2% malt and 2% agar was used, and cultures were stored at 21°C in 7 cm Petri-dishes.

Results

Symptoms

The first disease symptoms consist of single necrotic spots mainly on the current year's needles. Not all needles are damaged to the same extent, heavily damaged needles being found in the direct vicinity of green, apparently healthy needles (Fig. 1). However, as the disease progresses, the affected shoots turn completely brown and both the upper and under side of needles is covered by numerous black fruit bodies of the fungus (Fig. 2). In the presence of sufficient moisture, the conidia of the fungus become visible as a white to cream-colored pustule on the ripe acervuli which rupture the epidermis (Figs. 3, 4). During dry conditions, the fruit bodies have a black, shrunken appearance (Fig. 5).



Figure 1.

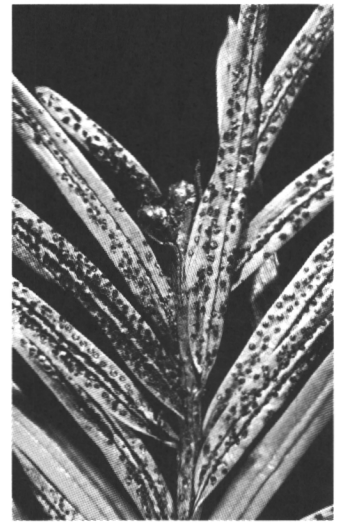


Figure 2.

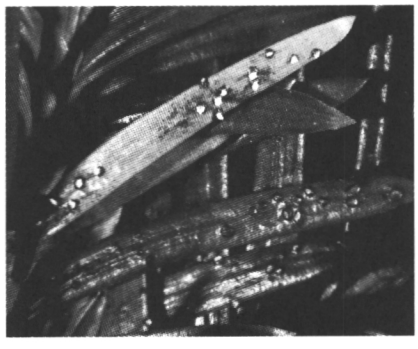


Figure 3.

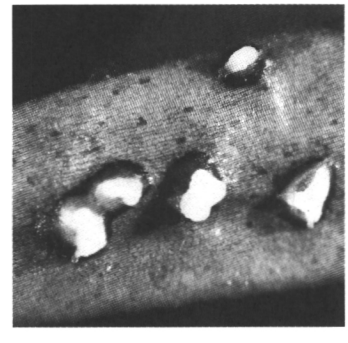


Figure 4.

Causal fungus

The dark gray to black, spherical fruit bodies, measuring 250-450 µm in diameter and up to 150 µm in height, develop beneath the epidermis, as shown in the cross section (Fig. 6). The needle epidermis is first pushed upwards in a pustular manner and then ruptures irregularly when the fruit bodies become ripe (Figs. 4, 5). During spells of wet weather the egg-shaped to elliptical, smooth-walled and hyaline conidia are produced, measuring 12-18 x 5-8 µm (Fig. 7). The determination of the fungus based on its demonstrated characteristics leads to *Cryptocline taxicola* (All.) Petr (Petrak 1925). On malt extract agar, the fungus exhibits felty to woolly, olive to gray-brown aerial mycelium (Fig. 8) and shows linear growth of 1,4 mm per day at 21°C on MEA.

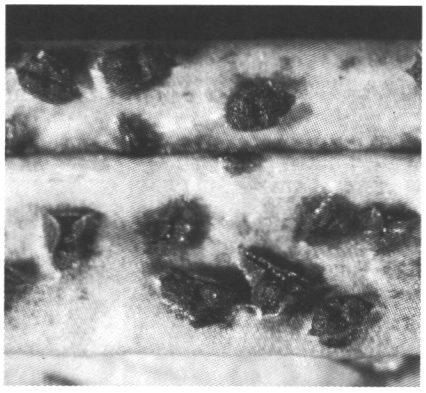


Figure 5.

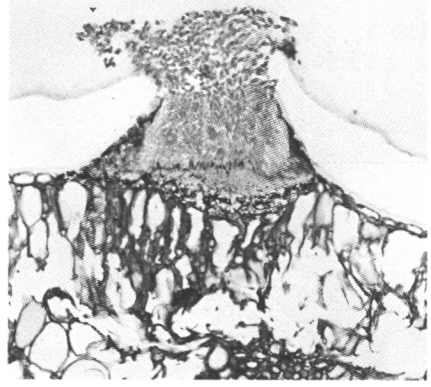


Figure 6.

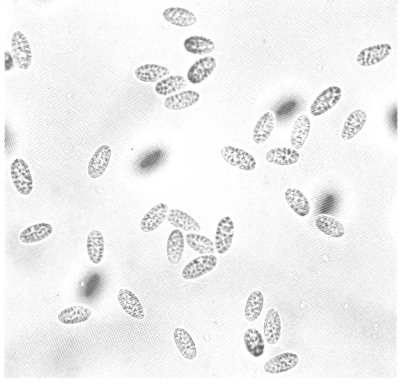


Figure 7.

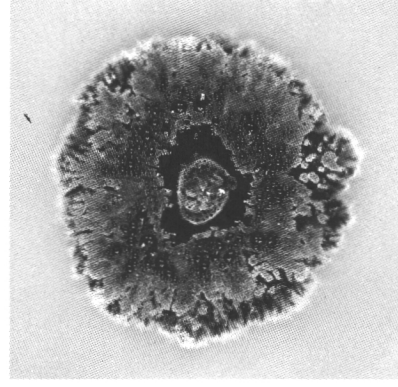


Figure 8.

Discussion

The spectrum of fungi reported specifically for English Yew in literature is small and limited mainly to the following species: *Botryosphaeria foliorum, Chaenothecopsis caespitosa, Diplodia taxi, Dothiora taxicola, Guignardia philoprina, Phacidium taxicola, Physalospora gregaria, Phytophthora cinnamomi* (Brandenburger 1985, De Vries et al. 1996, Ellis and Ellis 1997, Peace, 1962, Schütt et al. 1994, Von Arx and Müller 1954). Few of these species seem to be able to threaten their host tree seriously. Among the needle fungi only *Diplodia taxi* is reported to possibly have lethal effects (Schütt et al. 1994).

Cryptocline taxicola (All.) Petr. was first described in 1896 as *Gloeosporium taxicola* All., and PETRAK placed it in the genus *Cryptocline* in 1925 (Petrak 1925). Among the needle diseases it was judged to be of less importance. Recent observations show that it obviously possesses a larger degree of parasitic ability. Further investigations will show if this disease can be more harmful than previously thought.

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