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RECENT RESEARCH ON SCLERODERRIS CANKER OF CONIFERS

Neuere Forschungen über das Scleroderris-Triebsterben der Koniferen

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IUFRO

Working Party

S2.06-02

Canker Diseases - Scleroderris

Proceedings of Meeting in Salzburg/Austria and Ljubjana/Yugoslavia, September 1986

compiled by

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Canker Diseases - Scleroderris

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Abstract

An overview is given about the past activities of the IUFRO Working Party "Canker Diseases - Scleroderris" (S2.06.02) since the establishment in 1971. Between 1972 and 1986 five meetings were held. Papers and reports were published in proceedings, which represent the current knowledge of the respective year. Future activities of the Working Party will cover also other canker and shoot blight diseases. The name of the working party has been changed in "Canker and Shoot Blight of Conifers".

The fungus Gremmeniella abietina (Lagerb.) Morelet and the damage caused by it have been known in various European countries since about 100 years. In the sixties a first serious epidemic was observed on black pine (Pinus nigra Arnold) in the Netherlands and in northern Germany. A few years later, around 1972, heavy losses were caused by this fungus also on red pine (Pinus resinosa Ait.) in north-eastern USA and in eastern Canada. At the same time the disease was described also from Japan on Sachaline fir (Abies sachalinensis (Fr. Schmidt) Mast.)

It was at that time when several scientists met at the occasion of the XVth IUFRO World Congress, 14-20th March 1971, at the University of Florida, Gainesville, USA, and presented in a special working group of section 24, forest protection, papers about the Scleroderris canker disease of conifers (EJFP 1972). The papers dealt with the pathogen and

disease symptoms, the distribution and hosts in Europe and North America, the epidemiology and environmental factors influencing the outbreak, but also differences in attack between pine species and provenances, and control measures.

The importance of the Scleroderris canker disease for conifers of the temperate zone was emphasized by the fact that in conjunction with the reorganization of IUFRO at the XVth IUFRO World Congress at Gainesville, a new Working Party on Canker Diseases, Scleroderris, was proposed in Division 2, Subject Group S2.06. J. Gremmen, the Netherlands, was nominated as chairman, and C.E. Dorworth, Canada, as cochairman. Aim of the new working party was to promote contacts between scientists studying the Scleroderris canker disease, in order to discuss various aspects of this very important disease causing damage to conifer species throughout Europe, North America and parts of Asia.

The first session of the Working Party was held in conjunction with the 2nd International World Congress of Plant Pathology at Minneapolis, USA, September 1973. By 13 papers informations were presented about actual research activities of the Working Party members concerning infection trials, occurrence of the pathogen in the respective countries, resistance research, control measures, and especially the question, whether Scleroderris lagerbergii is an introduced pathogen in North America (Gremmen 1973).

A second session could be organized in conjunction with the 3rd International Congress of Plant Pathology at München, Fed. Rep. of Germany, August 1978. 14 members attended and presented reports about research activities in the various countries (Gremmen 1978). Gremmen summarized the actual situation of the disease in the world: "In general terms the Scleroderris situation in Europe does not give great concern during the past five years. The situation seems to be more or less stabilized. However, in North America the development is menacing since 1973, especially in the eastern part

of the USA. New information on Scleroderris was also received from Japan as well as a report on the occurrence of the fungus in western Canada." Special attention was directed on the occurrence of races of the fungus with different behaviour and pathogenicity in North America.

The third meeting of the Working Party was integrated into the International Symposium on "Scleroderris Canker of Conifers", held at Syracuse, USA, June 21-24, 1983, supported by the USDA Forest Service, the Canadian Forestry Service, and the State University of New York College of Environmental Science and Forestry. At this up to now largest Scleroderris Symposium 66 scientists from 10 countries participated and presented 40 papers in 6 sessions (Manion 1984). The proceedings of the symposium provide "(1) a review and update of the current and historical situation with regards to this disease on a worldwide basis, (2) a detailed look at the fungus causing this disease and a better understanding of the disease cycle and population dynamics of the disease, (3) new ideas on how to live with this disease considering the mangerial potentials available in light of the economic liabilities and assets of the forest system".

The fourth meeting of the Working Party was held in Austria and Ljubljana, Yugoslavia, in September 1986, and was attended by 32 participants of 13 countries. A 4 days tour in Austria was keyed to the Scleroderris problem in reforestations at high elevations. The papers presented during the tour and on a Working Party Meeting in Ljubljana on the occasion of the XVIIIth IUFRO World Congress are presented in these proceedings. We hope that they will provide further interesting informations on the Scleroderris disease and its pathogen Gremmeniella abietina.

It may be of interest to look at the publications about Scleroderris in the course of the last 100 years. More than 600 papers were published by about 330 authors (Stephan and Schulze 1987). The cumulative graphs of the number of publi-

cations between 1883 and 1986 show an obvious increase of papers since about 1965 (Figure 1). In the last 20 years

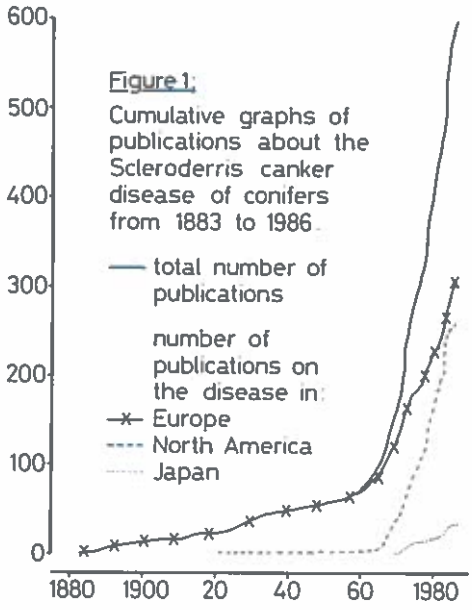

Figure 1:
Cumulative graphs of publications about the Scleroderris canker disease of conifers from 1883 to 1986.

about 85 % of all papers on Scleroderris canker disease were published. The distribution of publications on Europe, North America and Japan is interesting because it demonstrates the time of the first serious occurrence of the disease in the respective regions, and reflects the efforts to study both the disease and the pathogen, and to find possibilities for control measures. During the last 100 years European authors have published 307 papers, North American authors 260 papers, mainly since about 1965, and Japanese authors 34 papers since about 1970. In years with meetings (Minneapolis 1973, München 1978, Syracuse 1983) the highest number of papers were published, presumably stimulated by the meetings.

Until now the Scleroderris canker disease has been reported from 47 species within 7 genera of the conifer family Pinaceae. The disease occurs in about 25 countries in Europe, North America and East Asia.

All these facts mentioned above justify a continuation of activities of the IUFRO Working Party, for which the following chairmen and cochairmen were responsible since 1972:

1972 - 1976	chairman:	J. Gremmen, The Netherlands
	cochairman:	C.E. Dorworth, Canada
1977 - 1981	chairman:	J. Gremmen, The Netherlands
	cochairman:	D.D. Skilling, USA
1982 - 1986	chairman:	D.D. Skilling, USA
	cochairman:	B.R. Stephan, Fed. Rep. Germany
1987 - 1990	chairman:	B.R. Stephan, Fed. Rep. Germany
	cochairman:	T. Kurkela, Finland

During a business meeting of the Working Party at Ljubljana 1986 the members present at the meeting were of the opinion to enlarge the scope of the Working Party also to other canker diseases of conifers in order to give an opportunity of cooperation also to scientists, who work on canker diseases and pathogens others than Gremmeniella abietina and do not find a relevant IUFRO Working Party. The Working Party s new name is now "Canker and Shoot Blight of Conifers" (IUFRO S2.06.02).

Acknowledgement. The Working Party Meeting in Austria, September 1986, was organized by the Forstliche Bundesversuchsanstalt, Institut für Forstschutz, Vienna. For the highly interesting study tour to St. Martin, St. Michael and the Zillertal we are very grateful to Dr. E. Donaubauer and his staff. We want to thank also for the possibility to publish these proceedings by the Forstliche Bundesversuchsanstalt, Vienna.

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Stephan, B.R.; Schulze, I. (eds.), 1987: Scleroderris canker disease of conifers. Bibliography of the disease and its pathogen during the last 100 years (1883 - 1986).- IUFRO Working Party S2.06.02. Federal Research Centre of Forestry and Forest Products, Institute of Forest Genetics and Forest Tree Breeding, Grosshansdorf, Fed. Rep. of Germany. XIV + 111 pp.

Zusammenfassung

Die IUFRO-Arbeitsgruppe Rindennekrosen - Scleroderris

Es wird ein Überblick über die bisherigen Aktivitäten der IUFRO-Arbeitsgruppe "Rindennekrosen - Scleroderris" (S2.06.02) seit ihrer Gründung 1972 gegeben. Zwischen 1971 und 1986 fanden 5 Arbeitstreffen statt. Die Tagungsbeiträge wurden veröffentlicht und geben den jeweiligen Kenntnisstand über die Krankheit und ihren Erreger wieder. In Zukunft soll sich die Arbeitsgruppe auch anderen krebserregenden Rinden- und Zweigkrankheiten widmen. Daher wurde ihr Name geändert in "Krebs- und Sproßkrankheiten an Nadelholzarten".

II. The fungus Gremmeniella abietina, structure and disease symptoms

DESCRIPTION AND SYMPTOMS OF *GREMMENIELLA ABIETINA* ON *PICEA MARIANA*

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SUMMARY

The distribution of *Gremmeniella abietina* (Lagerb.) Morelet infecting *Picea mariana* (Mill.) B.S.P. in natural regeneration is restricted to an area 100 km north of Quebec City, Canada. Even with high rates of infection in two studied stands (38 and 80%), the disease is not conspicuous. This may explain the actual small known distribution area of scleroderris canker on natural spruce in North America. *G. abietina* infected one- and two-year-old shoots; cankers on branches and stems have been also observed. Fruit bodies of the fungus are usually numerous and symptoms have not been observed over 2 m from the ground. On black spruce, the fungus is morphologicaly identical to the same species on other hosts.

INTRODUCTION

The fungus *Gremmeniella abietina* (Lagerb.) Morelet (= *Ascocalyx abietina* (Lagerb.) Schläpfer-Bernhard) was first described under the name *Crumenula abietina* Lagerb. from a specimen collected on *Picea abies* (L.) Karst. (Lagerberg,1913). On this host, *G. abietina* is reported in a few European countries (Donaubauer,1972). In North America, the first and only report of *G. abietina* on *Picea mariana* (Mill.) B.S.P. and *P. glauca* (Moench) Voss was made by Smerlis (1967) in natural regeneration in the Laurentian Reserve 100 km north of Quebec city, Canada. This disease has not been reported elsewhere

in Quebec. Because spruce is widely used to reforest in Quebec, we are interested to know more about the description of the fungus and the disease on this host.

MATERIALS AND METHODS

All specimens of *C. abietina* on spruce deposited at Laurentian Forestry Centre (LFC) herbarium were studied. For each specimen, the type of fruit bodies (apothecia, pycnidia) were identified, the collection date was noted, and the following morphological characters were measured: the diameter of apothecia and pycnidia, the length and width of ascus, ascospores, and conidia.

Using information gathered by LFC Insect and Disease Survey personnel we located, on a map, the disease in spruce stands. After observation and sampling, two sample plots of 10 × 10 m were established among the regeneration in two infected black spruce stands, at an altitude of 800 m. Inside these plots, each spruce was examined for the presence of fruit bodies of *C. abietina* on diseased parts of the tree. The number of infected branches per tree, the height of the infection, and the location of infected shoots (apical or lateral) were recorded. Total height of each tree was also measured and other peculiarities were noted.

RESULTS

The fungus

Thirty-two specimens were studied: 26 specimens on *P. mariana*; 3 on *P. glauca* from natural spruce regeneration in the Laurentian Reserve; and 3 other specimens on *P. abies*, *P. rubens* Sarg., and *P. glauca* x *sitchensis* collected from trees in plantations located at the LFC forest station at Valcartier, near Quebec City.

Both types of fruit bodies were found throughout the growing season, together or separately on a given specimen. Apothecia measure about 1 mm in diameter, are dark brown becoming black when older, and

are orbiculate, sometime elongate when fruit bodies are crowded together. Ascus measure 50-116 × 4-9.5 µm. Ascospores measure 11.2-24.3 × 2-4.8 µm and are generally 3-septate, rarely more. Blackish pycnidia appear between scales of shoots and also on the peglike projections of twigs; their diameter is about 0.5 mm. Conidia are generally 3-septate, sometime up to 6-septate, and measure 15-56 × 1.8-3.7 µm. Cryptopycnidia (Cauchon and Lachance,1980) were not found.

Symptoms

All infected shoots observed did not bear needles, but fruit bodies were usually numerous. Both apical and lateral shoots were infected; the highest infection was observed at 2.3 m from the ground on an apical shoot. The fungus also causes cankers on the branches and the trunk, but we have not detected tree mortality that could be attributed to G. abietina.

In one sample plot, black spruce (20%) was dominated by balsam fir (80%). The height of spruce averaged 1.1 m (0.1-5.4 m). G. abietina was found on 10 spruce trees (38.5%) out of 26. An average of 6 branches were infected per tree (1 to 14 per tree) and 60% of the infected branches were below 0.5 m from the ground, 30% from 0.5 to 1.0 m, and 10% over 1.0 m.

In the second sample plot, black spruce (75%) was mixed with Larix laricina (Du Roi) K. Koch (25%). Spruce averaged 0.9 m (0.1-3.5 m) high. G. abietina was found on 70 spruce trees (80.5%) out of 87. Thirteen trees had their apical shoot infected. An average of 5 branches per tree were infected (1 to 15 per tree). Sixty-seven percent of infected branches were 0.5 m or less from the ground, 32% were 0.5 to 1.0 m, and 1% were higher than 1.0 m.

DISCUSSION

Morphological characters of G. abietina on black spruce in natural regeneration are similar to those described by Morelet (1980) for the same fungus on other hosts. On this basis, it is not possible to create a new form of the fungus even if Smerlis (1968b) observed differences in behavior between G. abietina on two hosts. For example,

maturation of fungal spores is very late in summer on spruce when compared with those on pine. This difference could not be fully attributed to climatic factors.

In our sample plots, G. abietina was mainly observed on shoots and branches, but the fungus also caused cankers on the trunk. Smerlis (1968a) reported that many spruce had cankers on trunks. These two observations may indicate two stages of an epedemic. In the first, shoots were infected; in the second, cankers form on the trunks. On P. abies, Barklund and Rowe (1981) demonstrated that initial infection by G. abietina takes place only on growing shoots.

Even if apical shoots are infected by G. abietina, the disease has been observed only on regeneration and more often on lower branches, the highest incident of infection was found at 2.3 m. The North American race of G. abietina on Pinus behaves the same way. On both hosts, cankers and apothecia are numerous. Further studies on race identification must be done with isolates of G. abietina on spruce.

Symptoms on Pinus are more visible than on Picea. Damages on black spruce are negligeable, even with a high percentage of the tree infected, compared to severe damages observed on Pinus. These two differences may explain the few reports of this disease on natural spruce regeneration in North America.

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ZUSAMMENFASSUNG

Beschreibung und Symptome von Gremmeniella abietina auf Picea mariana

Die Verbreitung von Gremmeniella abietina (Lagerb.) Morelet auf Picea mariana (Mill.) B.S.P. in Naturverjüngung ist auf ein Gebiet 100 km nördlich von Quebec City, Canada, beschränkt. Trotz hoher Infektionsraten in zwei untersuchten Beständen (38 und 80%) ist die Krankheit nicht auffallend. Dies mag eine Erklärung für das gegenwärtig kleine bekannte Verbreitungsgebiet der Scleroderris-Krankheit an natürlich vorkommender Fichte in Nordamerika sein. G. abietina infiziert ein und zwei Jahre alte Triebe; Krebsbefall auf Ästen und Stämmen wurde ebenfalls beobachtet. Fruchtkörper des Pilzes sind gewöhnlich zahlreich, und Symptome wurden über 2 m Höhe nicht beobachtet. An Schwarzfichte ist der Pilz morphologisch identisch mit derselben Art auf anderen Wirten.

ULTRASTRUCTURAL AND CYTOCHEMICAL CHARACTERIZATION OF *ASCOCALYX ABIETINA*

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SUMMARY

The fungus *Ascocalyx abietina* (Lagerb.) Schlaepfer-Bernhard is composed of regularly septate hyphae. Fungal cells are delimited by a thick wall which is itself surrounded by a dense fibrillar sheath. These structures may play an important role in the resistance of the fungus against unfavorable conditions. Sialic acid was detected in the sheath; this compound could be related to specific biological functions such as cell to cell repulsion and host-pathogen interactions. Mycoviruses were found in a majority of the *A. abietina* isolates tested. The ultrastructural and cytochemical peculiarities of *A. abietina* contribute to distinguish it from other Ascomycotina.

INTRODUCTION

Scleroderris canker, caused by the fungus Ascocalyx abietina (Lagerb.) Schlaepfer-Bernhard [= Gremmeniella abietina (Lagerb.) Morelet], is an important worldwide devastating coniferous tree disease (Dorworth,1972, Skilling,1981). This disease causes several management problems which have received much attention (Laidlaw,1983). However, past research efforts have been concentrated on determining the main properties of the pathogen; host range, physiological requirements, genetic variations, and epidemiology of A. abietina have been well documented (Donaubauer,1972, Dorworth and Krywienczyk,1975, Skilling, 1972). Sensitive methods of detection and strain differentiation have been developed (Benhamou et al.,1983, Dorworth et al.,1982, Ouellette et al., this issue) which improve significantly our comprehension of some biochemical properties of A. abietina. However, the role and mode of action of the fungus during disease process is still poorly understood. Knowledge of the ultrastructural features and chemical composition of the pathogen and of the mechanism of host/pathogen interactions are urgently needed.

Recent developments in cytochemical techniques has allowed cell investigations at the molecular level. Originally described by Faulk and Taylor (1971) for detecting specific antigens, these methods, which involve the affinity of a defined protein for a specific macromolecule, have been extended to various fields of cell biology (Bendayan,1984, Roth,1983, Vian et al.,1983). Colloidal gold used as an accurate probe for visualizing protein-macromolecule complexes is now well established. Gold-conjugated enzymes, lectins, and immunoglobulins are very useful in identifying various cell constituents (Chamberland et al.,1985, Vian et al.,1983).

We have recently reported on the ultrastructural properties and on some aspects of the chemical composition of A. abietina by means of gold cytochemistry (Benhamou and Charest,1986, Benhamou and Ouellette, 1986a, 1986b, 1986c, 1986d, 1986e, 1987). A general outline of the data from these various studies is presented. The discussion will be focused on the occurrence in A. abietina of unexpected constituents and on their biological function and possible role in host/pathogen interactions.

MATERIALS AND METHODS

Fungal isolates and culture conditions

Two isolates, one classified as the European race and the other the North American race by means of electrophoresis (Ouellette et al., 1987), were used in our studies. Isolates were grown at 14°C in a nutrient agar medium (Benhamou and Ouellette,1986c).

Processing of samples for electron microscopy

For transmission electron microscope investigations, pieces 1 mm³ collected from colonies of A. abietina were fixed in 0.1 M sodium cacodylate buffered 3% glutaraldehyde for 2 h at 4°C, post-fixed with 1% osmium tetroxide in the same buffer, dehydrated and embedded in Epon 812. Some samples were only fixed with glutaraldehyde. Sections collected on nickel grids, were either directly contrasted with uranyl acetate and lead citrate or further processed for cytochemical labeling.

For scanning electron microscope investigations, samples 1 cm³ cut from actively growing colonies were processed as described by Benhamou and Ouellette (1986d).

Preparation of the protein-gold complexes

Colloidal gold with particles averaging 8 nm or 15 nm in diameter was prepared according to Benhamou and Ouellette (1986a, 1986b, 1986c, 1986d).

Enzyme-gold complexes were prepared using the method outlined by Bendayan (1982). For all tests, 0.1 mg to 0.5 mg of enzyme was dissolved in 0.1 mL distilled water and mixed with 10 mL colloidal gold at appropriate pH (Table 1). After high speed centrifugation, red pellets were resuspended in 0.5 mL phosphate buffered saline (PBS) and the pH was adjusted according to the optimal pH for the enzyme.

Lectins were either directly complexed to colloidal gold as described above for enzymes or used in an indirect labeling procedure. In the latter, a protein with a high specificity for the lectin, was complexed to colloidal gold. The ovomucoid-gold complex was used for

Table 1. List of enzymes, conditions for complex formation and localization of the corresponding substrate in *A. abietina*

Enzymes	Source	Substrate specificity	pH of colloidal gold	Presence in *A. abietina*	Specific localization
α-amylase	Porcine pancreas	Glycogen	7.3	+	Electron-lucent intra-cytoplasmic vesicles
Chitinase	*Streptomyces griseus*	N-acetyl-D glucosamine	7.0	+	Cell wall
Cellulase	*Aspergillus niger*	Cellulose (Polymer of D-glucose	7.2	-	—
Exoglucanase	*Trichoderma harzianum*	β-(1→4)-D-glucans	9.3	-	—
β-galactosidase	Bovine liver	β-D-galac-tosides	7.9	+	Cell wall and cytoplasm
β-glucosidase	Almonds	Glucosides	9.3	+	Cytoplasm
Lipase	Wheat germ	Fatty acids	8.0	+	Cell wall
Pectin lyase	*Aspergillus japonicus*	Glycosidic bonds of esterified polygalacturonide chains	8.0	+	Cell wall
Pectin methyl esterase	Tomato	Methyl ester groups of polyga-lacturonic acids	7.5	+	Cell wall
Polygalactur-onase	*Aspergillus niger*	Polygalacturonic acids	8.2	+	Cell wall (external layers)

Table 2. List of lectins, conditions for complex formation and localization of the corresponding sugar residues in A. abietina

Lectins	Source	Sugar specificity	pH of colloidal gold	Presence in A. abietina	Localization
Concanavalin A (Con A)	Canavalia ensiformis (Jack bean)	α-D-mannose	8.0	+	Specific areas of cell walls and cytoplasm
Helix pomatia agglutinin (HpA)	Helix pomatia (Roman snail)	N-acetyl-D galactosamine	7.4	+	Specific areas of cell walls and cytoplasm
Lens culinaris agglutinin (LcA)	Lentil	α-mannose fucosy groups	6.9	+	Fibrillar network surrounding cell walls and "lipid bodies".
Limax flavus agglutinin (LfA)	Slug	N-acetyl neuraminic acid (Sialic acid)	-	+	Fibrillar matrix surrounding cell walls and "lipid bodies".
Lotus tetragonolobus agglutinin (LtA)	Asparagus pea	α-L-fucose	7.0	+	"Lipid bodies"
Ricinus communis agglutinin I (RcAI)	Castor bean	D-galactose	8.0	+	Cytoplasm
Ulex europaeus agglutinin (UeA)	Gorse (Triticum vulgare)	α-L-fucose	6.3	+	"Lipid bodies"
Wheat germ agglutinin (WgA)	Triticum vulgare aestivum	N-acetyl-D glucosamine	-	+	Cell wall

Protein	Source	Specificity	pH of colloidal gold		
Fetuin	Fetal calf serum	Limax flowus agglutinin	5.4	—	—
Ovomucoid		Wheat germ agglutinin	5.0	—	—

labeling with WGA and the fetuin-gold complex for labeling with the LFA (Table 2).

The indirect protein A-gold approach was used for labeling with antibodies. Protein A (1 mg) was diluted in 0.1 mL distilled water and mixed with 10 mL of colloidal gold pH 6.9. After centrifugation the red pellet was resuspended in PBS, pH 7.2 (Benhamou et al.,1986).

Cytochemical labeling and controls

In direct labeling with enzymes and some lectins, sections were incubated on a drop of PBS at the appropriate pH, then transferred on a drop of enzyme or lectin-gold complex for 30 min at room temperature. They were then washed with PBS, rinsed with distilled water, and contrasted.

In indirect labeling, sections were incubated on a drop of PBS then transferred on a drop of the unconjugated lectin (Benhamou and Ouellette,1986b) or on a drop of the antibody at the appropriate dilution (Benhamou et al.,1986, Benhamou et al.,1987). After washing with PBS, sections were incubated with the corresponding protein-gold complex.

RESULTS

Scanning and transmission electron microscope observations have shown that Ascocalyx abietina in culture is characterized by unique features. We ultrastructurally localized several components of A. abietina, some demonstrated for the first time not only in this organism but also in fungi in general, using cytochemical and immunocytochemical tests. Through use of monoclonal antibodies against a synthetic dsRNA, the occurrence of this component specific of mycoviruses has also been shown in a majority of the isolates tested.

These results are presented below as an overview. Further details are given in recent or in forthcoming publications (Benhamou and Ouellette,1986a-1986d, Benhamou et al.,1986, Benhamou et al.,1987).

General ultrastructure

A prominant feature of fungal cells in culture as seen in SEM, was that they were surrounded by an extracellular sheath giving them a warted or wrinkled appearance (Figs. 1, 2). In TEM, this sheath corresponded to a dense fibrillar network (Figs. 3-5). The sheath occurred equally consistently over cells of both the so-called North American (Fig. 3) and the European races of this pathogen. However, structure of this sheath varied greatly according to the portion of the colony sampled. In portions adjacent to the colony center, the sheath was continuous and uniform, it was very compact near the cell wall but looser marginally (Figs. 3, 5); at the colony margin, the fibrillar network was often discontinuous, more irregular, and loosely organized but in well delineated masses (Fig. 4, arrows).

Variation in cell wall thickness was observed not only between cells of supposedly diverse physiological conditions (compare Figs. 3 and 5) but also in the same cell where irregularities occurred at its junction with the sheath. Thicker walls often appeared multilayered. These were ordinarily electron translucent but frequently contained patches of aggregated or more disperse electron dense matter (Figs. 3, 4). Septa were typical of Ascomycotina, but often contained electron dense matter in their triangular junctions with the cell wall. As already described cells proliferated through or near the septa, to yield either larger or new cells.

Endocells (cells growing within another typical cell) were regularly observed in a high proportion of fungal hyphae (Figs. 5, 6). Walls of these endocells were often closely appressed or even seemed at times to be fused to the enclosing wall (Fig. 5, arrow head). Often cytoplasmic remnants of the mother cell appeared to be in continuity with the sheath through a rupture or an erosion of the enclosing wall (Fig. 6, arrow).

Cell organelles were typical of Ascomycotina (Beckett *et al.*, 1974) except that mitochondria were generally not as elongated and nuclei were irregular in shape due to apparent invaginations of the nuclear membrane (Fig. 7, arrow). The endoplasmic reticulum was often prominent, and arranged in parallel arrays that extended close to the plasmalemma giving often the impression of being fused with it.

Ultrastructural localization of cell constituents with enzyme and lectin-gold complexes

The enzymes and lectins tested are listed in Tables 1 and 2. Sugar residues such as chitin (a polymer of N-acetyl glucosamine), polygalacturonic acids, mannose, galactosides, and N-acetyl galactosamine were detected in the walls of *A. abietina* through the use of respectively gold-complexed chitinase or WGA, pectinases, Concanavalin A (Con A), β-galactosidase, and lectin from *Helix pomatia* (HpA). How-

ever, intensity and distribution of the gold labeling was somewhat different between these. Examples of the labeling obtained are shown in Figs. 7-12.

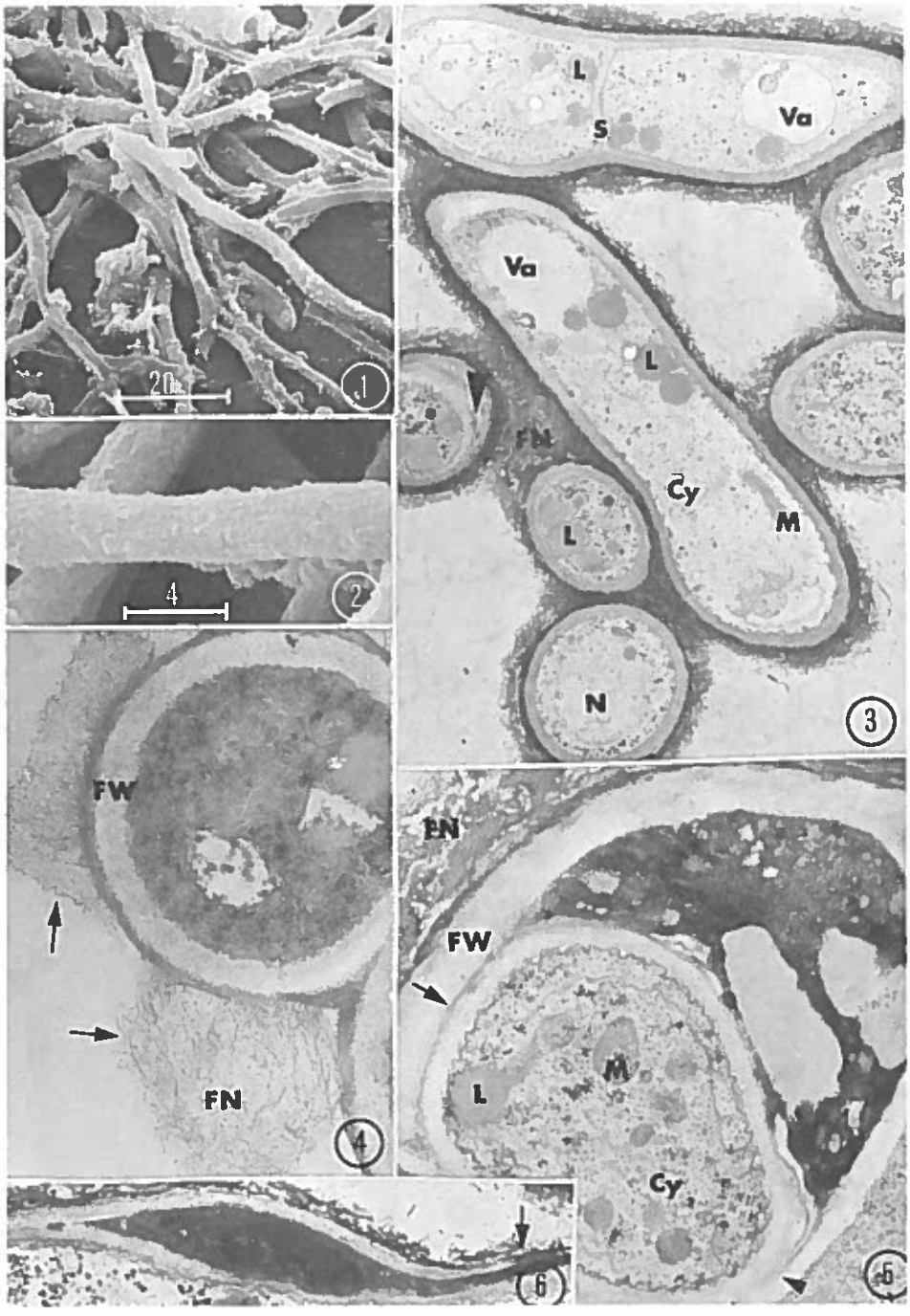
Tests for chitin indicated that labeling for this compound was specifically localized over the cell wall (Fig. 7). Tests with other complexes indicated a similar uniform, intense labeling over the wall, including a surprisingly significant strong positive reaction with pectinase-gold complexes (Benhamou and Ouellette,1986a) and with a lipase-gold complex (Benhamou and Ouellette,1986c). Contrarily, labeling with gold-complexed Con A (Fig. 9), HpA (Fig. 8), and galactosidase was more irregular and restricted to some wall areas such as septa (Fig. 8) or wall thickenings (Fig. 9). Con A binding sites were also abundant in the cytoplasm (Fig. 9). Tests with a cellulase or an exoglucanase-gold complex were negative (Benhamou and Ouellette, 1986c).

As expected, RNase-binding sites were general in the cell cytoplasm (Fig. 10). However, more unexpected was the intense labeling noted consistently over the fibrillar sheath (Fig. 10). The latter was also labeled with the lectin from Limax flavus (LfA) complex (Fig. 11, and Benhamou and Charest,1986, Benhamou and Ouellette,1986b). This lectin also specifically attached to structures identifiable as lipid bodies (Fig. 11). Such bodies but not the fibrillar sheath were also intensely labeled with lectins from Ulex europaeus (UeM) (Fig. 12), and with Lotus tetragonolobus (LtA) and Lens culinaris (LcA).

All control experiments including previous adsorption of the protein-gold complex with its corresponding substrate or sugar resulted in an absence of labeling.

Immunocytochemical tests with polyclonal antibodies produced against fimbriae from other fungi

Polyclonal antibodies against extracellular fibrillar material obtained from Ustilago violacea (AU) and Rhodotorula rubra (AR) (Gardiner et al.,1982) were used as probes to detect cross reactions with the cell surface of A. abietina (Benhamou et al.,1986). Antigen-antibody interactions were visualized by the protein A-gold approach (Bendayan,1984). With antibody AR, labeling was predominant over walls, septa, and plasma membrane of A. abietina cells. Numerous gold particles also occurred over the fibrillar sheath, particularly its inner dense layers. With AU, labeling was almost restricted to this sheath. Negative results were obtained with all control tests, including use of pre-immune serum instead of the antibodies.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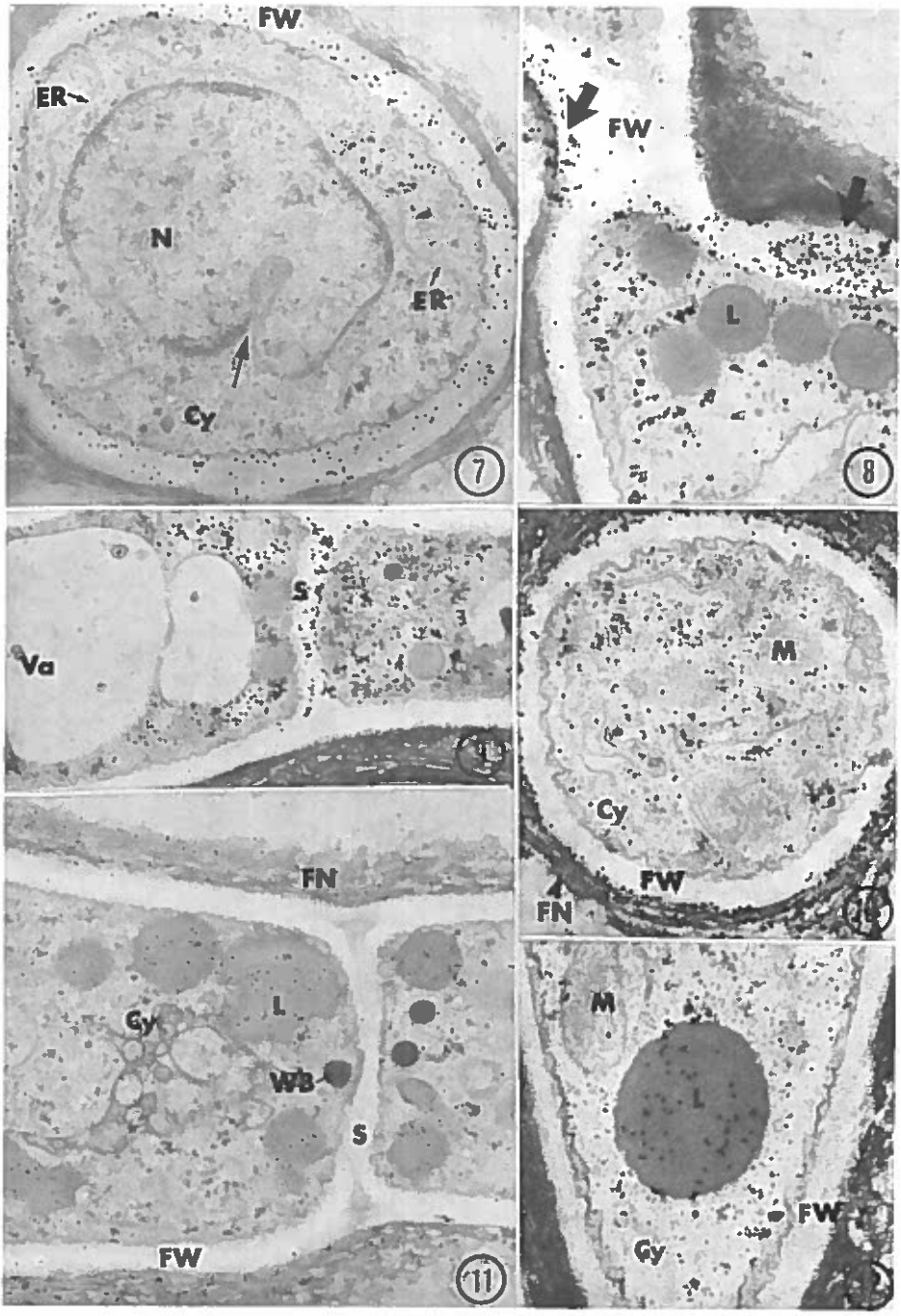
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Figures 1-12. Electron-microscope micrographs of A. abietina. Cy, cytoplasm; ER, endoplasmic reticulum; FN, fibrillar network; FW, fungal wall; L, Lipid body; M, mitochondrion; N, nucleus; S, septum; Va, vacuole; WB, Woronin body.

Figs. 1-2. Scanning electron microscope (SEM) micrographs of A. abietina mycelium. The cell surface of hyphae is warty. Bars are in micrometers 10^{-1}.

Fig.3. Transmission electron microscope (TEM) micrograph of an isolate of A. abietina, North American race. A dense fibrillar network surrounds the moderately thick wall of the cells. Walls, generally electron translucent appear to contain dense, material, sometimes in clumps (arrow heads). x 10 000.

Fig. 4. TEM micrograph of an isolate of A. abietina, European race. Samples were collected at the margin of the colony. Extracellular fibrils are organized in well-delineated masses (arrows). The cell wall contains electron dense fibrillar material which is at times perpendicularly oriented with the wall. x 30 000.

Figs. 5 and 6. TEM micrographs of endocells, European race-isolate. Wall of the endocell appears either appressed to that of the enclosing cell (Fig. 5, arrow) or fused with it (Fig. 5, arrow head). Cytoplasmic remnants appear to be in continuity with the sheath (Fig. 6, arrow). Fig. 5. x 30 000. Fig. 6. x 12 500.

Fig. 7. Localization of chitin in the wall by means of a chitinase-gold complex. x 30 000.

Fig. 8. Localization of N-acetyl galactosamine over parts of cell walls and cytoplasm by means of the gold-complexed Helix pomatia agglutinin. Wall thickenings are densely labeled (arrow). x 30 000.

Fig. 9. Localization of mannose by means of gold-complexed Concanavalin A. Labeling is restricted to the septum and the cytoplasm. x 22 000.

Fig. 10. Localization in the cytoplasm of RNA by means of a RNase-gold complex. Numerous gold particles are also present over the extracellular sheath. x 25 000.

Fig. 11. Localization in lipid bodies of sialic acid by means of a gold-complexed lectin from Limax flavus. Gold particles are also present over the fibrillar network. x 25 000.

Fig. 12. Specific labeling of lipid bodies with a gold complexed lectin from Ulex europaeus, indicating the presence of fucose. x 30 000.

Immunological detection of mycoviruses by means of monoclonal antibodies against Poly[I]: Poly[C]

In a screening program involving 100 isolates of A. abietina, the use in a dot-immunobinding procedure of monoclonal antibodies, against a synthetic double-stranded RNA (dsRNA) Poly [I]: Poly [C], revealed that mycoviruses were present in 64% of the isolates, of both the North American and European races. Specificity and sensitivity of such monoclonal antibodies were previously assessed through several immunobiochemical tests (Benhamou et al.,1987). Specificity of the dot-immunobinding assays was verified by the absence of reaction in control tests (Benhamou et al.,1987).

DISCUSSION AND CONCLUSION

The ultrastructural and cytochemical information obtained through our studies provide much new knowledge on the morphology and the chemical composition of A. abietina. These findings may have an important bearing for better understanding biological functions of this pathogen such as its resistance to unfavorable weather conditions and other functions in disease development such as cell to cell attachment and other host-pathogen interactions.

A. abietina resistance to deleterious environmental factors has been demonstrated (Blenis et al.,1983, Bergdhal,1983). The fungus was found to withstand extremely dry conditions despite the fact that moist weather was one of its optimal growth conditions (Bergdhal,1983). Similarly, Barklund et al. (1983) mentioned that A. abietina was highly resistant to adverse effects of acidity in locations subjected to acid rains. Our TEM observations revealed that cells of A. abietina were bordered by a very thick wall surrounded by a dense extracellular sheath. These peculiar features, which are now increasingly reported in fungi, are most probably involved in the resistance of A. abietina. Hess et al. (1985) also postulated this when he reported that the sheath bordering cells of a snowmold fungus could afford protection against dessication.

The chemical composition of A. abietina cell walls and of its fibrillar sheath may also be a major factor protecting the fungus against adverse conditions. Among the various polysaccharides found to occur in the walls and in the sheath, sialic acid residues were undoubtedly the most unexpected. These carbohydrates, known until now to occur mainly in animal cells, were shown to be implicated in a variety of biological functions (Schauer,1982). Sialic acid moieties

occupy a terminal position in glycoprotein or glycolipid chains and are negatively charged. Their accumulation in the fibrillar sheath of A. abietina may strongly influence the behavior of the fungus (Benhamou and Ouellette,1986b). They are probably involved in the attachment between fungal and host cells and moreover their repulsive electrostatic forces may prevent cell aggregation and influence transport of cationic compounds through the wall.

RNA in extracellular matrix was also unexpected. This may be explained by the liberation of cell cytoplasm constituents in the sheath through a ruptured wall (Fig. 6, arrow). Indeed, indications of ruptures were present at one point or another in the majority of the cells observed. Our cytochemical tests could not determine whether or not these nucleic acids are still active.

Cell surface proteins of A. abietina have been shown to cross-react with antisera to fimbriae of the anther smut fungus Ustilago violacea or extracellular fibrils of the basidiomycetous yeast Rhodotorula rubra. This adds to the list of fungi, particularly in Ascomycotina, that produce such extracellular fimbriae-like structures. These have been postulated as important factors in disease development (Day et al.,1986); they may also play a similar role in A. abietina. Present immunocytochemical observations indicated that these extracellular fibrils were probably assembled in the plasma membrane and extruded through the walls.

Finally, mycoviruses were detected in a high percentage of the A. abietina isolates tested. Mycoviruses in both the European and the North American races of this pathogen was an indication that pathogenicity was not apparently altered by virus infection as for other fungi (Hollings,1978). Their presence, however, may be related to some of the peculiar morphological features observed.

Our studies demonstrate that A. abietina, although similar in most respects to other Ascomycotina, presents typical features which are probably involved in specific biological functions.

ACKNOWLEDGMENTS

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ZUSAMMENFASSUNG

Ultrastrukturelle und cytochemische Charakterisierung von Ascocalyx abietina.

Der Pilz Ascocalyx abietina (Lagerb.) Schläpfer-Bernhard besteht aus gleichmäßig septierten Hyphen. Die Pilzzellen sind durch eine dicke Wand begrenzt, die selbst wiederum von einer dichten fibrillären Scheide umgeben ist. Diese Strukturen könnten eine wichtige Rolle spielen bei der Resistenz des Pilzes gegenüber ungünstigen Bedingungen. Sialinsäure wurde in der Scheide nachgewiesen; diese Verbindung könnte in Beziehung zu spezifischen biologischen Funktionen stehen wie etwa der Abstoßung zwischen Zellen und bei Wirt-Parasit-Interaktionen. Bei einer Großzahl der geprüften A. abietina-Isolate wurden Mycoviren gefunden. Die ultrastrukturellen und cytochemischen Einzelheiten von A. abietina tragen dazu bei, diese Pilzart von anderen Ascomycotina zu unterscheiden.

GREMMENIELLA ABIETINA ON PINE.

THE FUNGUS. SYMPTOMS.

MIXING UP WITH OTHER FUNGI[1]

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The fungus

Pycnidia and conidia

The pycnidia are usually formed earlier than the apothecia. Shoots from the last year can get brown bark and brown needles early spring. Pycnidia are often formed already in May. But these early pycnidia are usually very incomplete, formed in the bark and without any well-developed wall. The conidia may be pressed out in slimy drops, sometimes in tendrils. Later roundish, black pycnidia are formed, usually on the bark.

The macroconidia are narrow, more or less curved, Fusarium-like but without the foot cell, 1 - 7-septated, usually with 3 - 5 septa as described by Ettlinger (1945). In southeastern Norway they are usually very acute at both ends, often with 5 - 7 septa. In southwestern Norway and Great Britain most conidia do not have more than 3 septa.

Microconidia, described by Roll-Hansen & Roll-Hansen (1973) are not seldom formed, often in the same stroma as the macroconidia. There are all transitions between the microconidia and the macroconidia.

Apothecia

Apothecia are usually ripe one year later than the pycnidia. The blueish colour of the hymenium, seen in moist weather, is characteristic.

Gremmeniella abietina has formerly been mixed up with Cenangium ferruginosum. The apothecia with the greenish-

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[1] The paper was originally illustrated by 30 colour slides

yellow hymenium, and the conidiostromata (Kujala 1950) are characteristic to C. ferruginosum. But for example the German author Schwarz (1895) connected the teleomorph of C. ferruginosum with the anamorph of G. abietina, and the American author Weir (1921) did the same when he described a collection from Montana in U.S.A., thus indirectly reporting G. abietina from western North America.

The symptoms

Girdling at the base of the stem

Trees up to more than 2 m high may be girdled at the base.

Attack on the shoots

Brown-colouring of the needles from the base after killing of the bark by the fungus is a common symptom. Often is only the youngest shoot attacked. The characteristic browning of the needles starts often early spring. The brown colour of the base of the needles is especially in Pinus contorta in sharp contrast to the green upper part of the needles; the bark is brown, killed by the fungus. In P. sylvestris the colour contrast is less striking. Pycnidia are formed on such shoots, usually first in the bark, later on the bark. One year later apothecia may be formed.

Development after wounding

When branches have been pulled down by melting snow, cracks have often been formed and infection taken place through the cracks; weakening of the branches may have furthered the development of the fungus.

Development after weakening of the tree

Fatal development of G. abietina is often found after weakening of the tree, for example in too southern provenances or after attack by Phacidium infestans.

Attack by fungi that causes similar symptoms

Phacidium coniferarum (anamorph: Phacidiopycnis pseudotsugae) attacks sometimes pine shoots causing symptoms similar to those caused by G. abietina: brown colour on the

needles from the base upwards after killing of the bark. But this blue-stain fungus will soon give the wood a blueish-grey colour.

Sydowia polyspora (anamorph: Dothichiza pityophila) is a very weak parasite, but may give similar symptoms: browning upwards from the base of the needles. It sometimes develops after attack by insects as Blastesthia turionella.

Symptoms not directly caused by fungi

The bark will not be coloured brown when the shoots simply dry out. The needles will be brown-coloured from the tip downwards.

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Zusammenfassung

Gremmeniella abietina auf Kiefern. Die Pilz. Symptome. Verwicklung mit anderen Pilzen.

Für eine eindeutige Diagnose der durch Gremmeniella abietina hervorgerufenen Krankheit ist die genaue Kenntnis des Pilzes und der von ihm verursachten Symptome erforderlich. Beschrieben werden die Pyknidien, Makro- und Mikrokonidien sowie die Apothecien von G. abietina. Außerdem wurden die Krankheitserscheinungen an Pinus contorta und P. sylvestris charakterisiert. Von den Pilzarten mit ähnlichen Symptomen sind Phacidium coniferarum und Sydowia polyspora aufgeführt.

III. Occurrence and variation of *Gremmeniella abietina*

DETECTION OF CONIDIA OF *BRUNCHORSTIA PINEA* BY INDIRECT IMMUNOFLUORESCENCE

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SUMMARY

An indirect immunofluorescence staining procedure was developed for detection of conidia of *Brunchorstia pinea* using antiserum to these conidia and a commercially prepared FITC:protein A conjugate. This technique aids in the identification of *B. pinea* conidia collected in spore traps. Unfortunately, the immunofluorescence was not specific for *B. pinea*. Spores of *Fusarium solani, F. sporotrichioides, F. oxysporum, Sirococcus* sp., *Phialophora* sp., *Gliocladium* sp., and *Gelatinosporium* sp. all fluoresce to some degree, but can be distinguished from *B. pinea* by size, shape, and other morphological characteristics when examined by indirect immunofluorescence in combination with light microscopy. Therefore, this technique could be useful in the initial screening of a site for the presence of *B. pinea*, but collection of characteristic pycnidia from branches at that site will be required to confirm the results.

INTRODUCTION

In 1977, the New York State Department of Agriculture and Markets established a quarantine in northern New York State to limit the spread of Scleroderris canker caused by *Ascocalyx abietina* (Naumov) Schlaepfer-Bernhard [anamorph = *Brunchorstia pinea* (Karst.) Hohn.]. This quarantine is still in effect, even though the disease is not as widespread or severe today as it was in the mid-to-late 1970's. The quarantine is monitored periodically by NY State horticultural inspectors to determine its effectiveness and to insure compliance with quarantine restrictions by local Christmas tree growers and forest landowners. Monitoring is accomplished by visual inspection of plantations for characteristic disease symptoms followed by microscopic inspection of conidia of *B. pinea* from pycnidia collected in the field. The procedure is rather time consuming and relies to some degree on the skill and/or fortuitous combination of circumstances to identify the disease in its early stages.

To more systematically sample for the fungus, we have been exploring the feasibility of using monofilament line spore traps to collect fog and mist samples (Luley and Manion, 1984). During the summer of 1984, monofilament line spore traps were set up at 43 locations across New York State from Lake Ontario, on the west, to southern Vermont, on the east. Water samples were collected weekly for 14 weeks. Spores were concentrated from the water samples by centrifugation and characteristic *B. pinea* conidia were identified and tallied. However, identification of conidia of *B. pinea* was not an easy task because they resemble the spores of many other fungi, primarily *Fusarium* sp. and *Gelatinosporium* sp., which were often present in spore trap samples. Unfortunately, the spore sampling procedure identified *B. pinea* -like conidia from sites both inside and outside of the quarantined area.

Since visual inspection of spore samples presumably did not reliably identify *B. pinea* conidia, we attempted to develop and test an immunofluorescence procedure to verify the presence of *B. pinea*. The objective of this research was to develop a rapid, sensitive, and reliable assay procedure for identifying the presence of *B. pinea* conidia collected from spore samples in the field. The implimentation of this procedure will provide inspectors with a means of determining the presence and possibly quantifying the infection potential of the pathogen and would greatly facilitate their monitoring and management of the quarantine.

MATERIALS AND METHODS

Production of conidia

Ascocalyx abietina isolates SYRF 7, SYRF 9, SYRF 12, and SYRF 42 were obtained from SUNY College of Environmental Science and Forestry collections. These cultures were isolated originally from red or jack pine (*Pinus resinosus* Ait. or *P. banksiana* Lamb.) in New York State and represented the European strain (SYRF 9, SYRF 12) and Intermediate strain (SYRF 7, SYRF 42) based on serological tests. Cultures were maintained on V-8 juice agar (Wendler, 1980). Conidia were produced on *Gremmeniella* sporulation agar (GSA)(Hudler, *et. al.*, 1984), modified by omitting vitamin stock and trace elements, and incubated at 18 C under continuous fluorescent light at 2000 lux. Conidia were harvested by the method of Hudler *et. al.* (1984) and suspensions were filtered through a double layer of tissue paper to remove mycelial fragments. Spore concentrations were adjusted to 1 x 10^6/ml, and stored in 1.0 ml aliquots at -4 C.

Production of antisera

Antisera to intact conidia of *B. pinea* were produced in female New Zealand white rabbits. Conidia from the four isolates were combined, suspended in sterile distilled water at 1 x 10^6/ml, and emulsified with an equal volume of Freund's complete adjuvant (Difco Labs, Detroit, Michigan). Rabbits were immunized according to the protocol in Table 1. Intravenous injections were prepared without Freund's complete adjuvant and were injected into the marginal ear vein.

Table 1. Immunization protocol for production of antiserum to conidia of *B. pinea*

Day	Treatment	Route
1	Inject - 1 x 10^6 spores	Subcutaneous + intramuscular
7	Inject - 1 x 10^6 spores	Subcutaneous + intramuscular
14	Inject - 1 x 10^6 spores	Subcutaneous + intramuscular
21	Inject - 1 x 10^6 spores	Subcutaneous + intramuscular
51	Inject - 1 x 10^5 spores	Intravenous
58	Bleed	

Blood was extracted through the marginal ear vein and incubated at 37 C in hot water for one hour, followed by incubation at 4 C for an additional hour. The clot was removed by centrifugation at 9500 x g for 20 minutes. Antiserum was carefully removed. Antibody titer was determined using the microprecipitin test against intact conidia of *B. pinea* (Ball, 1973).

Immunoglobulin G (IgG) was purified from crude antisera by ammonium sulfate precipitation and dialysis according to the method of Shephard (1972), followed by anion exchange chromatography using diethylaminoethyl cellulose (DEAE-23) (Whatman, Inc., Clifton, NJ).

Immunofluorescence staining

An indirect immunofluorescence staining procedure was developed according to the method of Kawamura (1977), with modifications. Spores were fixed to glass slides by air-drying at room temperature. Approximately 25 µl of IgG at 1.0 mg/ml was added to each slide to cover the entire sample. After a rinse with phosphate buffered saline (PBS), pH 7.4, 50 µl of a commercially prepared FITC:protein A conjugate (Pharmacia, Piscataway, NY) at 0.1 mg/ml in PBS was added to each slide, and incubated for 2.5 hours followed by a second rinse with distilled water. Samples were observed with a Nikon (Optiphot) microscope equipped with epifluorescence.

Conidia of *B. pinea* produced in culture and those removed from pycnidial spore horns on red pine twigs collected from the quarantined region, as well as conidia of four species of *Fusarium* [*F. acuminatum* El and Ev., *F. oxysporum* Schlecht., *F. solani* (Mart.) Sacc., and *F. sporotrichioides* Sherb., obtained from Pennsylvania State University collections] and *Sirococcus* sp. were stained as described above to determine the specificity of the assay.

Spore collection

Monofilament line spore traps (Luley and Manion, 1984) were placed within and outside the New York State quarantined region in May, 1986. As a control, spore traps were established in red pine stands located in Indiana, Pennsylvania, and West Virginia, where Scleroderris canker has not been previously reported (Skilling, *et. al.*, 1986). In total, 40 spore traps were established and monitored throughout the summer of 1986.

Samples from the spore traps were filtered through a double layer of tissue paper, then centrifuged at 2000 x g for 20 minutes. The pellets were resuspended in 1.0 ml of distilled water and examined for conidia of *B. pinea* by indirect immunofluorescence staining, as described above.

To confirm results of immunofluorescence, single-spores resembling conidia of *B. pinea* were isolated from all spore trap samples and cultured on V-8 juice agar containing 0.05% tetracycline, and transferred to GSA. For isolation purposes, spore trap samples were spread on a 5% water agar medium. Once the droplet had dried, elongate spores were readily visible with a dissecting microscope at 225x. Samples of the elongate spores were picked up with a fine needle and transferred to culture media.

In addition, recently dead red pine twigs were collected from sites where *Brunchorstia* -like conidia were detected. These twigs were examined for pycnidia and spore horns characteristic of *B. pinea*. Spore trap samples containing a high number of fluorescent *Brunchorstia* -like conidia also were examined by phase-contrast microscopy for spores with characteristic size, morphology, and septation.

RESULTS

Production of conidia

Sporulation on GSA was observed in two-week old cultures. Numbers of conidia per plate ranged from 2.4 to 4.9 x 10^7 in four-week old cultures, depending upon the isolate used.

Production of antisera

Antibody titer as determined by the microprecipitin test was 32. Approximately 193 mg of IgG was purified from 10 ml of crude antiserum by ammonium sulfate precipitation and anion exchange chromatography. The purified IgG was stored at -4 C in 1.0 ml aliquots at 1.0 mg/ml, with the addition of 15 mg/ml bovine serum albumin (BSA) to stabilize the IgG.

Immunofluorescence staining

Conidia of *B. pinea*, grown in culture, fluoresced brightly by indirect immunofluorescence (Fig. 1.). The fluorescence did not fade rapidly over time. However, spore septations were not visible. Autofluorescence was minimal. Conidia from pycnidial spore horns on red pine twigs collected from sites within the quarantined region also fluoresced brightly.

Conidia of three *Fusarium* species tested also fluoresced. However, the fluorescence was not as bright as the fluorescence of *B. pinea* conidia, and it tended to fade away within one hour after staining. The fluorescence was spotty and faint in *F. solani* and spore septations were not visible. The fluorescence of conidia of *F. oxysporum* and *F. sporotrichioides* was brighter than that of *F. solani* spores, but spore septations were visible. Conidia of *F. acuminatum* did not fluoresce. Therefore, although the conidia of some *Fusarium* species fluoresced, they were distinguishable from those of *B. pinea* by this procedure. In addition, conidia of *Gliocladium* sp., *Phialophora* sp., and *Sirococcus* sp., which do not resemble *B. pinea* morphologically, but which were commonly collected in spore traps, also fluoresced.

Spore collection

Fluorescent spores indistinguishable by immunofluorescence from conidia of *B. pinea* were detected at several sites within New York State, both within and outside of the quarantined region during the summer of 1986 (Table 2). Pycnidia of *B. pinea* subsequently were observed on red pine twigs collected near the spore traps only from

previously positive Scleroderris canker sites (Table 2). Fluorescent *Brunchorstia* -like conidia were detected in spore traps from five of seven known infection sites, four of six suspect sites, and five of nine Scleroderris-free sites in New York State. Of these sites, 13 were within the quarantined region and nine were outside of the quarantined region. Fluorescent *Brunchorstia* -like conidia generally were not observed in spore traps located outside of NYS, with the exception of one trap located near Bartow, WV, in which one *Brunchorstia* -like conidium was observed (Table 2).

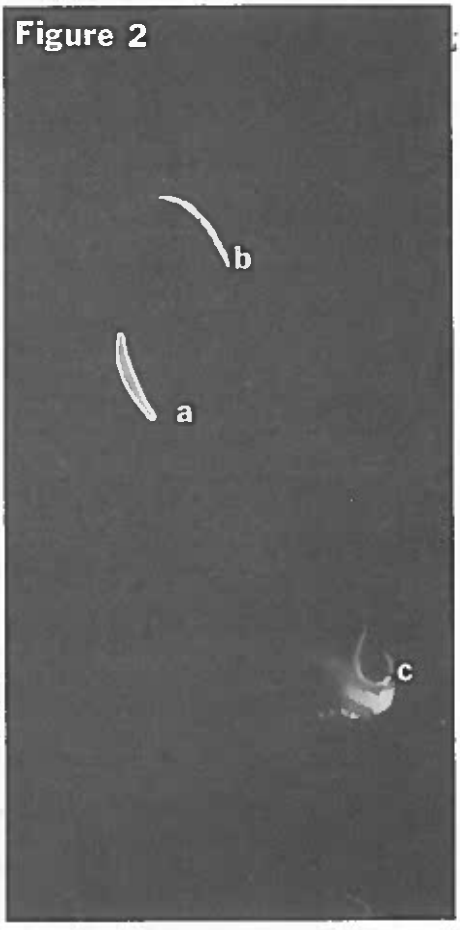

Figure 1. Fluorescence of conidia of *B. pinea* after staining with indirect immunofluorescence (x562.5)

Figure 2. Immunofluorescence staining of spores collected in a monofilament line spore trap located in Cold Brook, NY (x562.5). (a) resembles conidia of *B. pinea* in degree of fluorescence, size, and length-to-width ratio. (b) and (c) are distinguished from conidia of *B. pinea* by greater length-to-width ratio.

Table 2. Results of the indirect immunofluorescence assay for conidia of *Brunchorstia pinea* from spore trap samples collected during the summer of 1986

Prior Scleroderris Assessment[1]	Location	Immunofluorescence	Presence of Pycnidia
positive	Rockwood, NY	–	+
positive	Cold Brook, NY	+	+
positive	Prospect, NY	+	+
positive	Vienna, NY	–	+
positive	Williamstown, NY	+	+
positive	Pineville, NY (4 traps)	+	+
positive	Sullivan, NY	+	+
suspect	Saratoga, NY	+	–
suspect	Milton, NY	+	–
suspect	Mayfield, NY	+	–
suspect	Dolgeville, NY	–	–
suspect	Westernville, NY	+	–
suspect	Amboy, NY	–	–
negative	Cazenovia, NY	–	–
negative	Bridgewater, NY	–	–
negative	Cherry Valley, NY	–	–
negative	Duanesburg, NY	+	–
negative	Malta, NY	+	–
negative	Brewerton, NY	+	–
negative	Tully, NY (3 traps)	+	–
negative	Lafayette, NY (3 traps)	+	–
negative	Gilbert Lake State Park, Laurens, NY (6 traps)	–	–
negative	Indianapolis, IN	–	–
negative	Juniata Co., PA	–	–
negative	Perry Co., PA	–	–
negative	Bartow, WV (2 traps)	±	–
negative	Morgantown, WV	–	–

1. Prior assessment for New York State sites is based upon observations of disease by horticultural inspectors of NYS Dept. of Ag. and Markets. Out of state assessment based upon review of pertinent literature.

Other conidia, morphologically similar, but not identical to conidia of *B. pinea* , also fluoresced. These conidia did not fluoresce as brightly and their length-to-width ratio was greater than conidia of *B. pinea* (Fig. 2). Many spores of this type were observed in most of the spore traps. In addition, many traps contained spores morphologically similar to *Brunchorstia* conidia but which did not fluoresce with this technique.

Attempts to culture *A. abietina* from single spores resembling conidia of this fungus isolated from spore trap samples were not successful. Some cultures that resulted from single sporing yielded *Fusarium* sp. Spores of these cultures did not fluoresce. In non-sporulating cultures, the cultural morphology was not similar to *A. abietina*.

Attempts to verify the presence of *B. pinea* by collection of pycnidia from trap sites

were more successful (Table 2). Pycnidia of *B. pinea* were collected from all seven previously positive Scleroderris canker sites (Table 2) but not from any other location to date. The numbers of fluorescent *Brunchorstia* -like conidia collected in traps set up at these sites was also much greater than in traps set up at the other sites. These results are to be expected because Scleroderris canker is present in these seven locations, but not elsewhere. Therefore, in the four suspect sites and ten negative sites in which fluorescent *Brunchorstia* -like conidia were detected, detection of characteristic pycnidia would be expected to be more difficult. Nevertheless, these results suggest that it may be prudent to examine these sites closely for the presence of *Brunchorstia* pycnidia.

Pycnidia resembling those of *B. pinea* were collected from the trap site at Gilbert Lake State Park, Laurens, NY, which is approximately 30 miles outside the quarantine region. However, these pycnidia contained fluorescent conidia similar, but not identical, to conidia of *B. pinea* . These spores were seven to nine septate and the length-to-width ratio was much greater. This fungus has been identified as an isolate of *Gelatinosporium* sp. Length-to-width ratio is distinguishable under epifluorescent microscopy and the septations can be counted under phase contrast or brightfield microscopy, which makes it possible to distinguish these spores from those of *B. pinea.*

DISCUSSION

The monofilament line spore trap was used to monitor sites for the presence of *B. pinea* conidia. Because other spores may be confused with *B. pinea,* we developed an immunofluorescence procedure to help in the identification of *B. pinea.*

Conidia of *B. pinea* from culture or from pycnidia collected in the field fluoresced brightly by the indirect immunofluorescence procedure. Unfortunately, the technique was not specific for conidia of *B. pinea.* Conidia of *Fusarium solani, F. sporotrichioides, F. oxysporum, Sirococcus* sp., *Phialophora* sp., *Gliocladium* sp., *Gelatinosporium* sp., and probably many others, also fluoresce. However, these conidia could be distinguished from conidia of *B. pinea* by differences in size, shape, septation, length-to-width ratio, and degree of fluorescence. Therefore, all presumed *B. pinea* conidia from spore collections were observed both by indirect immunofluorescence and phase contrast microscopy.

Using these microscopy techniques, we identified *B. pinea* -like conidia from areas where we could not find and positively identify *B. pinea* pycnidia. These samples could represent long distance dispersal of *B. pinea* conidia or they could represent possible errors in identification. Conservative interpretation of these results would suggest that suspected positive sites need to be corroborated by pycnidial collections.

The preliminary results of this study, if verified by pycnidial collections, indicate that the quarantine currently in effect in NYS may need to expanded. *Brunchorstia* -like conidia were detected in five of nine sites located outside the quarantined area. Although three of these (Brewerton, Malta, and Duanesburg) are located only a few miles outside the quarantined area, the remaining two sites (Tully and Lafayette) are more than 20 miles from the nearest quarantine boundary.

With one exception, *Brunchorstia* -like conidia were not detected in spore traps established in Pennsylvania, Indiana, and West Virginia. These sites were chosen as control locations because they are well beyond the published range of Scleroderris canker (Skilling, *et. al.*, 1986). One spore, resembling a *Brunchorstia* conidium, was collected in a spore trap located near Bartow, WV. This occurrence has not yet been verified in other spore trap samples or by pycnidial collections.

Therefore, the indirect immunofluorescence staining procedure in conjunction with light

microscopy provides a useful tool for evaluating presence or absence of *B. pinea* as collected in spore traps in the field.

ACKNOWLEDGEMENTS

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ZUSAMMENFASSUNG

Entdecken der Konidien von Brunchorstia pinea durch indirekte Immunofluoreszenz

Ein indirektes Immunofluoreszenz-Färbeverfahren wurde für die Entdeckung der Konidien von Brunchorstia pinea entwikkelt, indem ein Antiserum gegen diese Konidien und ein kommerziell hergestelltes FITC: Protein A-Konjugat verwendet wurde. Diese Technik hilft bei der Identifizierung von in Sporenfallen gesammelten B. pinea-Konidien. Leider war die Immunofluoreszenz nicht spezifisch für B. pinea. Die Sporen von Fusarium solani, F. sporotrichioides, F. oxysporum, Sirococcus sp., Phialophora sp., Gliocladium sp. und Gelatinosporium sp. fluoreszieren ebenfalls zu einem gewissen Grad, können aber von B. pinea unterschieden werden durch Größe, Form und andere morphologische Merkmale, wenn sie durch indirekte Immunofluoreszenz in Kombination mit dem Lichtmikroskop untersucht werden. Daher kann diese Technik nützlich sein bei der ersten Prüfung eines Standortes auf Anwesenheit von B. pinea. Doch das Sammeln der charakteristischen Pyknidien von den Zweigen an diesem Standort ist erforderlich, um die Ergebnisse zu bestätigen.

OUTBREAK OF *GREMMENIELLA ABIETINA*, EUROPEAN RACE IN THE PROVINCE OF QUEBEC, CANADA

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SUMMARY

During a survey of 1,183 pine plantations in southwestern Quebec, scleroderris canker was found in 163 plantations, mainly on red and occasionally on Scots pine. The European race of *Gremmeniella abietina* was identified in 74% of the diseased plantations, the North American race in 18%, leaving 8% where tests were inconclusive or the isolates contaminated. This is the second large outbreak of the European race of this disease in North America after the one reported in New York State in the late 1970s. Plantations varied in age from 6 to 24 years and half of those diseased were severely damaged. Both races were well mixed geographically and no definite source of infection or cause for this outbreak could be identified.

INTRODUCTION

Since 1977, two races of *Gremmeniella abietina* (Lagerb.) Morelet, are known to occur in North America (Dorworth *et al.*,1977). The North American race is believed to be indigenous to North America and it is the one commonly encountered in pine plantations and natural stands. It usually does not cause appreciable damage to trees higher than 2 m

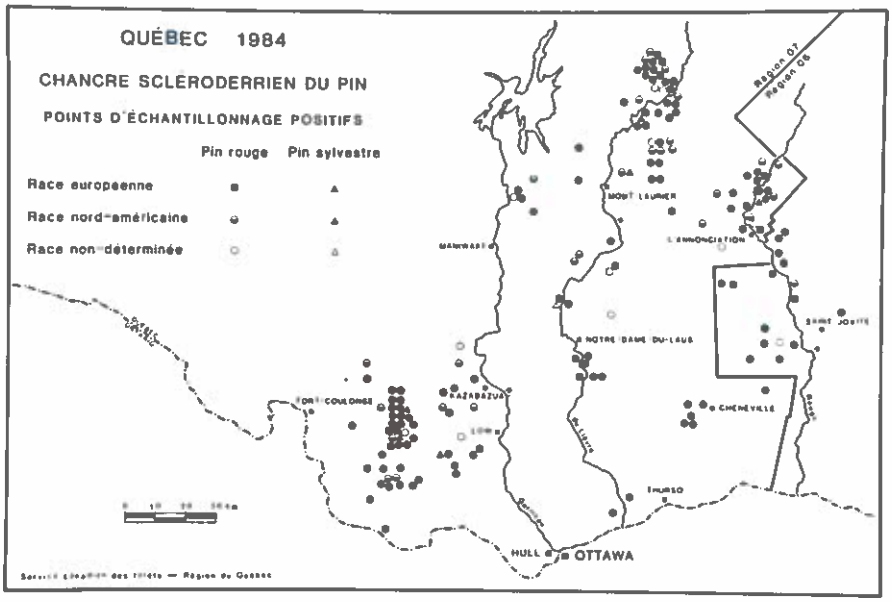

Fig. 1: Localisation of pine plantations infected with scleroderris canker in southwestern Quebec, Canada.

because it cannot infect branches above this height. It commonly produces cankers on the stem of affected trees and numerous apothecia. Contrarily, the European race is serologically related to European isolates of this fungus originating from Europe, it appears more virulent, and can and does infect branches at any height thus causing significant damage to plantations of any age. On the other hand, it seldom produces apothecia or cankering on the stem. It behaves in the field like an introduced pathogen, having a restricted known distribution and producing important local damage.

The distribution of the European race in the United States and particularly in New York State is fairly well documented (O'Brien, 1984; Setliff et al.,1975; Skilling,1977; Skilling et al.,1984). This European race was found for the first time in Canada in 1978 (Lachance, 1979) in a 10-year-old red pine plantation just a few kilometers north of the New York State-Quebec Province border. An intensive survey the

following year of all pine plantations located in Quebec within 10 to 15 km of the New York, Vermont, New Hampshire borders revealed 20 pine plantations lightly infected with the European race of G. abietina. There were 18 plantations of red pine, Pinus resinosa Ait., one of jack pine, P. banksiana Lamb., and one of Scots pine, P. sylvestris L. Clipping and burning of all infected branches in these plantations in 1979 and the following years, eradicated the disease from the area, at least temporarily.

In 1983, eight isolates of the fungus made from samples collected during a preliminary study in southwestern Quebec were tested as to their serologic races. Six of them proved to be European (Lachance et al.,1985). Then a major survey was planned the following year to find out the extent of the European race in the region (Lachance et al., 1985).

MATERIALS AND METHODS

The surveyed area covered approximately 18 000 km^2 of agricultural and private or public woodlands and was located in southwestern Quebec. It spread to about 150 km north and 100 km on each side (Fig. 1) of Hull-Ottawa.

The survey included all known 6 to 30 year-old pine plantations in the region plus any others that were discovered during the survey.

For each plantation, the location, tree species, age, and an estimate of the number of trees were noted. A random search up to a maximum of one-half hour was carried out for disease symptoms. The symptoms looked for were: a) dead twigs on lower branches with or without needles still attached; b) the presence on shoots of partly green needles with yellowing at the base and that could easily be pulled out from the shoot; c) greening of cambium underneath diseased twig bark; and d) the occurrence of scleroderris cankers on lower stems. When symptoms were found, three classes of disease severity were used: light when 1-5% of the trees were infected, moderate when 6-25% were infected, and severe when tree mortality was seen and/or more than 25% of the trees showed symptoms.

Disease samples were collected from each plantation when symptoms were present. They were examined in the laboratory with a stereoscopic microscope for the presence of pycnidia, apothecia, or cryptopycnidia in the bark (Cauchon and Lachance,1980). A plantation was noted as positively infected only when one of these fruit bodies was found. Disease symptoms alone were not sufficient proof.

Isolations were attempted from the fruit bodies. The medium consisted of 2% malt, 2.5% agar-agar, and 5% V-8 juice. Cultures were incubated for 2 weeks at 22-24°C and then purified before being stored at 18°C.

Race indentification of the isolates was made by Dr. C.E. Dorworth and his associates at the Great Lakes Forestry Centre, Canadian Forestry Service, using their standard immuno-serological method (Dorworth et al.,1977). Race determination of only one isolate per infected plantation was done, because the test is relatively time consuming.

RESULTS AND DISCUSSION

About 10 million trees were surveyed in 1,183 plantations. The disease, irrespective of race, was present in 163 or 13.8% of the plantations. Among these, 157 were red pine and they accounted for about 2 million trees. The other six plantations represented about 60,000 Scots pine trees.

The disease was found throughout the area surveyed and about half the infected plantations were severely affected according to the estimates used in this survey. However, the other half of the infected plantations were lightly affected.

Race determination of the isolates revealed that 74% (121 plantations) of the infected plantations were of the European race whereas 18% (29 plantations) were North American. Isolates from the remaining 13 infected plantations either did not react properly during the test or were contaminated.

The extent of this infection center certainly makes it the second largest known infection center of the European race in North America after that of New York State in the late 1970s. One major difference between the two situations is the age of the trees affected. In southwestern Quebec, the trees varied from 6 to 24 years of age while in New York State they varied between 35 to 50 years (Setliff et al.,1975).

Another significant difference is that symptoms typical of the North American race, such as apothecia and cankers, were relatively common in Quebec contrarily to the situation in New York (Skilling, 1977). This may be due however to a more intimate mix of the two races in several plantations surveyed. Additional isolates made the following year from a small number of these surveyed plantations often revealed a presence of both races at one location and, in a few cases, a predominence of the race other than the one obtained through the original single isolate. In a situation were both races are known or suspected to occur, more than one isolate is required to obtain a true picture of the situation. In this case, we can say that the European race was present in at least 74% of the infected plantations, knowing that this value can be higher.

The prevalence of the European race in this region is difficult to explain. We could not identify any definite or even suspect infection centers in the area. Most seedlings originated from nurseries where the fungus has been either absent up to now or found relatively recently and then most seedlings were destroyed. The scleroderris canker disease has been developing in the area only in the last 10 years. It seems that some climatic conditions favorable to the disease and only the presence of a relatively large number of susceptible red pine plantations could account for the damage observed. But this does not explain the presence of the European race in this area, which is 150 km away from the closest other known European race infected plantation.

Finally, it is interesting to note that no jack pine growing naturally or planted in this area was found infected with the disease even though both red and jack pine are indigenous and in many cases were both about the same age. We are continuing studies on the epidemiology and control technology of the disease.

ACKNOWLEDGMENTS

We sincerely thank Dr. C.R. Dorworth and his associates at the Great Lakes Forestry Centre of the Canadian Forestry Service, in Sault-Ste-Marie, Ontario who provided their time and expertise to make all the immuno-serological tests for race identification of the isolates referred to in this study.

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ZUSAMMENFASSUNG

Ausbruch der europäischen Rasse von Gremmeniella abietina in der Provinz Quebec, Canada

Während einer Erhebung in 1.183 Kiefernplantagen im südwestlichen Quebec wurde die Scleroderris-Krankheit in 163 Plantagen gefunden, hauptsächlich an Pinus resinosa und gelegentlich an P. sylvestris. Die europäische Rasse von Gremmeniella abietina wurde in 74%, die nordamerikanische Rasse in 18% der erkrankten Plantagen gefunden, mit einem Rest von 8%, wo die Tests nicht eindeutig oder die Isolate verunreinigt waren. Dies ist der zweite große Ausbruch der europäischen Rasse dieser Krankheit in Nordamerika nach dem ersten Ausbruch im Staate New York in den späten 70er Jahren. Die Plantagen variierten im Alter von 6 bis 24 Jahren, und die Hälfte dieser erkrankten Plantagen war stark geschädigt. Beide Rassen waren geographisch gut gemischt, und eine bestimmte Infektionsquelle oder Ursache für diesen Ausbruch konnte nicht festgestellt werden.

COLLEMBOLA ASSOCIATED WITH *ASCOCALYX ABIETINA* AND DIFFERNCES IN THE OCCURRENCE OF THE FUNGUS IN SOUTHERN AND NORTHERN FINLAND

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ABSTRACT: The apothecia of *Ascocalyx abietina* mature in Finland mostly in mid-June. Their development and spore production are often disturbed. In southern Finland only a few apothecia remain productive until the beginning of August. Instead of ascospores they produce microconidia. In northern Finland the same kind of disturbance is rare or not observed. The reasons for the disturbance are not known. It appears that some microfauna, especially Collembola, inhabiting bark are responsible for the phenomenon. In a feeding experiment, Collembola (*Xenylla maritima*) consumed all the hymenium of the exposed apothecia within one week.

INTRODUCTION

After killing pine shoots or branches, *Ascocalyx abietina* (Lagerb.) Schläpfer first produces pycnidia on bark. Apothecia usually appear two years after infection. However, the occurrence of mature ascocarps is irregular. In Central Europe, except for the Alps, apothecia are seldom found: and in Great Britain they are usually absent (Gibbs 1984). In North America as well, the lack of apothecia has been difficult to explain. One explanation presented has been that there are different strains of the fungus: one is European and usually does not produce apothecia; and one is an originally American strain with a more northerly distribution, which produces apothecia more often. Serological differences have been demonstrated for these two strains (Dorworth and Krywienczyk 1975). There are also some morphological characteristics which can be used in distinguishing strains, e.g. the size and septation of conidia (Ettlinger 1945, Uotila 1983). However, especially in Europe, this separation of the fungus into different strains is not very clear and does not explain why the perfect state of the fungus is lacking in many places where the conidial state is frequently seen.

In southern Finland, abundant early development of apothecia has been observed over several years. When the dispersal of

ascospores was expected, very few productive apothecia were found. These observations induced investigations of possible factors which may eventually destroy apothecia of *A. abietina* in an immature condition. On dead branches where pycnidia and apothecia of the fungus were developing, numerous microfauna were found. The three most interesting groups were *Xenylla* spp. (Collembola with a regenerated springtail), Nematodes, and mites.

This paper presents observations on the behaviour of Collembola living on pine or spruce shoots killed by *A. abietina*, and on the development of the apothecia of the fungus, in different parts of Finland. Dr. Larry Huldén, Department of Zoology, Univ. Helsinki, identified Collembola species, for which I am indebted to him.

MATERIALS AND METHODS

Observations and collection of samples

The samples for this study were collected mainly in July of 1984, 1985, and 1986. The maturation of the apothecia of *A. abietina* occurs and the liberation of ascospores begins in Finland during July. In southern Finland, the samples were collected in diseased pine stands the age of which were 10 - 25 years. Usually the samples were taken from the upper crown. In northern Finland *A. abietina* was mostly found on young, naturally regenerated pines, usually less than ten-year-old.

Feeding of *Xenylla maritima* on *Ascocalyx abietina*

Killed shoots of Norway spruce with a rich population of *Xenylla maritima* Tullberg were collected in Kuru, Itä-Aure (Grid 688-31). In the shoots, sporocarps of *A. abietina* were probably destroyed by the *X. maritima* inhabiting the shoots. For the experiment, these shoots were moistened and put into plastic bags. Some moistened pieces of pine bark covered tightly with mature apothecia of *A. abietina* were added to the same bags and kept in contact with the spruce shoots. The bags were filled with air and shut tightly. Every two days the bags were opened and shut again. The bags were kept at room temperature varying between 20 - 22 °C.

For the control, some moistened pieces of pine bark with apothecia of *A. abietina* were put in a plastic bag without *Xenylla* Collembola. The apothecial samples used for the feeding experiments were collected in Inari, Köysivaara (Grid 758-48) July 16, 1984. The apothecia were ripe for ascospore production. Another feeding experiment was conducted with some adults and larvae of *X. maritima* placed on mycelial cultures of *A. abietina* on agar plates. During the experiments the behaviour of *X. maritima* was observed regularly.

RESULTS AND DISCUSSION

Collembola on dead branches

Collembola without, or with a very regenerated springtail were seen on both Norway spruce (*Picea abies* Karst.) and Scots pine (*Pinus sylvestris* L.). Different species were found, but all of them belonged to the same genus *Xenylla* (Figure 1). Usually they were associated with microfungi on the dead branches. The first observation of Collembola was made on the top shoots of spruce which had been killed one or two years earlier by *A. abietina*. They occupied bark cracks and insect holes. Collembola also appeared in tunnels made through and under pycnidia. It seemed that they had consumed the inner substances and the bottom of the pycnidia. If the bark was intact, they were hiding in depressions or under the bark scales and lichens. Collembola had left exuviae and laid eggs in the colonized holes or tunnels, or sometimes on exposed sites on the underside of horizontal branches (Figures 2 and 3). In living branches of older pines, Collembola were found among the nodal bud scales with a great mass of attached pollen grains.

Collembola are primarily known as forming a part of soil microfauna. Some studies have been published on their feeding behaviour. Generally, the most preferred food is plant debris. However, some groups appear to prefer fungal material (Takeda & Ichimura 1983).

Distribution of Collembola

Xenylla Collembola were found in southern Finland, from the southern coast up to Lake Oulujärvi in the North. However, living adults or larvae were not seen regularly. Apparently their occurrence and population density depends on local moisture conditions. Their habitats were detectable, however, because of the exuviae left there. To the north of Oulujärvi, no signs of *Xenylla* were found. However, one cannot conclude that they do not occur in northern Finland, although they were not found on the pines or pine branches killed by *A. abietina*.

Feeding experiments with *Xenylla maritima*

A great number of *X. maritima* individuals moved from the pieces of spruce shoots to the pine bark during the experiment. They were regularly observed grazing on the apothecia. In one week these Collembola had totally consumed the hymenium from the apothecia, although spruce and pine bark tissue were also available during the experiment. *X. maritima* clearly preferred the fresh, soft material of hymenium to the rough outer excipular tissue in the apothecia (Figures 4 and 5). It was not

diseased seedlings, but apothecia are seen only in taller trees and develop after one crop of pycnidia. Climatic factors may also account in part for this different development. The area around Lake Oulujärvi, where these features of A. *abietina* change, coincides with the southern border of the northern boreal vegetation zone (Ahti et al. 1968) as well as with the southern limit of the occurrence of some common forest diseases in boreal forests (Kurkela & Norokorpi 1975, 1979). Studies are planned in the near future to more thoroughly investigate the observed interaction between A. *abietina* and the microfauna of the forest canopy.

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ZUSAMMENFASSUNG

Collembolen in Verbindung mit Ascocalyx abietina und Unterschiede im Auftreten des Pilzes im südlichen und nördlichen Finnland.

Die Apothecien von Ascocalyx abietina reifen in Finnland meist Mitte Juni. Ihre Entwicklung sowie die Sporenbildung sind oft gestört. Im südlichen Finnland bleiben nur einige Apothecien bis Anfang August produktiv. Anstelle von Ascosporen produzieren sie Mikrokonidien. Im nördlichen Finnland ist dieselbe Art von Störung selten oder noch nicht beobachtet worden. Die Gründe für die Störung sind unbekannt. Es scheint, daß die Mikrofauna, vor allem Collembolen, die die Rinde bewohnen, für dieses Phänomen verantwortlich ist. In einem Fütterungsversuch fraßen Collembolen (Xenylla maritima) das gesamte Hymenium der dargebotenen Apothecien innerhalb von einer Woche.

were then visited by BAZZIGHER (unpublished results). Out of nine sites with Larix decidua six were affected with A. laricina, but Lachnellula willkommii (Hartig) Dennis and L. flavovirens (Bres.) Dennis were also present at all sites. Three out of four sites with Pinus cembra were infected with A. abietina whereas in three sites with Picea abies, only Herpotrichia juniperi (Duby) Petrak was present. The latter being a major disease in subalpine conifer plantations.
Based on a phytopathological and entomological survey in 1985 we can assume that in recent years no new and heavy infestations occured. Fifteen reforestations sites were listed with shoot dieback of Larix decidua, Pinus cembra, P. montana or Picea abies distributed in all alpine regions. The presence of Ascocalyx sp. being known in 8 sites for several years.

Ascocalyx abietina (Lagerb.) Schlaepfer-Bernhard in Switzerland

From 1965 to 1984 BAZZIGHER and KANZLER (unpublished) collected material with A. abietina from 27 locations throughout the Swiss alps. Pinus cembra, P. montana, P. sylvestris and Picea abies were hosts. In spring, the anamorph Brunchorstia pinea (Karst.) v. Höhn. was present, whereas from July the teleomorph was also found. This contrasts with data from CAPRETTI (1984), who never found the teleomorph in the Italian alps, and with DONAUBAUER (1984) who found the teleomorph only on P. cembra, and rarely on other hosts.

There are still doubts whether Brunchorstia pinea forms races. ETTLINGER (1945) describes the alpine race with 7 septate conidia and compares it to material from Denmark with 3 septa. Based on the size and the septation of the conidia, MORELET (1980) distinguishes between the race Brunchorstia pinea var. cembrae (5-7 septa) and Brunchorstia pinea var. pinea (0-7 septa, mostly 3). Although the 7-septate spores are very common in our Brunchorstia pinea collection, 3-septate spores are often present, even on P. cembra. Random samples of a reforestation site in the Bernese Oberland yielded pycnidia with 3- to 7-septate conidia (tab.1). These different conidia are present in all hosts examined and may occur in the same pycnidia or in different pycnidia on the same branch, negating the hypotheses that fungal races or the substrate may be responsible for the septation.

Disease development

After the heavy outbreak of Ascocalyx laricina in a plantation of Larix decidua (Chilchenberg, Andermatt,1600-2000m above sea level) in 1969, Bazzigher observed the health of the trees during 8 years. Already in 1970 the epidemic seemed to wane. Ninety-six larch trees were then photographed every year. The health and vigour of 44 trees improved, remained constant for 18 trees and decreased for 32 trees. The general improvement can be attributed to a cessation of the epidemic, but also to

the increase in size of the trees. Additionally the trees that got worse were mainly located in the highest part of the plantation above 1800 m. Climatic reasons and reduced general vigour may have accounted for their poor health.

Tab.1
Brunchorstia pinea conidia. Percentage of conidia with 1-8 septa in one pycnidium. Highest percentage underlined.

Tree species	tree no	twig	septa 0	1	2	3	4	5	6	7	8
P.cembra	1858	1	2	5	2	18	18	16	11	28	0
		2	2	5	2	36	20	18	10	7	0
P.montana	1124	1	0	0	0	1	3	13	34	49	0
		2	2	1	0	43	23	27	4	0	0
	1229	1	1	1	0	4	12	16	28	38	0
		2	0	1	0	8	9	22	25	35	0
	1388	1	0	0	2	22	20	30	12	12	2
			0	0	0	62	12	14	10	2	0
		2	0	0	0	28	20	20	14	18	0
			0	4	2	38	14	22	10	10	0
Picea abies	1787	1	0	0	1	5	4	11	20	59	0
			0	2	0	10	2	12	27	47	0
		2	0	0	0	47	33	10	6	4	0
			7	0	1	17	20	15	18	22	0
	1779	1	0	7	5	73	8	6	1	0	0
		2	0	2	1	25	32	15	16	9	0

Discussion

So far in Switzerland there are no shoot dieback problems in Pinus nigra stands. Two reasons may account for this: 1) less than 0.01% of the Swiss forest area is planted with P. nigra, and 2) most of the P. nigra grow in the Jura and south of the alps on dry sites (BUERGI and DIEZ,1986), conditions that do not favour the spread of the disease.

Ascocalyx sp. often endangers new plantations in the subalpine regions. BAZZIGHER (1986) reports that in a Pinus cembra plantation in Avers 92% of the plants were killed by Ascocalyx abietina within 10 years. In a comparable plantation at Andermatt P. cembra is still in good health whereas Larix decidua suffers heavily from Ascocalyx laricina. In turn this fungus does not create problems in Avers (LAWRENZ and HEINIGER, in preparation). The infection pressure from infected old Pinus cembra trees may be the reason for the heavy attack in Avers.

There are many reasons for an afforestation to fail at the subalpine sites: planting shock, deer and black grouse (Lyrurus tetrix) damage, mechanical damage caused by snow, wind, rolling stones, frost, insects etc. Several fungi may also be involved in dieback that are commonly found on young plants like Lachnellula sp., Phacidium infestans, Herpotrichia sp., Cenangium sp. and fungi, the role of which is not yet elucidated, e.g Cytospora sp., Tympanis sp.. SCHNELL et al.(1985) even doubts whether Ascocalyx laricina is responsible for the larch dieback. He failed to produce the disease with artificial infections on larch and believes that frost has the main impact. He isolated Brunchorstia pinea from dead larch shoots. This fungus also occurs as endophyte in healthy Pinus cembra twigs (CANAVESI,1983). The role of the endophytes, which seem to be present in many plant tissues, is still not elucidated. They may either incite defence mechanisms in plants and protect them from further infections or they may be weak parasites waiting for the plant vigour to decrease in order to create an outbreak of disease.

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Zusammenfassung

Triebsterben in subalpinen Aufforstungen in der Schweiz.

Das Ascocalyx-Triebsterben ist in allen subalpinen Regionen der Schweiz verbreitet. Vor allem junge Bäume in Aufforstungen sind betroffen. Ascocalyx laricina (Ettlinger) Schlaepfer-Bernhard befällt Larix decidua und Ascocalyx abietina (Lagerb.) Schlaepfer-Bernhard Pinus cembra, P. montana und Picea abies. Nach einem heftigen Krankheitsausbruch im Jahre 1969 scheint sich die Krankheit im Moment abgeschwächt zu haben. Im Frühjahr bildet sich zuerst die Nebenfruchtform Brunchorstia pinea auf den Pinus-Arten und auf Picea abies aus, erst im Sommer ist die Hauptfruchtform zu finden. Brunchorstia pinea hat Sporen mit 0 bis 8 Septen, wobei Sporen mit 3 oder 7 Septen vorherrschen. Auf dem gleichen Ast können beide Sporentypen vorkommen.

INVESTIGATIONS ON NEW MEANS OF IDENTIFYING RACES OF *ASCOCALYX ABIETINA*

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SUMMARY

Results of testing isolates of *Ascocalyx abietina* (Lagerb.) Schlaepfard-Bernhard with electrophoresis (PAGE) on a large scale are presented. Using US11 as reference, characteristic bands of the 155 isolates tested were obtained that permitted separating these into two distinct groups. When methods of identification by serology and electrophoresis were compared, a 96% concordance was obtained between 129 isolates tested by the first and then by the second method, and 100% concordance in a reciprocal test using 26 other isolates. Variations of protein profiles in the group classified as North American call for further investigations. The value of PAGE for routine testing of isolates to differentiate the European race is discussed. A report is also given of the successful use of some of the specific protein bands eluted from the gels, to produce monoclonal antibodies.

INTRODUCTION

Results of our previous investigations using polyacrylamide gel electrophoresis (PAGE) as an alternate test and an easier procedure than serology for identifying races of Ascocalyx abietina, were reported at the International Symposium on Scleroderris canker of conifers held in Syracuse, 1983 (Benhamou et al.,1983). We found from the protein profiles obtained that the isolates could be classified in six categories. The first three corresponded to the known races, the so-called North American, European, and Asian, as proposed according to serology tests (Dorworth et al.,1977). The other categories were: spruce isolates from Quebec, balsam fir isolates from Quebec, and an intermediate group of isolates combining characteristics of both the European and North American races on pine. The isolate F4 was used as reference for these tests. In subsequent work isolate US11 (European race) was used as reference and techniques were slightly modified to confirm and perfect the test. Another approach for differentiating races is to obtain monoclonal antibodies against proteins that are specific to the North American and European races.

This is a brief report on the current results obtained in these two types of investigations.

MATERIAL AND METHODS

A total of 129 isolates from pine that had been classified by serology were again tested with electrophoresis to confirm the concordance between race identification by both methods. Also, 26 isolates chosen randomly from a group of over a 100 classified first by electrophoresis were then tested by serology (thanks to Mr. C. Davis, Great Lakes Forestry Centre, CFS, Sault Ste-Marie, Ontario). In addition to testing these isolates obtained from infected tissue on pine or mass isolates from conidia and ascospores, 75 monoascospore isolates (chosen randomly from a collection of 425) were tested. The monoascospore isolates originated from four apothecia collected on red pine in plantations that were affected by both the North American and European races (isolates furnished by Dr. Michel Dessurault, Faculté de foresterie et de géodésie, Université Laval, Québec).

The culture medium used was: KH_2PO_4, 0.5 g; Na_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; KCl, 0.25 g; Asparagine (D-L) 2.0 g; glucose (D-) 10.0 g; $FeCl_3$, 2 mL (0.5% sol); HCl 0.1N, 4.5 mL; V-8 juice, 50 mL of supernatant after centrifugation; H_2O dist. 943.5 mL. The pH was adjusted to

7.0 with NaOH and after sterilization of the medium was around 6.2. The medium was used at the rate of 125 mL per Erlenmeyer flasks. Incubation was at 14°C for 4 to 5 weeks. Methods of protein extraction and of electrophoresis were as described in Benhamou et al. (1983). The protein reagents used were mostly the Bio-Rad protein assay dye reagent concentrate.

For monoclonal antibody production, some of the specific protein bands were eluted, ammonium sulfate precipitated, dialysed, and resuspended in buffer. A sample was run again through electrophoresis to ensure it contained the desired protein before we injected it into mice. Benhamou and Ouellette (1986) give the details of this procedure and that for hybridoma production appears in Benhamou et al. (1985).

It is to be noted that the operators were not aware of previous culture identification in conducting the present tests.

RESULTS

The electrophoresis profiles obtained in the present tests contained bands that matched those already reported in Benhamou et al. (1983). The bottom bands (Fig. 1A) (towards anode end) of the gel were, however, always more pronounced and better demarcated than those observed previously (Benhamou et al.,1983). Although differences were also observed in other bands, only these two bottom bands were used as a basis for differentiating the isolates. Thus using the bands obtained from US11 as a reference the isolates could be separated into two distinct groups, the first group similar to it, being identifiable as the European race. Isolates of the other groups were considered as of North American race. However, some isolates in this first group yielded differences in some of the other band profiles that might indicate the presence of variants in the group. All the monoascospore isolates tested were uniform, except for one, and were identified as North American.

When methods of identification by serology and electrophoresis were compared, a 96% concordance between the 129 isolates tested by the first and then by the second method was obtained, and 100% congruence in a reciprocal test with 26 randomly sampled isolates. In two cases, discrepencies were registered in a first test, but clarified in a second test. Many of the cultures identified as uncertain by serology were clearly identified as North American group in the present tests.

For the production of monoclonal antibodies the specific, bottom protein bands were eluted and treated for mouse injection. Unfortunately, uncertain results were obtained probably because molecular weight of these proteins was too low. In other tests, protein bands were taken from the middle or higher portions of the gel that were also

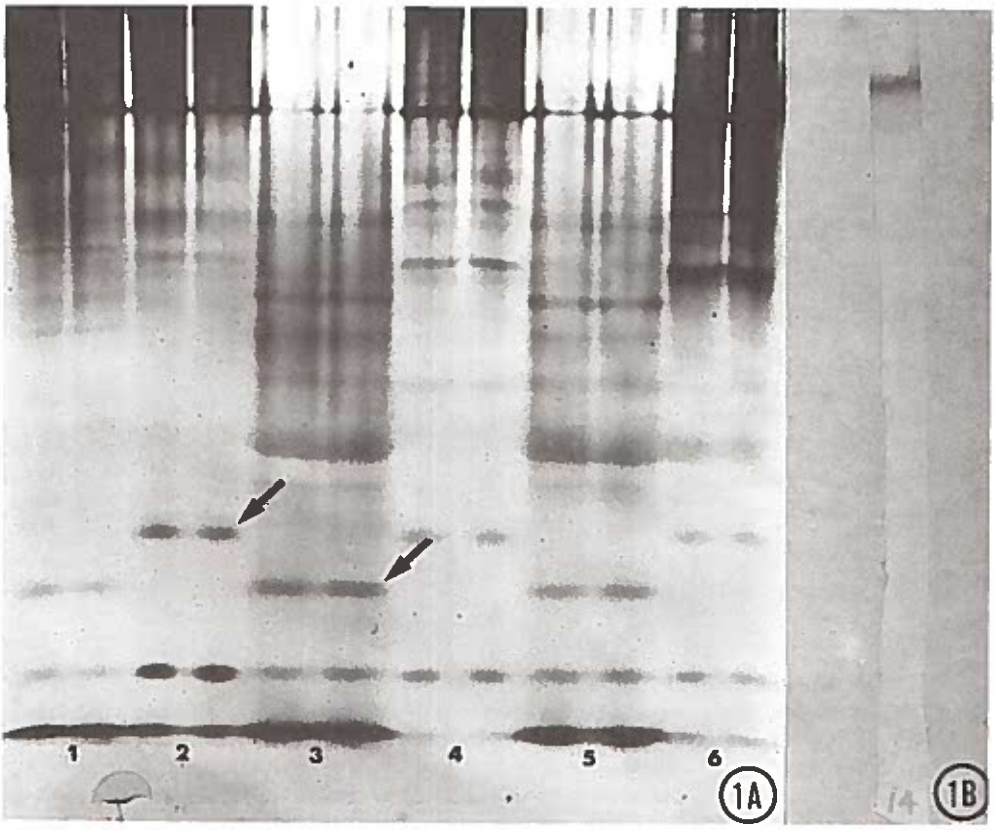

Fig. 1A. Characteristic protein profiles obtained by polyacrylamide gel electrophoresis using extracts from isolates of Ascocalyx abietina. Differences are noticeable in many of the bands, particularly in the bottom ones (arrows). Comparing the patterns of those bottom bands for six of the isolates (represented by two lanes each) isolate 1(US11), 3 and 5 are similar, and thus of the European race, whereas isolates 2, 4, 6 are different; these are of the North American race group.

Fig. 1B. Shows a specific positive reaction with Western blot (transfer of protein bands from polyacrylamide gels to nitrocellulose paper strips, treatment with antibodies and revealing reagents, see Benhamou et al.,1985) for the protein band that was used originally for monoclonal antibody production.

considered specific for either the North American or the European race. As with previous tests, the antigen solution obtained from the latter proved to be highly toxic to mice. However, as determined by ELISA tests (Ouellette and Benhamou,1986) several hybridomas were obtained for the North American race using culture filtrates as capture antigen. Some of these hybridomas were tested in western blot and found to give a strong, specific reaction (Fig. 1B).

CONCLUSION

PAGE is an easy method to sort the European race isolates of Ascocalyx abietina from other races. Except for the necessary long waiting period while cultures grow, the test is rapid, easily reproducible, and possibly less onerous than serology, at least for a large screening. We are confident therefore that this test could be advantageously used without further refinement for routine identification of cultures. However, refinements such as the use of two dimension electrophoresis, or study of comparative enzyme profiles (as is now conducted by Dr. Margarete Breitenbach, Forstliche Bundesversuchsanstalt, Vienna, Austria, Personal communication), would be greatly beneficial to further sort out the possible variants within a group, particularly, presented here as North American. Also, clarifying the status of isolates obtained from hosts other than pine, i.e. spruce, balsam fir, and larch, calls for such refinements. In attempting to establish a typical pattern for the North American race, the numerous ascopore isolates obtained would undoubtedly be a great asset.

Specific monoclonal antibodies used for race differentiation would provide an ideal method of diagnosis for A. abietina isolates. Although good progress has now been made by obtaining antibodies against the North American race, it is hampered by the difficulties in obtaining antibodies against the European race. Further efforts to settle this problem by using other European isolates as test organism are planned. In the meantime, the present monoclonal antibodies are being used to attempt following the development of the pathogen in its host.

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ZUSAMMENFASSUNG

Untersuchungen über neue Wege zur Rassen-Identifizierung bei Ascocalyx abietina

Dargestellt werden Ergebnisse aus Versuchen, in denen in großem Umfang Isolate von Ascocalyx abietina (Lagerb.) Schlaepfer-Bernhard mit Elektrophorese (PAGE) getestet wurden. Mit US11 als Referenz erhielt man bei den 155 geprüften Isolaten charakteristische Banden, die eine Trennung in zwei unterschiedliche Gruppen erlaubten. Verglich man Methoden der Identifizierung durch Serologie und Elektrophorese, so ergab sich eine 96%ige Übereinstimmung zwischen 129 mit der ersten und sodann mit der zweiten Methode getesteten Isolaten, und eine 100%ige Übereinstimmung in einem reziproken Test mit 26 anderen Isolaten. Variationen der Proteinprofile in der als nordamerikanisch klassifizierten Gruppe erfordern weitere Untersuchungen. Der Wert von PAGE für Routinetests von Isolaten zur Unterscheidung der europäischen Rasse wird diskutiert. Berichtet wird auch über die erfolgreiche Verwendung einiger spezifischer Proteinbanden nach Eluierung aus den Gelen zur Bildung von somaklonalen Antikörpern.

DIFFERENTIAL BEHAVIOUR OF *ASCOCALYX ABIETINA* FROM TEST INOCULATIONS IN ITALY

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SUMMARY

Inoculations on young trees of *Pinus nigra*, *P. sylvestris*, and *Picea abies* with conidia suspensions of *Brunchorstia pinea* obtained from different host species in different locations (Alps, Apennines and Adriatic coast), exhibit strong differentiations among hosts and isolates behaviour.

All the isolates were virulent in respect to the tested species although one of the Alpine isolates caused only very litte damage on all the hosts.

The susceptibility of infection was more pronounced on *P. nigra* followed by *P. sylvestris* whereas *P. abies* exhibited the fewest infections.

INTRODUCTION

Ascocalyx abietina (Lagerb.) Schläpfer and the anamorph *Brunchorstia pinea* (Karst.) Höhn. is a fungus widely distributed in North Temperate Zones. In Europe it occurs from the circumpolar areas to the Mediterranean Region (Dorworth, 1981; Muller and Dorworth, 1983).

This parasite is known in Europe in two different conidial forms (Donaubauer, 1974; Morelet, 1980). One form is *B. pinea* with 2 to 3 septa per spore and relatively short conidia. It is widespread in Europe and is known to infect a large number of conifers. A second type, *B. pinea* var. *cembrae*, produces conidia which are longer and less broad than those of the first type and which may contain 7 or 8 cells. Also, the number of known host species is smaller than in the case of the first variety.

The distribution of *Brunchorstia pinea* in Italy is quite interesting in that it normally occurs in high elevation zones. This parasite, however, is present in the Mediterranean area along the Adriatic coast, in the Apennines, which is in the central part of the peninsula, and in the Alps from

the valley floor to the top of the timber line (Moriondo, 1963; Capretti, 1984). The biological characteristics of the isolates that are present in the previously mentioned areas are still to be investigated. Many tests have been made to determine and compare the pathogenicity of this fungus. However, the differences in testing procedures have made these tests difficult to compare.

In the present test, it seemed most useful to compare the susceptibility of three different conifers of special importance in Europe with respect to isolates collected from different geographical regions. Proper replication of all the experimental elements were insured and data obtained were subjected to analysis of variance.

METHODS

Six year-old transplants of Pinus nigra (Austrian pine), Pinus sylvestris (Scots pine), and five year-old Picea abies (Norway spruce) were obtained for inoculation tests with Brunchorstia pinea. The trees were planted in rows one metre apart and 2.5 - 3.0 long. A factorial experimental design was adopted with 9 replicates, including the control group and 25 trees for each sub-plot. Trees were inoculated on the evening of June 14, 1984 with spore suspensions derived from monoconidial cultures of 4 different isolates of B. pinea (Table 1). They were obtained from a variety of different host species in different locations. Conidia, used for the inoculations, were produced on autoclaved wheat seeds in Petri dishes and test tubes. They were incubated for 7 - 8 weeks at a temperature of 20 degrees C. with alternating 12-hour exposures to ultraviolet light and darkness. More than 80% of the conidia germinated in water agar within 24 hours.

The conidia used in inoculations (Table 2) were dispersed in 0.1% water agar at a concentration calculated to deposit 5 x 10 (to the fifth power) conidia per plant when applied with a hand sprayer held 15 cm distant from the shoots. A shield was employed to reduce or eliminate drift of the inoculum. Controls were treated in the same manner as the test plants but were inoculated with 0.1% water agar alone. All trees were wetted with tap water several hours before and one day after inoculation.

At the time of inoculation, the length of the terminal shoots of the Norway spruce were 1.5 - 5 cm long. The Austrian Pines were 2 to 10 cm long and the Scots Pines were 2 to 13 cm in length.

By the end of April 1985 all infected trees showed the characeristic symptom: discolouration of needle bases. All trees were recorded as infected or noninfected on that basis. Terminal shoot infections were recorded in every instance as well as lateral meristem infections in the cases of Scots and Austrian pines. The number of shoots infected

on each species was also recorded. When pycnia were present on the shoots, spores were collected and measured. Twenty percent of trees that exhibited symptoms were returned to the laboratory and placed in culture to confirm the presence of the pathogen.

Data were subjected to factorial analysis of variance and the means were compared with L.S.D. method.

RESULTS

All isolates of B. pinea proved to be virulent with respect to spruce and pine but the percent of infection differed among the various hosts and isolates of B. pinea (Table 3). We never observed infections on the control. For this reason no further information on the control is given.

Irrespective of host species, there is no statistical difference between isolates I-6 and I-8 which are statistically inseparable with respect to percentage of meristems infected following inoculation (Table 3). In every instance, however, the Austrian pine was infected to a greater extend than were the other two species (Table 4). The Norway spruce was the species least frequently infected (Table 4), which confirms the previous experience of numerous investigators. The alpine isolate I-7, by contrast, exhibited only a low degree of virulence toward all the host species. Isolate I-5 was intermediate with respect to percentage of infections on all host species. With respect to total meristems infected, I-5 did not differ significantly from I-6 (Table 3-a). Isolate I-5 was responsible for approximately twice as many infections on lateral meristems than on terminal meristems or shoots (Table 3-b,c). Additionally, approximately twice the number of lateral shoots were infected on Austrian pine compared with the number infected on Scots pine. Spore septation and length of the conidia obtained from the infected shoots were similar with those used for the inoculations (Table 2). One exception is isolate I-7 which did not produce any pycnia on the shoots.

DISCUSSION AND CONCLUSIONS

Results of this test indicated a pronounced difference in virulence of the isolates of B. pinea from different geographical areas and on different hosts. Differences in susceptibility of various host species have been observed in the past (Donaubauer, 1974; Skilling and Riemenschneider, 1984) which have been confirmed by the present test. Notably, the Alpine isolate, I-7, exhibited only slight virulence in the Apennines area in contrast with other isolates from the same area. This is the first confirmed observation of this type, although further isolate replication would be required

to make a generalization. Similar observations could be made with respect to the other isolates, with their relative success as pathogenes in this area of somewhat elevated summer temperatures.

There is some indication that virulence and host range are related to serotype in North America, with the North American serotype found almost exclusively in Pinus spp., and on tissues two metres or less above ground level. A thoroughly replicated test of isolate/serovar virulence correlated with disease has not yet been reported from North America. The European serotype, conversely, exhibits the disease syndrome familiar in Europe in all of its manifestations. The present test represents our first effort to gain a correlation among isolate geographic source, host species of the isolate origin , and test species inoculated in terms of quantitative and qualitative disease impact.

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ZUSAMMENFASSUNG

Unterschiedliches Verhalten von Ascocalyx abietina in Inokulationsversuchen in Italien.

Inokulationen junger Bäume von Pinus nigra, P. sylvestris und Picea abies mit Konidiensuspensionen von Brunchorstia pinea verschiedener Wirtsarten an unterschiedlichen Standorten (Alpen, Apennin, Adriaküste) ergaben deutliche Unterschiede zwischen Wirtspflanzen und dem Verhalten der Isolate. Alle Isolate waren in Bezug auf die geprüften Wirtsarten virulent, obwohl ein Alpenisolat nur sehr geringe Schäden an allen Wirtspflanzen verursachte. Die Anfälligkeit gegenüber einer Infektion war ausgeprägter bei Pinus nigra, gefolgt von P. sylvestris, während Picea abies die wenigsten Infektionen aufwies.

Table 1. Isolates of Ascocalyx abietina = Brunchorstia pinea used in inoculation tests in the northern Apennines, Italy.

Isolate	Host	Date of isolation	Geographic origin	Altitude m
I-5	Pinus Pinea	1982.03	Volano (Ferrara) Adriatic coast	0 - 5
I-6	Picea abies	1982.02	Ortisei (Bolzano) Alps	1.500
I-7	Pinus cembra	1981.06	Ortisei (Bolzano) Alps	2.000
I-8	Pinus nigra	1980.05	Centocroci Pass (Parma) Apennines	1.000

Table 2. Distribution of mean lenght, range of lenght and septation of the conidia: a) used for inoculations, b) obtained from the infected shoots. (*) no spore production.

Isolate	Mean length um	Range of length um	Septation of conidia(%) 1	2	3	4	5	6	7
I-5 a	31.0 ± 0.6	21.6 - 48.0	9	3	88				
b	27.4 ± 0.4	19.4 - 38.8	2	3	95				
I-6 a	28.1 ± 0.3	21.7 - 36.3		1	98	1			
b	27.3 ± 0.4	15.5 - 38.8	4	2	94	1			
I-7 a	34.2 ± 0.3	25.4 - 47.7		1	43	36	13	5	3
b	(*)								
I-8 a	34.3 ± 0.3	19.1 - 47.7		5	90	3	2		
b	27.9 ± 1.0	19.4 - 38.8	4	5	91				

Table 3. Percent of a) trees, b) top leaders and c) new conifers shoots infected by four isolates of *Brunchorstia pinea* after inoculations.

a) Infected trees

Isolates (%)	I-7 (7.4)	I-5 (47.6)	I-6 (63.2)	I-8 (70.8)
F=0.01				

Species (%)	*Picea abies* (21.4)	*Pinus sylvestris* (51.3)	*Pinus nigra* (70.1)
F=0.01			

b) Infections on the top leader only

Isolates (%)	I-7 (1.1)	I-5 (19.3)	I-8 (39.6)	I-6 (43.5)
F=0.01				

Species (%)	*Picea abies* (0.3)	*Pinus sylvestris* (25.2)	*Pinus nigra* (53.4)
F=0.01			

c) Infections on lateral shoots only

Isolates (%)	I-7 (1.4)	I-5 (37.9)	I-8 (50.3)	I-6 (58.1)
F=0.001				

Species (%)	*Pinus sylvestris* (26.6)	*Pinus nigra* (55.4)
F=0.01		

Table 4. Results of inoculation tests with different isolates of *Brunchorstia pinea* on young trees of *Picea abies*, *Pinus nigra* and *P. sylvestris*. Percentage of damaged trees. Total no. 2,524.

a) *Infected trees*

Isolates/ Hosts	*Pinus nigra*	*Pinus sylvestris*	*Picea abies*
I-5	83.4	37.1	21.6
I-6	99.0	81.3	14.6
I-7	7.1	10.1	5.4
I-8	92.9	77.2	43.9

b) *Infection on the top leader only*

Isolates/ Hosts	*Pinus nigra*	*Pinus sylvestris*	*Picea abies*
I-5	43.8	13.3	0.4
I-6	88.9	44.4	0.4
I-7	0.4	3.0	-
I-8	82.8	40.5	0.4

c) *Infections on lateral shoots*

Isolates/ Hosts	*Pinus nigra*	*Pinus sylvestris*
I-5	61.0	15.4
I-6	82.2	37.2
I-7	1.2	1.7
I-8	75.2	37.8

IV. Stress factors and infection

THE EFFECT OF LOW TEMPERATURE ON THE EPIDEMIOLOGY OF SCLERODERRIS SHOOT BLIGHT

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SUMMARY

The effect of winter temperature and snow cover on the development of Scleroderris shoot blight was examined. Red pine seedlings were artificially inoculated with the North American serotype of Gremmeniella abietina. In three separate experiments repeated over two seasons, symptoms developed on seedlings which for at least 51 days between 1 November and 28 February were exposed to ambient temperatures between -6 C and +5 C for the entire day, or were covered by enough snow to maintain a similar temperature throughout the canopy. Results were consistent under natural field conditions, artificially manipulated field conditions, or completely artificial conditions. Thus, a relatively mild canopy temperature during the winter appears to favor disease development. The apparent latent period in the disease cycle, the occurrence of symptoms primarily on lower branches, and the restriction of the disease to latitudes that receive sustained snow cover are all consistent with this observation.

Additional key words: Ascocalyx abietina, Brunchorstia pinea, Pinus resinosa

INTRODUCTION

Scleroderris shoot blight, caused by the North American serotype of Gremmeniella abietina (Lagerb.) Morelet, is a problem on red pine (Pinus resinosa Ait.) in the northern Lake States and northeastern regions of the United States, and in eastern Canada. Our studies in northern Wisconsin and the Upper Peninsula of Michigan in the United States have focused on the effect of the environment on tissue colonization by the fungus and eventual symptom development. Three unique characteristics of the epidemiology of the disease as it occurs in this area have been identified:

(1) Although initial penetration by the fungus typically occurs in late spring or early summer, the fungus appears to remain latent in bract tissue throughout the summer and fall, rapidly colonizing tissue only in late January or early February (Patton et al. 1984). Initial symptoms, typically a red discoloration of needle bases, appear by early spring, approximately one year after infection. Such a long disease cycle makes it difficult to assess the relative importance of environmental factors on different stages of disease development.

(2) Symptoms on large trees usually occur within about 2m from ground level and such trees survive the infection (Anonymous 1981). Mortality is observed only in trees less than 2m in height (Anonymous 1978, Dorworth et al. 1977). These characteristics are in contrast to symptoms observed in the disease caused by the European serotype of the fungus, which include death of pole-sized trees.

(3) Worldwide distribution of the disease incited by the North American serotype of the fungus is restricted to areas north of about 45° N latitude (Nicholls 1979). The northern half of Wisconsin and the Upper Peninsula of Michigan fall above this line. Madison, Wisconsin lies south of the natural range of the disease.

Several observations pertinent to this disease provided us with clues to understanding these unique epidemiological characteristics. The literature indicates that the fungus may grow at temperatures as low as -5.8 C (Ettlinger 1945), and suggests a relationship between disease occurrence and snow depth and cover (Martin 1964, Yokota 1975, Yokota et al. 1974). Under a snow cover temperatures may remain near 0 C, regardless of ambient air temperature. We have verified that temperatures in red pine seedling canopies under snow remain between 0 and -5 C (Alberga et al. 1987 and personal observation, unpublished). Both northern and southern Wisconsin and Michigan experience extremely cold winter temperatures, but the northern areas usually have significantly more snowfall than does southern Wisconsin.

Given this information it seemed reasonable to hypothesize that snow cover provides a more temperate thermal microenvironment which favors colonization by the fungus. We suggest the term "conducive day" as a day in which the fungus can grow, but the trees remain quiescent. We then propose that disease will occur only after a critical number of conducive days has accumulated. We have defined a conducive day as one in which the ambient air temperature is between -6 C and +5 C, or the snow cover is enough to completely cover the experimental trees (20 cm for the containerized seedlings used). The lower temperature limit was chosen as that above which growth of the fungus has been observed. The upper limit was chosen somewhat arbitrarily as a temperature below which physiological activity of the tree is severely reduced (Vegis 1973). We present here three experiments designed to test this hypothesis. The first attempted to correlate disease development with naturally varying microclimates; the second was designed to artificially manipulate the thermal microenvironment to prevent disease development where it occurs naturally or induce disease development where it does not occur naturally; and the third attempted to replicate, in an artificial situation, conditions conducive to disease development.

MATERIALS AND METHODS

Local Climate Experiment

In June 1984, nine plots were established in Wisconsin and Upper Michigan, both north and south of 45° N latitude. Each plot consisted of 98 containerized red pine seedlings obtained from the Potlatch Corporation nursery in Cloquet, Minnesota. The seedlings were transplanted to a 2.0 X 0.6 m area of ground at each location. Half of these were artificially inoculated by brushing 0.1 to 0.2 ml of a 1 x 10^6 conidia/ml suspension onto the stem, bract, and lower needle surfaces of the current year's expanding shoots; the remaining half were "naturally" inoculated by suspending infected branches above the seedlings (Skilling and Waddell 1974). Throughout the spring and summer of 1985 the plots were assessed for symptom development at monthly intervals by the "pull test" technique (Patton et al. 1984). All needles which easily pulled off were counted and labelled according to plot, seedling, and date of collection. Ten loose fascicles per tree (if available) were plated out on V-8 agar + 100 mg/l streptomycin and incubated at either 4 C or 18 C under a 16-hr photoperiod at 100 µmoles $m^{-2}s^{-1}$ light intensity to induce sporulation. Based on preliminary isolation trials, a given seedling was considered infected if a sporulating isolate was obtained, or if at least five non-sporulating isolates were obtained from the ten isolation attempts. Trees with more than ten loose fascicles were considered infected without attempts at isolation. For each location, National Weather Service temperature and snow data for the previous winter months at each location were compiled and the number of conducive days determined. The data were analyzed for correlations between infection and conducive days. The experiment was repeated with new seedlings in 1985 and trees assessed in 1986.

Snow Cage Experiment

Four plots of 25 three-year-old red pine seedlings were established in 1984, two at Blackhawk Ski Club near Madison and two at Copper Falls State Park 420 km north of Madison. At Blackhawk, the "snow" plot was completely covered with natural or artificial snow between 8 January 1984 and 2 March 1985; on the "no snow" plot, all of the natural snow that fell was manually removed so that the crowns of the seedlings were always exposed to ambient conditions. At Copper Falls, a wire-mesh cage (18 X 14 mesh) was placed over the "no snow" plot to prevent snow accumulation, while the "snow" plot remained covered by natural snow throughout the same period. Average hourly and daily maximum and minimum temperatures were sensed with thermistors placed within the canopy 40 cm above ground in the center of each plot, recorded with a CR-21 (Campbell Scientific) data logger, and stored in a solid state storage module (Campbell Scientific model SM64). An automatic photographic system (Alberga et al. 1987) was used to record snow cover at each plot. Trees were assessed for symptoms the following summer. The experiment was repeated in 1985 with 49 containerized red pine seedlings per plot, with thermistors placed at 20 cm above ground. Data were collected and analyzed as in the local climate experiment.

Controlled Temperature Experiments

Six containerized seedlings each were planted in 23.5 X 19.0 X 19.0 cm wooden flats which were buried in the ground in northern Wisconsin. Half were artificially inoculated in June 1984 and half placed under natural inoculum.

These were left in northern Wisconsin until 10 December to ensure hardiness. One artificially and one naturally inoculated flat were then transferred to controlled temperature rooms at each of the following conditions: (1) -30 C dark, (2) -20 C dark (45 days) followed by +4 C dark (60 days), (3) 16 C, 8-hr photoperiod, (4) 20 C, 8-hr photoperiod, (5) 24 C, 8-hr photoperiod, and (6) 26 C day/21 C night, 12-hr photoperiod. Two flats were also transferred to Blackhawk and reburied and two were left at the site of inoculation. After 105 days, flats from the -30 C and +4 C treatments were transferred to 16 C, 8-hr photoperiod, to induce bud break and allow any further symptom development. Seedlings in all growth rooms were then assessed daily for symptom development. Flats at Blackhawk and northern Wisconsin were assessed for symptom development after 121 and 147 days, respectively. Infection was determined as in previous experiments.

In early 1985 twelve similarly planted trees were inoculated in the laboratory, placed in an unlighted dew chamber at 20 C and 100% relative humidity for three days to allow for initial infection, then transferred directly to -4 C without light. Symptoms were assessed at monthly intervals.

RESULTS

Local Climate Experiment

Disease incidence and mortality observed on the artificial inoculation plots ranged from 0 to 100% in both 1985 and 1986. (The natural inoculation technique led to much less consistent infection over two years, and therefore only the results of the artificial inoculations will be presented.) The number of conducive days accumulated between 1 November and 28 February in the various plots ranged from 18 to 80 in 1985 and from 41 to 113 in 1986. There did appear to be a critical number of conducive days, somewhere between 44 and 59, before which disease did not develop but after which disease was observed (Tables 1 and 2).

Table 1. 1985 infection and mortality as related to number of conducive days.

Location	Days	% Infection	% Dead
Janesville	61	6	0
Hancock	44	0	0
Sturgeon Bay	68	42	7
Trout Lake	42	0	0
Mellen	65	61	52
Spooner	18	0	0
Alberta	80	93	73
Newberry	66	100	100

Table 2. 1986 infection and mortality as related to number of conducive days.

Location	Days	% Infection	% Dead
Janesville	41	0	0
Sturgeon Bay	101	25	25
Trout Lake	59	70	43
Mellen	101	61	52
Spooner	102	88	75
Alberta	101	100	100
Newberry	107	54	50
Calumet	113	86	58

We were also able to follow five of the original nine plots through a second season and relate infection to the accumulation of conducive days for the two seasons. Percent infection increased in all cases (Figure 1). In one instance (Trout Lake) a plot that was exposed to 42 conducive days in the first season, and showed no infection in 1985, had 88% infection in 1986, after accumulating 59 additional conducive days in the second season.

Snow Cage Experiment

In southern Wisconsin in 1985 the "no snow" plot was exposed to 21 conducive days and all seedlings were symptom free. Artificial snow cover increased the number of conducive days on the "snow" plot to 65, and 80% of the seedlings developed symptoms. In northern Wisconsin in the "no snow" plot, the snow cage reduced the number of conducive days to 21, and no symptoms were observed. The "snow" plot was naturally exposed to 51 conducive days, and disease developed in 80% of the seedlings. In 1986 our southern Wisconsin plots were severely damaged by meadow mice and therefore could not be evaluated. In northern Wisconsin, we obtained some disease development in the "no snow" plot (23 conducive days) as well as in the "snow" plot (100 conducive days).

We observed a dramatic increase in disease in 1986 on the seedlings inoculated in 1984. These trees were not reinoculated in 1985; however, any natural snowfall was allowed to accumulate on the Blackhawk "no snow" plot and the snow cage was removed from the "no snow" plot at Copper Falls, so that all plots were exposed to at least 46 additional conducive days. Infection observed in both "snow" plots increased to 100%, while that in the "no snow" plots increased from zero to over 70% (Figure 2). In addition, symptoms on these trees were found first on two-year-old needles, rather than on the one-year-old needles typically affected.

Controlled Temperature Experiments

Of the six artificial overwintering regimes to which the seedlings were exposed, only 60 days at 4 C allowed symptom expression. All of the trees at this temperature as well as all of those trees overwintered at Blackhawk ("snow" plot, 65 conducive days) and at northern Wisconsin were infected.

All seedlings inoculated under laboratory conditions were asymptomatic after one month at 4 C, but exhibited initial symptoms after two months at this temperature.

DISCUSSION

The data from our local climate, snow cage, and controlled temperature experiments between 1984 and 1986 support the hypothesis that a critical number of conducive days is necessary for disease to develop from initial infection through to symptom expression. This critical number seems to lie between 44 and 51 (Figure 3). Under laboratory conditions, a summer latent period was not necessary for disease development as long as the proper overwintering conditions were met. We artificially induced the entire disease cycle, from inoculation through symptom development, in only two months, by inoculating trees and then placing them at +4 C for eight weeks. The hypothesis in its present form does not fit one point, the "no snow" plot in northern Wisconsin in 1986, but does account for this point if the definition of a conducive day is altered, for example by increasing the upper temperature limit to +9 C. Further work is being done to refine the hypothesis in this way.

Our results allow us to address all three unique characteristics which have posed problems to us in our study of the disease. First, since conducive days as defined here do not begin accumulating until late fall, if over 44 such days are needed for tissue colonization and symptom expression, the existence of a long latent period between initial infection and symptom expression the following spring is quite understandable. We found that the fungus can in fact remain latent for more than one year, if enough conducive days are not accumulated in the first season. Second, since branches closest to the ground are covered with snow for longer periods in a given season, and therefore exposed to more conducive days, one would expect to find symptoms here more often than on higher branches. When such symptoms are observed, they may be due to infections which occurred more than one year ago and have remained latent until exposed to enough days of the proper environmental conditions. The fungus has in fact been reisolated from asymptomatic trees up to 23 months after artificial inoculation (Blenis et al. 1984). Third, since northern latitudes have a much higher likelihood of exposure to over 51 conducive days in any given season, allowing the disease to persist at endemic if not epidemic levels, it is here that the disease would be able to maintain itself over time. Such an understanding of the field conditions conducive to disease development, and knowledge of how to provide similar conditions in a controlled environment, will expand the potential of future investigations into the etiology, epidemiology, and control of this disease.

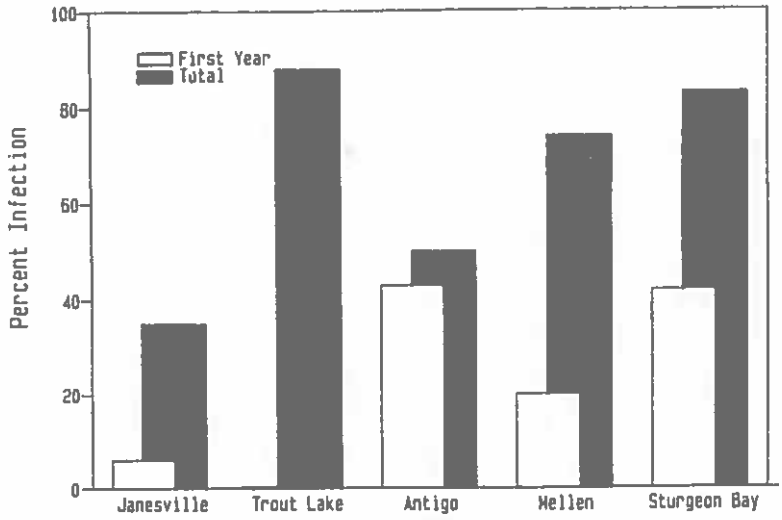

Figure 1. Increase in disease incidence (percent infection) in local climate experiment plots from 1985 (□) to 1986 (■).

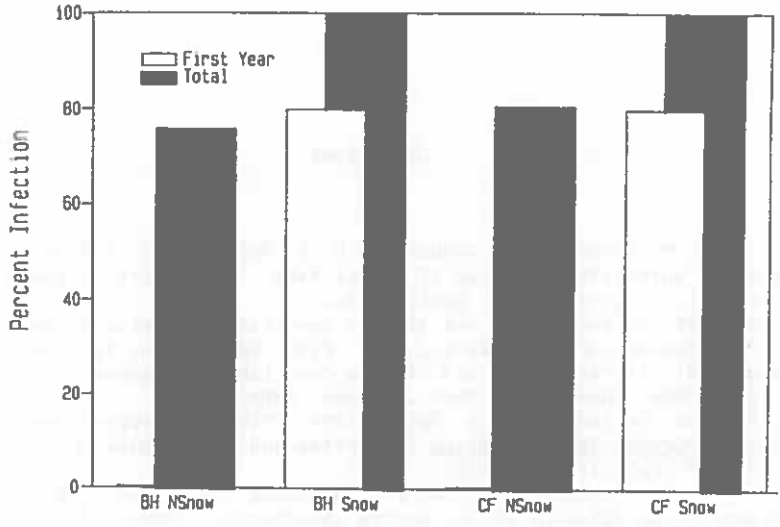

Figure 2. Increase in disease incidence (percent infection) in snow cage experiment plots from 1985 (□) to 1986 (■).

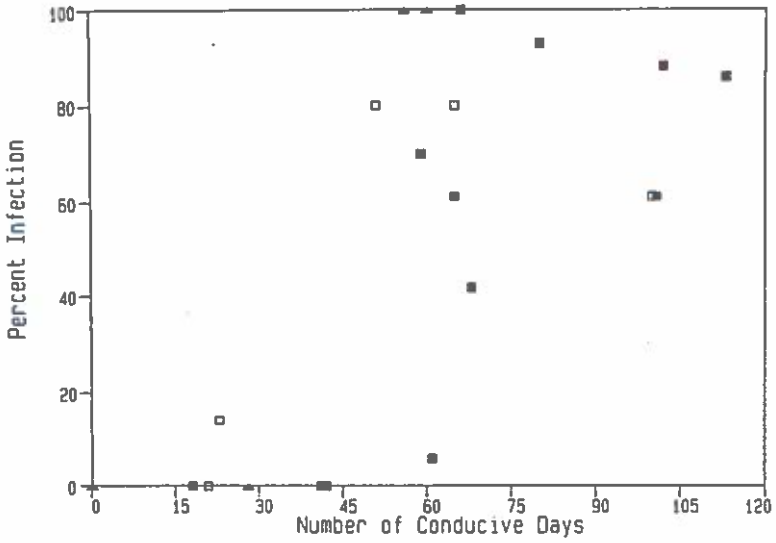

Figure 3. Disease incidence (percent infection) as a function of number of conducive days in 1984 and 1985 local climate experiment plots (■), snow cage experiment plots (□), and controlled temperature experiment flats (▲). A day is conducive if the ambient air temperature is between -6 C and +5 C or snow cover > 20 cm.

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ZUSAMMENFASSUNG

Die Effekte von Wintertemperatur und Schneebedeckung auf Scleroderris "shoot blight" wurden durch künstliche Inokulation von Pinus resinosa-Sämlingen mit dem Nordamerikanischen Serotyp von Gremmeniella abietina überprüft. In drei verschiedenen Experimenten, die über zwei Jahre wiederholt wurden, entwickelten sich Symptome an Sämlingen. Diese Sämlinge waren, für mindestens 51 Tage zwischen dem 1. November und dem 28. Februar, ganztägig Umgebungstemperaturen zwischen -6 und +5 C ausgesetzt, oder von mindestens 20 cm Schnee bedeckt, wodurch die Temperatur im Blattbereich ähnlich hoch war. Die Resultate unter natürlichen Feldbedingungen, künstlich beeinflussten Feldbedingungen, sowie unter vollständig künstlichen Bedingungen stimmten überein. Sie legen nahe, dass relativ milde Blattemperaturen während des Winters die Krankheitsentwicklung fördern, und das Auftreten von Symptomen vorwiegend an den unteren Zweigen sowie die Beschränkung der Krankheit auf Breitengrade mit anhaltender Schneebedeckung erklären.

STRESS COMBINATIONS AND THE SUSCEPTIBILITY OF SCOTS PINE TO ASCOCALYX ABIETINA

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ABSTRACT

The simulated "unfavourable" growing season - low temperature and low light intensity - made the pine seedlings more susceptible to Ascocalyx abietina. The cold treatment had a smaller effect on the seedlings growing in favourable conditions than on those in unfavourable ones. In the unfavourable season the inoculations right after the cold treatment caused more damage than the corresponding inoculations without the cold treatment. The needle retention values characterized the condition of the seedlings and at the same time the influence of the inoculations.

INTRODUCTION

Cool and rainy growing seasons, which are unfavourable conditions for pine, have been found to increase the occurrence of Ascocalyx abietina (Lagerb.) Schläpfer-Bernhard damage. Frost damage is also supposed to favour fungus infection (Yokota et al 1974, Teich 1968). The effect of various combinations of environmental factors, as well as the timing of such factors, on the susceptibility of pine to disease is an interesting topic from the point of view of, for instance, carring out preventive measures in forest nurseries.

Favourable and unfavourable growing seasons were simulated in greenhouse conditions in this study. The effect of cold stress and fungus inoculation carried out at the beginning and end of the growing seasons was investigated in pine (Pinus sylvestris) seedlings growing in different conditions.

MATERIAL AND METHODS

The study was carried out at the Suonenjoki Research Station of the Finnish Forest Institute. One-year-old pine seedlings were subjected in the spring to "favourable" and "unfavourable" growing seasons. The temperature in the favourable growing season was + 20 °C and the light intensity about 200 $uEm^{-2}s^{-1}$. The corresponding values for the unfavourable season were + 10 °C and 90 $uEm^{-2}s^{-1}$. In the unfavourable growing season (160 days) the temperature sum reached 890 d.d. and in the favourable growing season (160 days) 2 200 d.d. The origin of the seedlings was a region where the temperature sum of the growing season (155 days) is 1 100 d.d.

One group of seedlings from those grown in the two different types of growing seasons were subjected to the cold treatment in the spring, and the other group in the fall the same year. The cold treatment - inoculation groups were:

1. Cold treatment in the spring of the growing season, inoculation before the treatment
2. " " of the growing season, inoculation right after the treatment
3. " " of the growing season, inoculation in fall of the growing season
4. Cold treatment in the fall of the growing season, inoculation in the previous spring
5. " " "inoculation right after the treatment

The experiment also included seedling groups not subjected to the cold treatment. The inoculation dates for these were the same as in the earlier groups.

The cold resistance of the seedlings was estimated using a freezing test.

The impedance (f= 1 kHz) and the diameter of the previous year's shoot were measured before and after the frost treatment. The specific impedance difference (Δz) was calculated according to the formula

$$\Delta z = A/l \, (Zj - Ze)$$

where A= the cross-sectional area of the shoot
l= the distance between the electrodes

Ze, Zj = the absolute impedance of the shoot before and after the frost treatment

The temperature response of the specific impedance difference of the shoot was determined ($\Delta z=f(T)$, Fig.1) (Repo & Pelkonen 1986). The temperature equivalent to a value of $\Delta z = -10\ \Omega$ m was estimated from the curve. This was the target temperature for the seedlings in the frost treatment. The survival rate corresponding to the specific impedance difference was estimated using the results of an earlier study (Repo & Pelkonen 1986). This gave the temperature response of the survival rate of the seedlings in the different frost treatments (Fig. 2).

The rate of freezing and warming during the treatment was 4 - 5 $^{o}C\ h^{-1}$. The overall length of the treatment was 12 - 14 h. The minimum temperatures and their duration are presented in Table 1. The expected survival of the experiment seedlings as a result of the freezing test was determined from curves in Figure 2 in order to compare the different cold stresses (see Tables 1 and 2).

Inoculation was performed by dripping a suspension of Ascocalyx conidia onto the terminal buds of the seedlings. 0.22 ml of suspension/seedling (density of germinating conidia, 5 x 10^{6} / ml) was used on two successive days in inoculations carried out in the spring. In inoculations carried out in the fall, 0.51 ml of suspension was used/seedling (density of germinating conidia 6 x 10^{6}/ml) and the inoculation given on one day only. The seedlings grown under favourable conditions were kept for 2 days in unfavourable conditions before and after inoculation.

The retention value of a single needle pair on the upper and lower part of the new shoot was measured before overwintering. Corresponding measurements were made the following spring.

After the growing season had terminated the seedlings were hardened. The hardening, winter and dehardening programme took 140 days. The winter temperature was $0^{o}C$.

The experiment was terminated after dehardening. The new height growth of the seedlings was measured. The seedlings were classified according to their external appearance into four classes:

1. healthy
2. weakened

3. clearly diseased
4. dead

The shoots of the seedlings were then moistened, put into plastic bags and stored at + 13 °C. After three weeks the shoots were examined for the development of Ascocalyx conidia mass or mycelium tufts (cf. Hudler et al 1983).

RESULTS AND DISCUSSION

The unfavourable growing season made the seedlings susceptible to infection by Ascocalyx abietina. Inoculating the seedlings resulted in a number of dead and weakened seedlings (Fig. 3). Seedlings grown in the favourable growing season rarely showed any clear effects of inoculation. The difference between the susceptibility of seedlings grown in different conditions could, according to Read (1968), be a result of the varying amount of soluble carbohydrates in the seedlings. The amount of soluble carbohydrate is low in seedlings grown in low light intensity. Provenances which are too southern with respect to the growing area have been found susceptible to Ascocalyx (Roll- Hansen 1972, Uotila 1985). This could be connected to successful infections of the seedlings subjected to the growing season with a low temperature sum in this study. However, temperature sum does not account for all the changes in growing conditions associated with transferring seed from south to north.

Inoculations carried out in the spring had a stronger effect (proportion of dead seedlings 85 %, 57 %) than inoculations in the fall (proportion of dead seedlings 16 %) (Table 3, Fig. 3). One reason for this may be that, after inoculations in the spring, the fungus had a longer time to become established on the seedlings. Such establishment could be possible because of the unfavourable condition. Another reason could be that inoculation in the spring was done on two days and inoculation in the fall during one day. The weaker effect of inoculation in the fall was, however, surprising. The unfavourable growing season was expected to make the seedlings more susceptible to fungus. Kurkela and Norokorpi (1979) have found that Ascocalyx inoculation done in the fall more frequently produced cankers on Scots pine than inoculation in the spring. In the experiment carried out by Yokota et al (1974) in Japan, Ascocalyx inoculation in the fall had a stronger effect than inoculation in spring in Abies sachalinensis.

The calculated effect of cold treatment on the seedlings was exceeded more often in the unfavourable than in the favourable conditions (Table 2). Recovery from damage has been found to depend partly on the plant's condition (Mullick and Jensen 1976). In unfavourable conditions, the effect of the cold treatment in the fall considerably exceeded the calculated effect (Table 2). Hardening was started ten days after the treatment, and hence there was not much time for the damage to heal. The time required for healing, for the formation of a protective zone, increases towards the fall, and healing stops completely during the winter (Mullick and Jensen 1976).

The cold tolerance of plant depends, among other things, on the development stage and depth of hardening of the plant. Ice crystals, which develop in or outside cells may cause dehydration of the cells and the cell structures to break down (i.e. Burke et al 1976). Plants weakened by cold stress are also susceptible to less aggressive pathogens (Crist and Schoeneweiss 1975, Schoeneweiss and Wene 1977). There is much data available about Ascocalyx abietina damage initiating from frost pockets (Ohman 1966, Dorworth 1972, Skilling et al 1966). Yokota et al (1974) found that cold damage increases the effect of inoculation. On the other hand, Skilling (1972) did not find that subjecting the needles to cold treatment had any effect on infection.

The temperatures used during this experiment did not prevent the fungus from surviving. The fungus is killed within one week if the temperature is + 33 °C or more (Sletten 1971). The optimal temperature for fungus growth is +13 - + 20 °C (Dorworth and Krywienzyk 1975, Sletten 1971, Uotila 1982). The fungus is found to grow at 0 °C and even at -5.8 °C (Ettlinger 1945). Sletten (1971) did not find any growth below - 2 °C. The fungus withstands the cold treatment used in the experiment discussed here (Petäistö & Repo 1985, unpublished).

In this study, cold treatment had no clear effect on the susceptibility of the seedlings to the fungus in all treatment-inoculation combinations. However, inoculation made immediately after the cold treatment in the spring had a stonger effect than the corresponding inoculation without the cold treatment. Inoculation after the cold treatment in the fall had a stronger effect than the corresponding inoculation without the cold treatment, also (Table 3, Fig. 3). New tissue damage may possibly help the fungus infection.

All seedlings inoculated before the cold treatment died, correspondingly dead seedlings proportion was 85 % in the inoculated seedlings not subjected to the cold treatment (Table 3). The cold treatment in the spring had no effect on the susceptibility of the seedlings to the inoculation done in the fall. The corresponding inoculation without the cold treatment had nearly the same result (Table 3). The seedlings inoculated in the spring (the latter inoculation) before the cold treatment carried out in the fall had a very high proportion of dead seedlings (93 %), the dead seedlings proportion of uninoculated seedlings was 62 %. Cold treatment given in the fall was too strong in one seedling group, the seedlings given the former inoculation in the spring and their controls all being killed. That is why there are no inoculation results for this treatment- inoculation group (Fig. 3, Table 3).

Incubating the shoots in plastic bags produced black sporangia, conidia mass and green mycelium tufts on 18 seedlings only. Of these, 14 seedlings were from those grown under unfavourable conditions. The fungus developed most frequently on shoots from weakened and dead seedlings and least from healthy seedlings.

The needle retention values of seedlings grown in unfavourable conditions were smaller both in the fall of the growing season and in the following spring (0 - 77 x 10^{-3} N), than the values for seedlings grown in favourable conditions (in spring 78 - 236 x 10^{-3} N). The difference between these conditions was statistically highly significant.

There was no difference between the retention values of the needles of inoculated and control seedlings in the fall of the growing season, but in the following spring the difference was highly singnificant in seedlings grown under unfavourable conditions, and fairly significant in seedlings grown under favourable conditions (Fig. 4). The values for the inoculated seedlings were smaller.

In the spring following the growing season, there were highly significant differences between the needle retention values of healthy, weakened and dead seedlings. The healthy seedlings had the highest values.

Ascocalyx abietina causes the most damage to plant tissue during the dormant period of the plant. In this study, too, the damage did not become visible until after winter. The needle retention value as well as the growth of seedlings in spring after the growing season characterized the condition of the

seedlings. Healthy seedlings had a mean value of 34 mm (dev 19.5) and the weakened 6 mm (dev 17.1).

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ZUSAMMENFASSUNG

Streß-Kombinationen und die Anfälligkeit der Waldkiefer gegenüber Ascocalyx abietina.
Eine simulierte "ungünstige" Vegetationsperiode - niedrige Temperatur und geringe Lichtintensität- machte die Kiefernsämlinge anfälliger für Ascocalyx abietina. Die Kältebehandlung wirkte sich auf Sämlinge, die unter günstigen Bedingungen wuchsen, weniger aus als auf solche unter ungünstigen Bedingungen. In der ungünstigen Vegetationsperiode verursachten Inokulationen direkt nach der Kältebehandlung größere Schädigungen als entsprechende Inokulationen ohne Kältebehandlung. Die Anzahl festsitzender Nadelpaare am Langtrieb charakterisierte den Zustand der Sämlinge sowie den Einfluß der Inokulationen.

Table 1. The minimum temperatures, their duration and the dates of the cold treatments. A = the unfavourable, B = the favourable growing season.

Cold treatment in the spring	T_{min} °C	Duration of T_{min}	Date of treatment	Temperature sum, d.d.
A Inoculation before treatment	-10.7	1 h 30 min	12.5.-84	80
B — " —	-12.0	40 min	11.5.-84	156
A Inoculation after treatment	-12.0	30 min	10.5.-84	70
B — " —	-6.5 - -7.7	1 h	9.5.-84	128
A Inoculation in the fall	-5.5 - -6.2	40 min - 4 h 20 min	21.-23.5.-84	95 - 105
Cold treatment in the fall				
A Inoculation in the spring and the fall	-5.0 - -8.0	1 h - 2 h 40 min	2.-5.10.-84	790 - 805
B — " —	— " —	— " —	— " —	2055 - 2090

Table 2. The expected and the actual survival proportions of various seedling groups. A = unfavourable, B = favourable growing season.

Cold treatment in the spring	Expected survival proportion %	Survival proportion in experiment %
A Inoculation in the spring before treatment, control	93	64
B — " —	92	88
A Inoculation in the spring after treatment, control	92	90
B — " —	98 - 100	91
Cold treatment in the fall		
A Inoculation in the spring and the fall, control	92 - 100	0 - 38 - 60
B — " —	60 - 100	100 - 96 - 72

Table 3. The proportion of dead seedlings in various treatment combinations and the proportion remainders (inoculated minus control). Unfavourable growing season.

		The proportion of dead seedlings % Inoculations		
		The former in the spring	The latter in the spring	The one in the fall
No cold treatment	inoculated	85	64	16
	control	0	7	0
	remainder	85	57	16
Cold treatment in the spring	inoculated	100	91	22
	control	36	10	7
	remainder	64	81	15
Cold treatment in the fall	inoculated	100	93	74
	control	100	62	40
	remainder	0	31	34

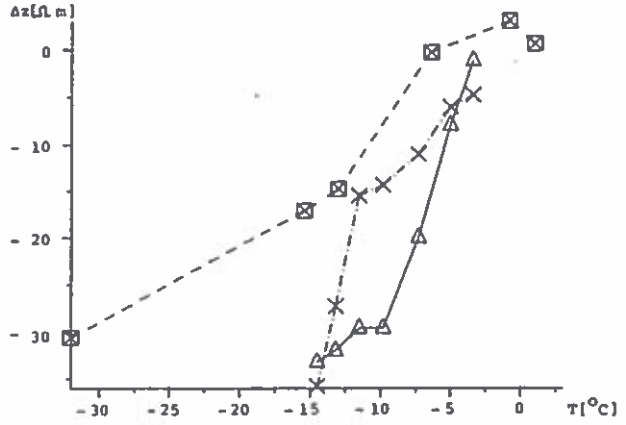

Fig. 1. Determination of the frost resistance of the test seedlings using the temperature response of the specific impedance difference in the current year shoot: cold treatment was given in spring 1984 (- ⊠ - ⊠ -) and in autumn 1984 (favourable —△—△—△— and unfavourable - · X · - · X · growing season). Each point is the mean of 6 - 12 seedlings in the spring, and the mean of 6 seedlings in the autumn.

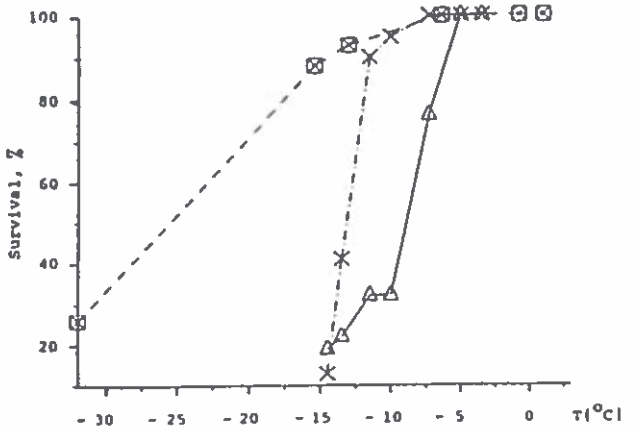

Fig. 2. The specific impedance difference - survival of the test seedling estimated as a function of the cold treatment temperature. The estimation is made using the relationship between specific impedance and survival (Repo & Pelkonen 1986). The explanations as in Fig. 1.

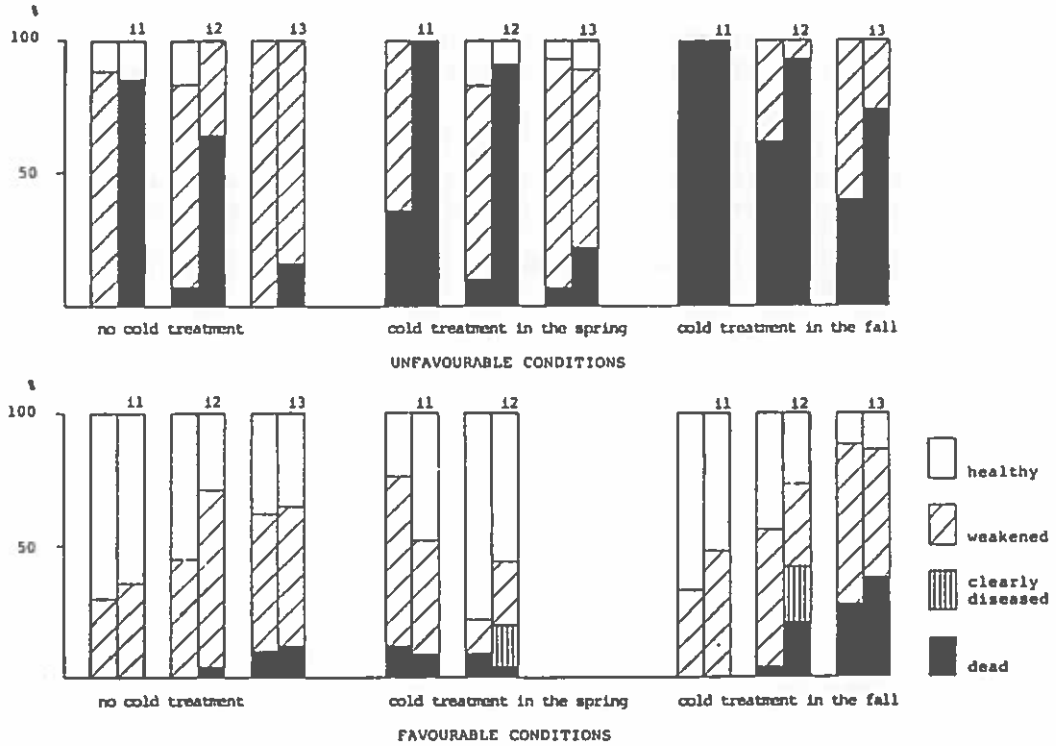

Fig. 3. The proportions of condition classes in the different treatment combinations i1 and i2 = the former and the latter inoculation in the spring, i3 = the inoculation in the fall, the left column = control

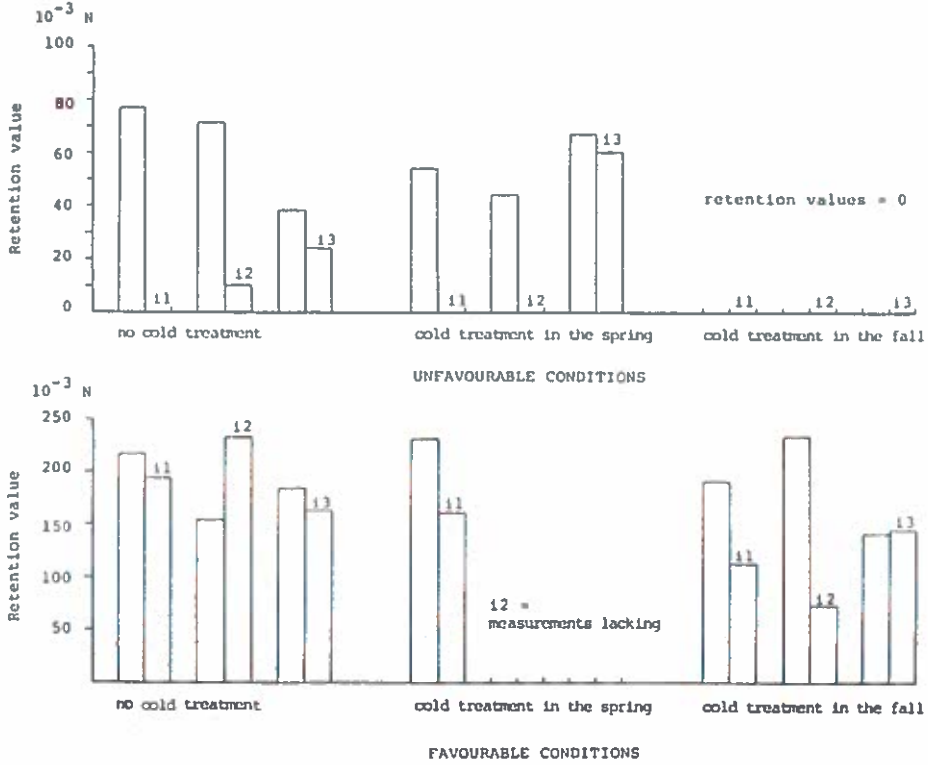

Fig. 4. The needle retention values in the spring in the different treatment combinations. The explanations as in fig. 3.

THE INFLUENCE OF DURATION OF LEAF WETNESS, PINE PROVENANCE AND POST-INFECTION MODE OF OVERWINTERING ON THE DEVELOPMENT OF GREMMENIELLA ABIETINA

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SUMMARY

The effects of the duration of leaf wetness after infection, and of the provenance and mode of overwintering of the host were studied by artificially inoculating young potted pine plants with conidia of Gremmeniella abietina from pure culture. No significant differences were found between periods of 6, 12, 18,24, 48, 60 and 72 hours of leaf wetness. In all cases the trees became seriously diseased. Plants that had been kept wet for 30 minutes were subsequently lightly attacked. In a second experiment the susceptibility of one P. sylvestris provenance and 4 P. nigra provenances was tested. After infection the plants overwintered in a greenhouse or outdoors. A significant difference in attack was observed between P. sylvestris and P. nigra and between one of the P. nigra provenances and the other three. The differences between provenances did not depend on the mode of overwintering. In all cases, however, the plants overwintered outdoors were attacked significantly more severely.

Additional keywords: Brunchorstia, Scleroderris, resistance, epidemiology.

INTRODUCTION

Without exception, climate and weather play a prominent role in the infectivity and epidemiology of fungal pathogens. This is especially obvious in case of Gremmeniella abietina. Many researchers have reported on the supposedly positive effects of wet springs and cold winters on the development of Brunchorstia. The disease is more severe in topographic depressions (Dorworth, 1973), in too dense plantations and in the shelter of older plantations (Gremmen, 1966), on northern slopes (Read, 1966) at high altitudes (Donaubauer, 1984) and in provenances planted too far north of their original location (Navelainen & Uotila, 1984). It should be mentioned that the disease pattern in the field in many of the above-mentioned examples can be explained both by low temperatures (or frost damage) and by moister conditions in the plantation and leaves remaining wet for longer. Research to date has made clear that the disease syndrome of Brunchorstia is not determined by a single factor. Pinus nigra in the Netherlands is both sensitive to frost and susceptible to Brunchorstia. So, given the supposedly additional effect of frost on the development of the disease, or even a mutual effect, there is only one way to unravel the separate effects: by experiments under controlled conditions.
In this paper we report on two inoculation experiments that were done to study the effects of duration of leaf wetness, mode of overwintering and provenance on the disease progress of Brunchorstia.

EXPERIMENT 1: THE EFFECT OF LEAF WETNESS

Material and methods.

Plant material. In March 1981 72 three-year-old plants of Pinus nigra ssp. maritima (Corsican pine) were potted in 3-litre pots and left outdoors. At the time of inoculation in June the new shoots were developing, the needles had just emerged from the needle sheaths and the green unlignified bark was visible.

Inoculum. G. abietina was isolated in March 1981 from a pycnidium on a shoot of P. nigra killed by the fungus in 1980. To increase the inoculum the isolate was inoculated on sterilized wheat grains and incubated at room temperature. Production of conidia started 5 weeks after inoculation. A conidial suspension was prepared in demineralized water at a concentration of 1.75 x 10/5 per millilitre. The viability of the spores was checked on malt agar at 24 degrees Celcius. The maximum time that elapsed between preparation of the suspension and inoculation was 2 hours.

Inoculation method. On 15 June 1981 the plants were brought into a greenhouse, where 48 plants were sprayed with 400 ml of suspension and 24 other plants were sprayed with demineralized water, to serve as controls. The plants were then kept wet for various periods.

Duration of leaf wetness. The plants were divided into 8 groups, each group consisting of 6 inoculated and 3 control plants, unless otherwise indicated. Immediately after inoculation the first group of plants was

brought into a room, where they were allowed to dry. The drying period did not exceed 30 minutes.
The same procedure was subsequently carried out with the other groups after 6, 12, 18, 24, 48, 60 or 72 hours. When the plants were dry they were put into a greenhouse made with plastic film where they remained until the end of the growing season, protected from rain. Water was supplied on the pot soil. On 28 October the pots were brought into the open and left there until the end of the experiment in March 1982.

Assessment. From Februari 1982 onwards the plants were regularly checked for symptom development. The final assessment was carried out at the end of March 1982. The response of a plant was considered to be positive if one or more shoots showed typical disease symptoms (see below).

Results.

Viability of the inoculum. When the inoculum was inoculated on malt agar the conidia germinated readily and produced abundant colonies of G. abietina. It was therefore concluded that the inoculum was viable at the time of inoculation.

Symptom development. In February 1982 the first symptoms became visible. The needles of many inoculated plants turned greyish and could easily be removed from the plants. In March the bases of such needles turned brown and many needles dropped. During the course of the summer numerous pycnidia of Brunchorstia arose from the needles and the killed bark tissue. When such symptoms were observed they were attributed to G. abietina. Table 1 shows the number of plants showing Brunchorstia symptoms, in relation to duration of leaf wetness.

Table 1: Attack of P. nigra by G. abietina in relation to leaf-wetness period, assessed 9 months after inoculation.

leaf wetness	inoculated plants		control plants	
period/hours	total	diseased	total	diseased
0.5	6	4	3	0
6	6	6	3	0
12	6	6	3	0
18	6	6	3	0
24	6	6	3	0
48	6	6	3	0
60	5	5	3	0
72	4	4	3	0

Four of the six plants from the series "0.5 h wet" showed only a few diseased shoots, but the plants in all other series were seriously diseased: in those series at least 50% of the shoots had been killed and many plants had even been totally killed.

EXPERIMENT 2: THE EFFECT OF PROVENANCE AND MODE OF OVERWINTERING

Material and methods.

Plant material. In Spring 1983 four provenances of P. nigra and one provenance of P.sylvestris were planted as one-year-old stock in 2-litre pots. The pots remained outdoors during a whole year to allow them to recover from the plant shock. The provenances used were: P. sylvestris 'Grubbenvorst', P. nigra ssp. maritima 'Wouw', 'Koekelaere',' Texel a' and 'Texel 16e'. (In this paper "provenance" is defined as the locality in the Netherlands from which the seed was collected). The numbers of inoculated and control plants are given in Table 2. At the time of inoculation the plants were 2 years old: the needles had just emerged from the needle sheaths and the green unlignified bark tissue of the new shoots was visible.

Inoculum. In March 1984 an isolate was made from a pycnidium present on a shoot of Corsican pine killed the year before by G. abietina. Inoculum was prepared as described above. The inoculum density was 10/5 spores per millilitre.

Inoculation method. On 23 May 1984 inoculation was carried out as follows. A total volume of 5 litres spore suspension was sprayed over 234 plants, so that each plant received an average amount of 21 ml inoculum (= 21.10/5 spores per plant). Control plants were sprayed with demineralized water. After inoculation the plants were kept moist by being covered with plastic for 16 hours. The cover was then removed and the plants allowed to dry. The plants remained outdoors until 4 January 1985.

Effect of overwintering. In the night of 3 to 4 January 1985 the temperature dropped from minus 5 to minus 18 degrees C and about 20 cm snow fell. Such conditions are very exceptional in Holland and, as we suspected low temperatures as being a factor that predisposes Pinus to Brunchorstia (see introduction) this seemed to provide an opportunity to study the effect of winter temperatures on the disease development. Therefore we decided to dig up a number of plants from each provenance from the snow and bring them into a greenhouse where the temperature varied between +5 and +10 degrees C. They remained there until the end of the experiment. In spring, the temperatures in the unheated greenhouse increased considerably when the weather was sunny. Flushing started in March, while flushing of the plants outdoors did not start until the end of April.
The other plants remained outdoors. Temperatures outdoors were not recorded, but the winter 1984-1985 was severe by Dutch standards. The numbers of plants that were placed in the greenhouse and left outdoors are given in Table 2.

Table 2: Number of plants in the various treatments

	overwintered outdoors		overwintered indoors	
provenance	inoculated	control	inoculated	control
Grubbenvorst	36	16	10	4
Wouw	41	16	10	4
Koekelaere	25	11	10	4
Texel a	40	21	11	4
Texel 16e	41	20	10	4

Assessment. Assessments were carried out on 21 January, 5 February, 13 February and the final assessments from 19 to 29 April 1985. All top shoots that could have been infected during inoculation, which were those that developed in 1984, were individually examined.
A shoot was considered to be killed by G. abietina if all its needles had a brown base and the whole bark tissue of the shoot was necrotic. When only part of the needles and the bark showed necrosis, but other parts were still green, shoots were classified as "diseased". A shoot was classified as "attacked" if it was dead or diseased.

Statistical analysis. The effect of provenance and overwintering on the degree of shoot attack was examined using logistic regression analysis (Nelder & Wedderburn, 1972). In this analysis it is assumed that the number of attacked shoots has a binomial distribution, depending on provenance and mode of overwintering. It was also assumed that each plant of a provenance has an individual resistance that may differ from the general resistance of the provenance as a whole (so-called extra binomial variation: McCullogh & Nelder, 1983).
The analysis was carried out using the GENSTAT computer programme (Alvey et al., 1983). The analysis answers the question whether the effects of provenance and mode of overwintering are significant or not and whether there is an interaction between these effects. It also gives estimates of these effects, with matching standard errors. To examine whether the conclusions with respect to the whole plant are also applicable to the leader shoot alone, the same analysis was also carried out for the leader shoots.

Results.

Viability of inoculum. When the inoculum was introduced on malt agar and incubated at 24 degrees Celcius it germinated readily, and produced normal colonies.

Symptom development. No external symptoms could be found on any of the plants inside or outside the greenhouse on 21 January 1985. On 5 February no symptoms could be found in the plants outdoors, but on a number of plants that had been placed in the greenhouse the base of the needles had turned brown and the bark under such needles appeared to be necrotic. The same symptoms were found outdoors on 13 February. The disease did not progress further after the end of March 1985. The results of the assessments carried out from 19 to 29 April are given in Table 3.
In the control plants no symptoms developed, either outdoors or in the greenhouse.

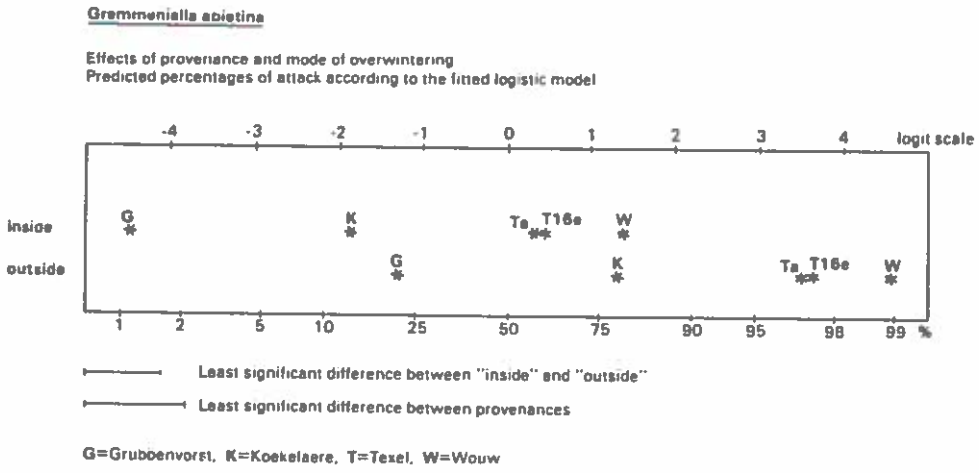
Statistical analysis. Logistic regression analysis applied to the data gave the following results. The outdoor overwintering yielded a significantly ($P<0.001$) higher percentage of shoot attack then the indoor overwintering. The effect of provenance on shoot attack was significant ($P<0.001$). The interaction between provenance and mode of overwintering was not significant. Thus, (on logit scale) the differences between outdoor and indoor overwintering may be regarded as almost equal for all provenances (or, equivalently: the differences between provenances do not depend on the mode of overwintering).
In Figure 1 the predicted percentages of shoot attack are given according to the logistic model fitted to our data. The least significant differences are also given, using yardsticks for provenances and mode of overwintering.

Table 3: Percentage of attacked shoots of pine, 10 months after inoculation, in relation to provenance and mode of overwintering.

provenance	overwintering	dead	attacked[1)]	n
Grubbenvorst	outdoor	8.9	21.7	36
	greenhouse	0.0	0.0	10
Wouw	outdoors	92.6	96.0	41
	greenhouse	79.7	91.5	10
Koekelaere	outdoors	42.7	78.9	25
	greenhouse	6.3	12.5	10
Texel a	outdoors	89.8	97.5	40
	greenhouse	44.0	57.1	11
Texel 16e	outdoors	87.0	98.7	41
	greenhouse	34.7	54.7	10

1) attacked = dead plus diseased.

Figure 1: Mean percentage of attack in relation to provenance and mode of overwintering



Thus, from Figure 1 it may be concluded that 3 groups of provenances may be distinguished. Firstly, P. sylvestris 'Grubbenvorst', which shows the lowest degree of attack; secondly, P. nigra 'Koekelaere' showing an intermediate degree of attack and, finally the group consisting of P. nigra 'Wouw', 'Texel a' and 'Texel 16e' showing the highest degree of attack (the latter three provenances did not differ significantly from each other). The above results are based on the data of side shoots. For the leader shoots logistic regression analysis yielded the same conclusions.

GENERAL DISCUSSION AND CONCLUSIONS

In the first experiment, duration of leaf wetness and inoculum were the only variables. After the treatment the plants were kept dry during the whole vegetative period. Nevertheless, they became seriously diseased by Brunchorstia in the following spring. Even in the series that had been kept wet for only 30 minutes, typical symptoms developed (Table 1). So, it may be concluded that under the experimental conditions a long period of leaf wetness during infection or during the vegetative period following infection is not a prerequisite for disease expression.

However, if it is not moisture, what other factors determine disease expression? Experiment 2 gives some indications.

Variables were inoculum, provenance and mode of overwintering. The effect of inoculum was obvious, as in Experiment 1: no disease symptoms without inoculation. The effect of provenance was equally obvious: the degree of attack depended greatly on species and provenance. Finally, the mode of overwintering was apparent: plants in the greenhouse were less diseased than those overwintered outdoors, even though the plants were not moved indoors before January and had been exposed one night to severe frost. It is true, that temperature was not the only factor that differed between "indoors" and "outdoors". At least two other factors differed as well, viz. radiation and relative humidity. However, we suspect frost as the most important factor predisposing pine to an attack by Brunchorstia, because of the overwhelming circumstantial evidence reported in the literature.

Furthermore, relative humidity was the same for all plants during the whole vegetative period until the plants were separated and it is unlikely that relative humidity still plays an important role after the pathogen has penetrated into the host.

Radiation has been reported to correlate negatively with disease expression (Read, 1966). In our experiment, radiation was lower in the greenhouse (which was not illuminated) so, if there were an effect, it would have been positive.

Our results support those of Blenis, Patton and Spear (1984), who kept inoculated plants at various temperatures and concluded that freezing probably favours colonization of P. resinosa by Brunchorstia.

The determining role of winter temperatures and inoculum will probably explain most of the serious epidemics in the Netherlands, but this has yet to be confirmed.

We found, that under the experimental conditions P. sylvestris 'Grubbenvorst' was attacked by Gremmeniella less severely than the P. nigra provenances and that P. nigra 'Koekelaere' was attacked less severely than the other 3 P. nigra provenances. The degree of damage on the plants overwintered outdoors and indoors followed the same sequence. Apparently, environmental conditions affected only the degree of attack, but not the relative susceptibility of the provenances, suggesting that these differences are defined genetically. Prospects for disease-resistance breeding therefore seem to be promising. However, experiments to test disease resistance cannot be performed in the open in The Netherlands, because nobody can predict whether the temperatures in the winter following infection will be sufficiently low to allow Brunchorstia to colonize the host. Such experiments should be carried out in climatic chambers, or, alternatively, at a more Northerly latitude. Until more resistant provenances of P. nigra are available, the establishment of P. nigra in the Netherlands must be discouraged, except in the coastal region, where winters

are mild. The provenance 'Koekelaere' is promising, but more experiments are needed to confirm its usefulness for large-scale plantations.

Acknowledgement

The assistance given by ir. P. van der Zweep in experiment 1 is acknowledged.

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ZUSAMMENFASSUNG

Der Einfluß von der Dauer der Blattbenetzung, der Kiefernherkunft und der postinfektionellen Überwinterungsart auf die Entwicklung von Gremmeniella abietina.

Die Auswirkung der Dauer der Blattbenetzung nach der Infektion, sowie der Herkunft und der Überwinterungsart der Wirtspflanzen wurden untersucht, indem junge getopfte Kiefernpflanzen mit Konidien von Gremmeniella abietina aus Reinkulturen künstlich inokuliert wurden. Eine Blattbenetzung von 6,12, 18, 24, 48, 60 und 72 Stunden ergab keine signifikanten Unterschiede. In allen Fällen erkrankten die Bäumchen sehr stark. Pflanzen, die für 30 Minuten feucht gehalten wurden, wurden nachfolgend nur leicht befallen. In einem zweiten Versuch wurde die Anfälligkeit von einer Pinus sylvestris - und 4 P. nigra-Herkünften geprüft. Nach der Infektion überwinterten die Pflanzen in einem Gewächshaus oder im Freien. Dabei waren signifikante Befallsunterschiede zwischen P. sylvestris und P. nigra bzw. zwischen einer P. nigra-Herkunft und den anderen 3 Herkünften festzustellen. Die Unterschiede zwischen den Herkünften hingen nicht von der Art der Überwinterung ab. Dennoch waren in allen Fällen die im Freien überwinterten Pflanzen signifikant stärker befallen.

VOLE DAMAGE AS A PREDISPOSING FACTOR TO INFECTION BY *Gremmeniella abietina*

Margareta Karlman [1]

ABSTRACT

During a ten-year period 19 provenances of *Pinus contorta*, introduced into Sweden from western North America, and 4 provenances of the indigenous *Pinus sylvestris*, were investigated each spring and autumn with respect to different kinds of damage - primarily parasitic fungi. A high correlation was found between vole damage and infection by *Gremmeniella abietina* even among northern provenances of *Pinus contorta*. The rapid spread of *Gremmeniella* within two other provenance trials in the same series was associated with unfavourable weather conditions in northern Sweden during the summers and autumns of 1984-1986.

INTRODUCTION

Sweden has only two indigenous conifer species of economic importance, the Scots pine (*Pinus sylvestris* L.) and the Norway spruce (*Picea abies* (L.) Karst.). During the past decades an introduced species, the lodgepole pine (*Pinus contorta* Dougl. ex Loudon) has become increasingly important to Swedish forestry. Eighty million seedlings of *Pinus contorta* are planted in northern Sweden every year compared to 2 million in Norway and 1.5 million in Finland. No other introduction of an exotic has been so carefully followed as that of *Pinus contorta* in Sweden. Pathogens and other threats to lodgepole pine have been studied by the author since 1976 (Karlman 1980 a, 1980 b, 1981, 1982, 1984). The introduction of this North American conifer species has been preponderatingly successful, especially in the southern and central parts of northern Sweden.

So far the most severe threat to *Pinus contorta* in northern Sweden has been vole damage in connection with peak years in the vole populations (Hansson & Lavsund 1982, Karlman 1982, 1984, Hansson 1985). Experiences are similar in Norway (Roll-Hansen & Roll-Hansen 1977) and in Finland (Annila et al. 1983, Routsi 1983). Voles prefer lodgepole pine to Scots pine providing the vole population is moderate. At times of high vole populations even Scots pine suffers damage. Studies during the past few years indicate that the well documented capacity of *Pinus contorta* for recovery even from severe vole attacks, has been reduced considerably by secondary infection by *Gremmeniella abietina* (Lagerb.) Morelet two to three years after vole attack (Karlman 1984). As a consequence of three extremely wet summers (1984-86) the spread of *Gremmeniella* has increased rapidly in provenance trials and progeny tests in the northernmost parts of Sweden, where extensive planting of lodgepole pine has been recommended.

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MATERIALS AND METHODS

In 1974 a series of provenance trials with lodgepole pine, Scots pine and Norway spruce were laid out in northern Sweden by the Institute for Forest Improvement. The entire series consists of seven sites, the results for three of which - Hornmyr, Nattavaara and Tärendö - are presented in this paper (Fig. 1). Early results covering survival rates, height increment and tree damage have been published by Rosvall & Strömberg (1980, 1984) and results from the Moskosel and Sävar trials in the same series have been thoroughly discussed by the author (Karlman 1982, 1984, 1986).

Figure 1. Location of provenances in western North America and trial sites in Sweden planted in 1974 (modified from Rosvall et al. 1984).

Table 1. Description of trial sites

Site	Latitude	Longitude	Alt. m.	Exp.	Number of seedlings	P.c.	provenances P.s	
Hornmyr	64°25'N	18°23'E	450	NW	6 144	19		4
Nattavaara	66°47'N	21°17'E	425	SW	4 608	14		3
Tärendö	67°08'N	23°02'E	270	W	4 608	14		3

The Hornmyr trial lies on a gentle NW-facing slope on a good soil and is bounded in the north by an older stand of Norway spruce (Picea abies L.) and on the other sides by a grassy Norway spruce plantation of trees now about 70 cm high. The local vegetation is dominated by Dechampsia flexuosa (L.) Trin. In the centre of the trial there is a frost pocket. The Hornmyr trial was planted with 1-year-old seedlings in four replicate blocks with 64 seedlings per plot. Nineteen provenances of lodgepole pine were compared with 4 provenances of Scots pine (Table 2). The Hornmyr trial has been regularly inspected for the occurrence of parasitic fungi and other damage, from 1976 onwards, after 1978 mainly blocks I and III. Vole damage was recorded in 1977/78, 1980/81 and 1981/82 within all four blocks.

In the Tärendö and Nattavaara trials 14 provenances of lodgepole pine were compared with 3 provenances of Scots pine (Table 2). These trials have been regularly inspected since 1983.

Table 2. Provenances used in the trials at Hornmyr (H), Nattavaara (N) and Tärendö (T).

ORIGIN								
Identification no		Name	Lat. N	Long. W	Alt m	H	N	T
						Number of seedl		
Is	727	Rusty Creek, Yukon	63°30'	136°25'	800	256	256	256
Is	728	West Summit Lake, Yukon	63°03'	136°25'	740	256	256	256
Is	729	Tantalus Butte, Yukon	62°08'	136°15'	680	256	256	256
Is	597	Whitehorse, Yukon	60°52'	135°15'	750	192	256	-
Is	599	Mile 864, A. Hw., Yukon	60°20'	134°00'	800	64	-	256
Is	730	Watson Lake, Yukon	60°03'	128°43'	725	256	256	256
Is	731	Skagway, Alaska, Nordl. kust.	59°27'	135°18'	0-60	256	256	256
Is	592	Toad River, B.C.	58°52'	125°21'	760	256	256	256
Is	591	Ft. Nelson, B.C.	58°50'	122°42'	460	256	256	256
Is	600	Juneau, Alaska, Nordl. kust.	58°25'	134°38'	30	256	256	256
Is	732	Trutch Mountain, B.C.	57°40'	122°55'	1150	256	256	256
Is	733	Blueberry River, B.C.	56°35'	121°27'	860	256	256	256
Is	734	Hudson Hope, B.C.	56°04'	121°53'	530	256	-	-
Is	735	2034 Kispiox, B.C.	55°38'	127°54'	610	256	-	-
Is	602	Kitwanga, B.C.	55°11'	127°49'	300	256	240	256
Is	736	Windy Point, B.C.	55°06'	123°28'	770	256	256	256
Is	705	Chuculty Lake, B.C.	53°50'	123°30'	730	256	-	-
Is	583	Quesnel, B.C.	53°04'	122°26'	670	256	256	256
Is	737	Sunchild Reserve, Alta	52°38'	115°23'	1150	256	-	-
Is	581	Fly Hills, Salm. Arm., B.C.	50°39'	119°24'	1430	256	-	-
P. sylvestris								
AC	562	from seed orchard	65-66°	E	3-500	256	-	-
BD	428	from natural stands	66°		3-400	256	-	-
AC	531	"	65°		3-400	256	-	-
AC	532	"	64°		3-400	256	-	-
BD	427	from seed orchards	65-67°		50-450	-	256	256
BD	432	from natural stands	68°04'		330	-	256	256
BD	429	composite seed samples	67-67°45'		300-350	-	256	256

Damage caused by voles and parasitic fungi was recorded and classified on a 4-degree scale, in which degree 1 indicates a slightly damaged or infected plant and grade 4 a dying plant, according to Karlman et al. (1982). The method is described in detail in Karlman 1986.

RESULTS

Up to 1983 high survival rates were recorded for all the northern provenances of lodgepole pine. Within the southern provenances a high mortality rate was recorded already during the initial year after planting, due to poor winter acclimatization of the seedlings in the nursery. During the first few years after planting, the frequency of damage was in general very low. Damage was recorded primarily to the southern provenances. All three trials suffered, however, from severe vole attacks during the late 70's and the early 80's.

The Hornmyr trial, which has been followed since 1976, was seriously damaged during the winter of 1977/78, which was a peak year for voles in the province of Västerbotten in northern Sweden (Fig. 2, App.). When the frequency of damage within all four blocks in the Hornmyr trial is compared, a clear tendency is seen for the most northern provenances of lodgepole pine to have a lower frequency of vole damage than the more southern ones. When the results for the individual plots in each block are considered separately, it is clear that even the most northern provenances did suffer a relatively high degree of damage.

The vole population was considerably smaller in the next peak year, during the winter of 1980/81. The plots attacked this time were those situated adjacent to a grassy clear-felled area with the ground partly littered by twigs and branches, in other words a favourable biotype for voles. For those plots in block II and IV, situated in the vicinity of an older stand of Norway spruce, low frequencies of vole damage were recorded. When the results for individual plots are considered separately (Fig. 3. App.), then in that year too, also northern provenances of lodgepole pine suffered a high degree of damage within blocks I and III:

Rusty Creek	(63°30')	59% (III:20)
West Summit Lake	(63°03')	51% (III:17)
Watson Lake	(60°03')	93% (III:2)
Whitehorse	(60°52')	64% (I:8)

The type of vegetation and the situation of the plots in the trial was of decisive importance regarding the intensity of vole damage.

Those seedlings which suffered severe vole damage during the winter of 1977/78 and had become bushy in consequence appeared in the winter of 1980/-81 to be less attractive to voles, which seemed to prefer to attack still intact plants. The low frequency of vole damage recorded in 1980/81 for the Tantalus Butte provenance (62°08') is worthy of mention. This was also the case in the peak vole period of 1977/78.

In 1980/81 the Scots pine provenances were wholly unaffected by voles in all four blocks. The data from 1981 indicate that, providing the vole population is only moderate, voles prefer lodgepole pine to Scots pine. At times of high vole populations even Scots pine suffers damage.

A more serious consequence of the vole attacks in 1977/78 than bushy trees, was the fact that the majority of the trees damaged by voles were infected by Scleroderris canker (Gremmeniella abietina) two to three years after the vole attack (Fig. 4). The most serious fungal attacks in 1982 were recorded for the local provenances of Scots pine (Pinus sylvestris) and for the lodgepole pine provenance Whitehorse (60°52'). Infection by Gremmeniella only occurred in connection with vole damage caused in 1977/78.

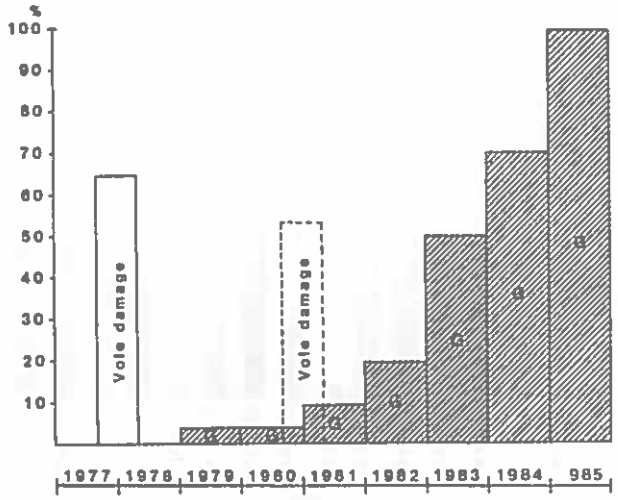


Figure 4. Infection by Gremmeniella abietina (G) following vole damage during the winters of 1977/78 and 1980/81 within the Whitehorse (60°52') provenance plots in blocks I and III at Hornmyr. Initially 128 seedlings.

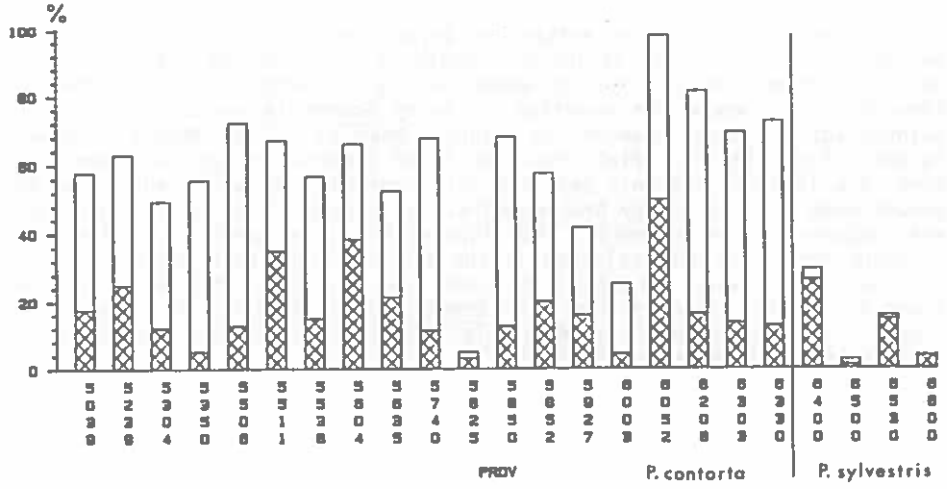


Figure 5. The frequency of Gremmeniella abietina at Hornmyr in 1983 and 1985 Blocks I and III. Initially 3 072 seedlings.

During 1984 even trees that had not been attacked by voles were infected by Gremmeniella. Up to 1984 the northern lodgepole pine provenance Tantalus Butte (62°08') was the most vole repellent provenance and thus the least damaged provenance in the Hornmyr trial. During 1984 also this provenance was infected by Gremmeniella. In Figure 5 the spread of Gremmeniella from 1983 to 1985 is demonstrated and in Figure 6 the severity of infection. The most northern provenances were those with the highest frequency of infection.

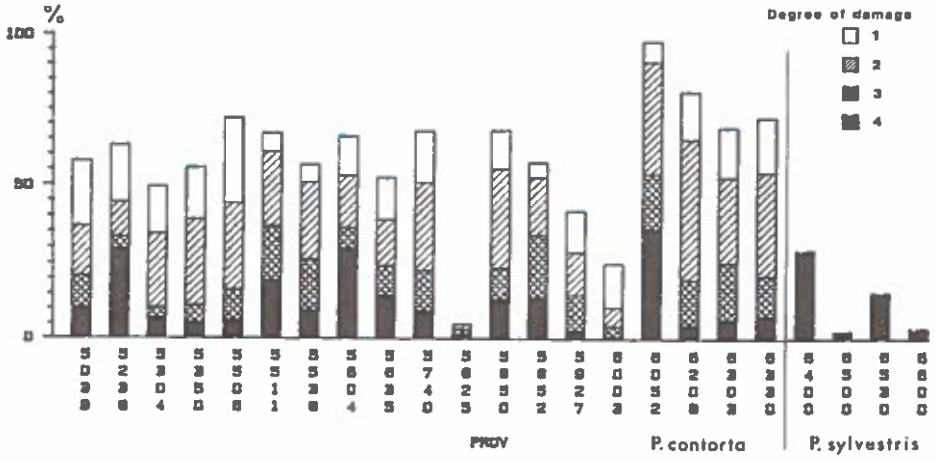


Figure 6. The frequency of Gremmeniella infection at Hornmyr in September 1985. Blocks I and III. Initially 3 072 seedlings.

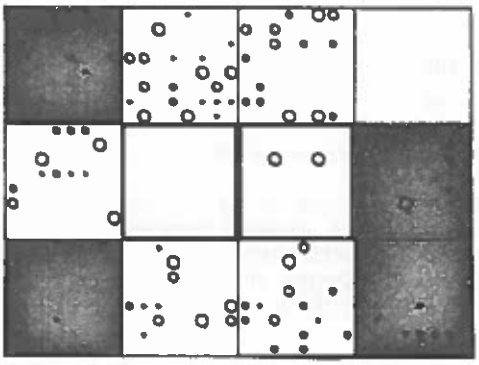
In the Tärendö and the Nattavaara trials the northern provenances were not so seriously infected as the more southern ones. The rapid spread of the infection from 1983 to 1985 is shown in Fig. 7 (Figs. 8-9 App.) for the Tärendö trial, where the position of every Gremmeniella-infected tree is pointed out. It must, however, be observed that so far the degree of damage is only slight in this trial, just the lower branches of the 3-4 metre high trees are infected and only severely vole damaged trees with reduced height growth have been killed by Gremmeniella. The results from the progeny tests are, however, more alarming. The interesting provenances i.e. the most northern ones, are dark-coloured in the figure. Scots pine plots are lined in blue. There were originally 64 seedlings per plot. Missing trees and trees killed by other reasons than Gremmeniella-infection are recorded in Figure 7 c. The spread of Gremmeniella within the trial will be statistically evaluated (Karlman unpublished).

Figure 7.

a) Degree of infection by Gremmeniella abietina at Tärendö in August, 1983. Ten provenances of Pinus contorta are compared with two provenances of P. sylvestris. The most northern lodgepole pine provenances are dark coloured. Every square represents one plot with originally 64 seedlings. Detail from Fig. 8.

b) The spread of Gremmeniella abietina in 1985. Detail from Fig. 9.

c) Missing trees and trees killed by other reasons than Gremmeniella- infection.



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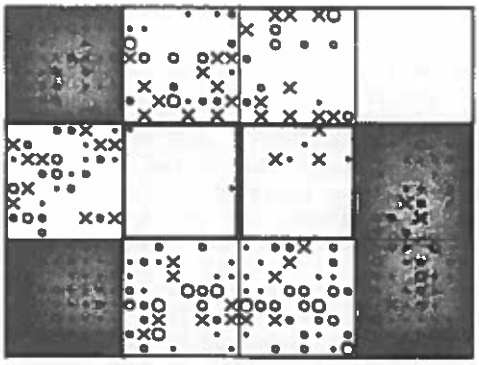

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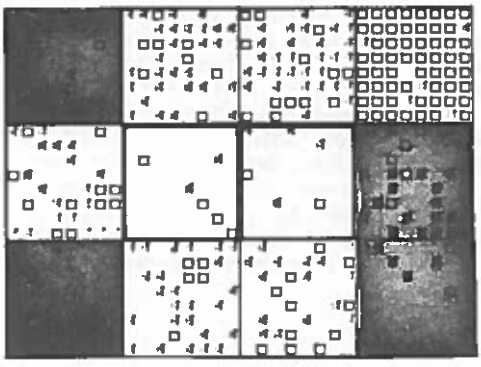

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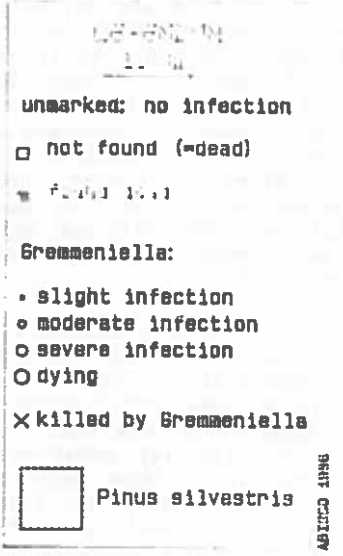

An obvious correlation has been documented between Gremmeniella-infection and vole damage in the Tärendö trial:

	Vole 83+	Vole 83-	
Grem 84+	217	1 569	$X^2 = 38.64^{***}$
Grem 84-	44	894	$p < 0.001$

The correlation is even more evident in the Hornmyr trial.

The Nattavaara trial was as severely damaged by voles during the 70's as that in Hornmyr, but no obvious correlation between Gremmeniella-infection and vole damage could be found in the Nattavaara trial, owing to the fact that the investigations of that trial started in 1983, and it was not always possible to record vole damage from the 70's.

DISCUSSION

The observations made during the vole peaks in 1977/78 and 1980/81 in the province of Västerbotten (1976/77 and 1979/80 in central Norrland) have shown quite clearly that voles prefer Pinus contorta to Pinus sylvestris. This situation was neither expected, nor foreseeable, when Pinus contorta was first introduced on a large scale in Sweden during the 1970's. Even though lodgepole pine is capable of surviving repeated attacks by voles, their growth is hindered, and wood quality suffers, and the injuries to the bark often provide ingress for secondary pathogens. Such damage by small mammals does not represent any serious threat to survival of Pinus contorta trees growing in western North America. Damage caused by pocket gophers and porcupines has, however, been reported (Cook & Hamilton 1957, Tackle 1959, Barnes 1973) as well as damage by squirrels and snowshoe hares (Sullivan & Sullivan 1982) although the number of published reports of such damage is low (Lindsey 1975). The Swedish results, on the other hand, are wholly unequivocal.

A plant with reduced vitality is often attacked by secondary pathogens. A clear correlation has been documented between severely weather-damaged lodgepole pine of southern provenance and infection by Gremmeniella abietina (Karlman 1984, 1986). During the last few years even northern provenances of lodgepole pine have thus been infected by Gremmeniella. In the Hornmyr trial the infection was spread from vole-damaged trees, which were infected by the fungus two to three years after the vole attack. Owing to a high inoculum level among susceptible trees, the infection soon spread all over the trial and also to trees, which had not been attacked by voles, for example the Tantalus Butte provenance ($62^{o}08'$). Both Roll-Hansen (1964) and Kurkela (1981) have found as well that Gremmeniella sometimes invades the tissue through wounds.

A provenance trial is presumably a very favourable environment for the spread of pathogens. Southern more susceptible provenances are mixed with more northern provenances. The results from progeny tests in northern Sweden with lodgepole pine of exclusively northern origin (62^{0} - $63^{0}30'$) indicate, however, a rapid spread of infection from 1983 to 1986 and only minor differences in susceptibility among progenies (Karlman unpublished). Earlier results (Karlman 1984) show, that all provenance trials attacked by voles are not necessarily infected by Gremmeniella and vole damage is not the only predisposing factor to infection.

As stated above, severe weather damage is another predisposing factor to infection by Gremmeniella on lodgepole pine in northern Sweden. Weather conditions were very extreme during 1984 in northern Sweden with great variations in temperature during May-June (below zero temperatures mixed with high summer temperatures). In addition July-September 1984 and 1985 were months with very high precipitation and thus favourable for the spread of infection. The summer and autumn of 1986 were extremely humid and the spread of Gremmeniella within both provenance trials and progeny tests increased disquietingly. Several researchers have observed a correlation between Gremmeniella and unfavourable weather conditions (cf. Karlman 1986 and the literature cited therein).

During 1986 infection by Gremmeniella has also been observed in conventional plantings of lodgepole pine in the province of Västerbotten in northern Sweden (own observations). Damage to 25-30-year-old stands of Scots pine was recorded in southern and central Sweden as well during 1985 (Barklund pers. comm.). Also in Finland damage to young plantations of Scots pine has been reported and correlated to unfavourable climatic factors (Kurkela 1983).

In the extreme north of Sweden, in areas which are difficult to regenerate, because of a very harsh climate, there is a lack of enough hardy seed of the indigenous Scots pine. Here the lodgepole pine is a very interesting alternative and has been recommended for years by the National Board of Forestry. So far most young lodgepole pine plantations in this area seem to be healthy and productive. The results of provenance and progeny tests are, however, alarming. These indicate that extensive use of lodgepole pine in the very north of Sweden must be questioned until unequivocal results of more long-term investigations are presented.

ACKNOWLEDGEMENT

The author thanks Miss Ann-Kathrin Persson for typing the manuscript and Mr. Evert Jeansson and Mr. Adriaan de Jong for statistical advice and data processing. The plotted "diagrams" (Figs. 7-9) have been made in collaboration with de Jong.

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ZUSAMMENFASSUNG

Wühlmaus-Schäden als prädisponierender Faktor für die Infektion durch Gremmeniella abietina

Während eines zehnjährigen Zeitraums wurden 19 aus dem westlichen Nordamerika nach Schweden eingeführte Herkünfte von Pinus contorta sowie 4 Herkünfte der heimischen Pinus sylvestris jedes Frühjahr und jeden Herbst auf unterschiedliche Schäden hin untersucht, in erster Linie auf parasitische Pilze. Zwischen Wühlmaus-Schäden und Infektion durch Gremmeniella abietina konnte eine hohe Korrelation nachgewiesen werden, auch unter nördlichen Pinus contorta-Herkünften. Die rasche Ausbreitung von Gremmeniella in zwei anderen Herkunftsversuchen derselben Serie stand in Verbindung mit ungünstigen Wetterbedingungen in Nordschweden während der Sommer- und Herbstmonate 1984 bis 1986.

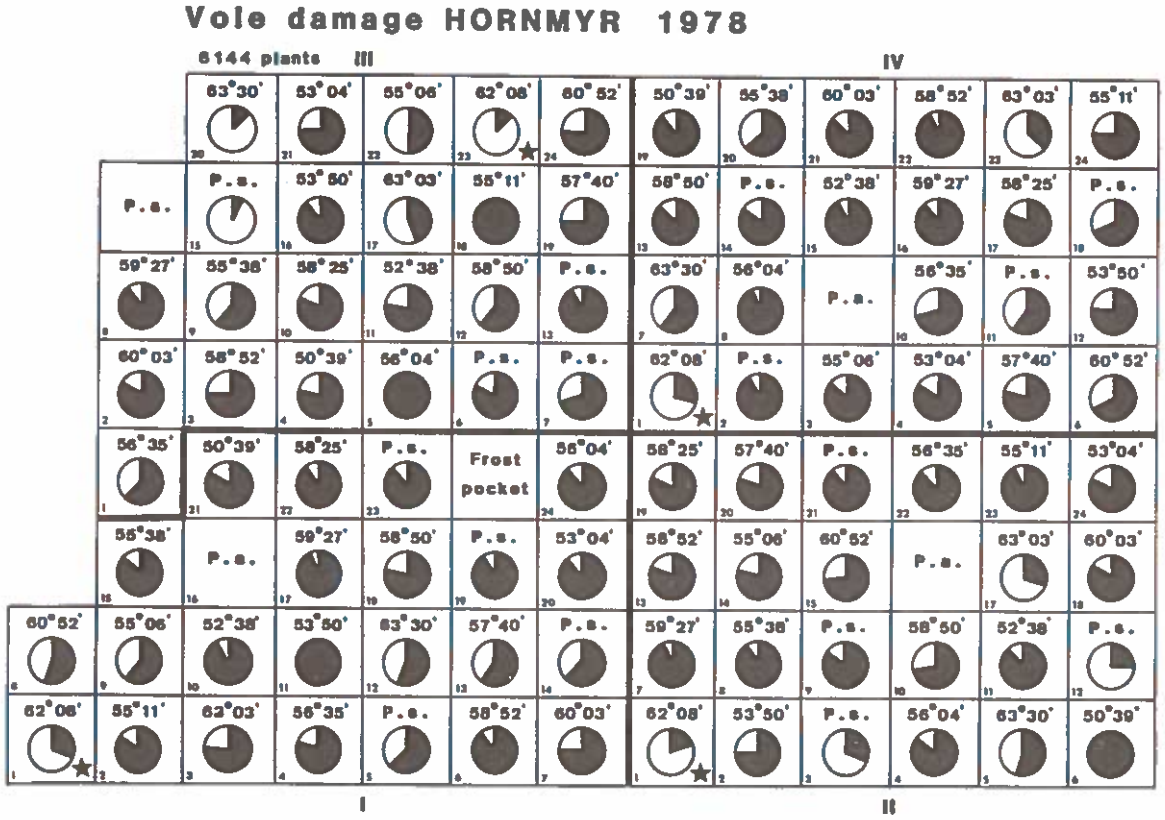

Figure 2. The frequency of vole damage (%) at Hornmyr in 1978. Every square represents one plot with initially 64 seedlings. The Tantalus Butte (62°08') provenance is marked with a star. P.s. = Pinus sylvestris.

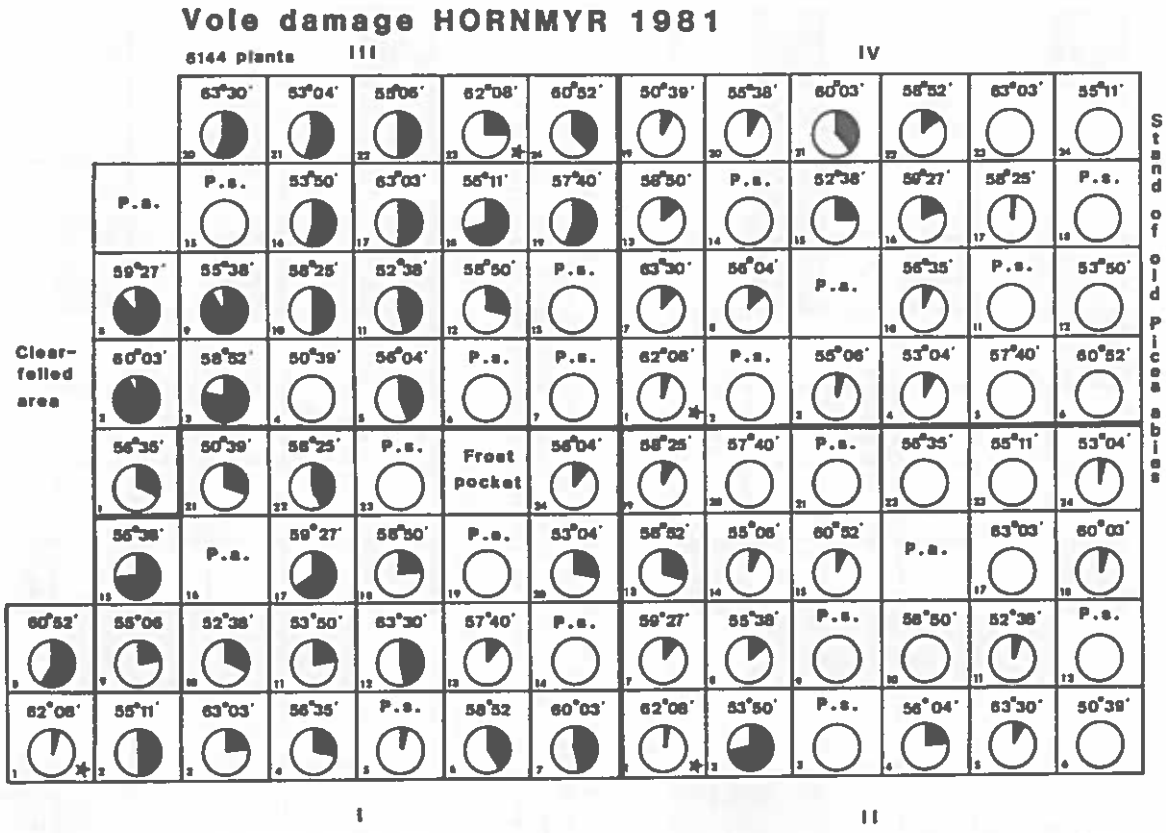

Figure 3. The frequency of vole damage at Hornmyr in 1981. The Tantalus Butte ($62^{o}08'$) provenance is marked with a star. P.s. = Pinus sylvestris.

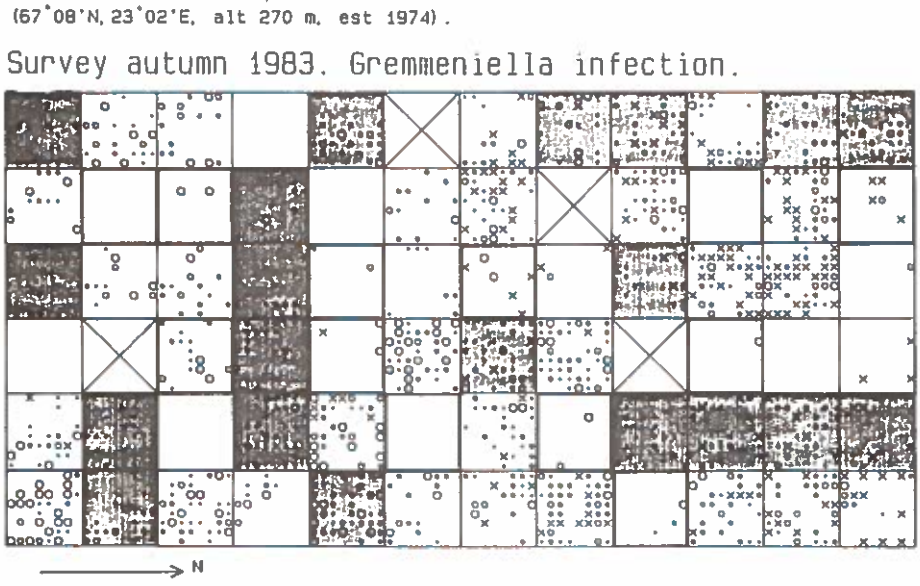

Figure 8. Trees infected by Gremmeniella abietina in a provenance trial at Tärendö in August 1983. 14 provenances of Pinus contorta are compared with 3 provenances of P. sylvestris. Every square represents one plot with initially 64 seedlings. The most northern provenances are dark-coloured.

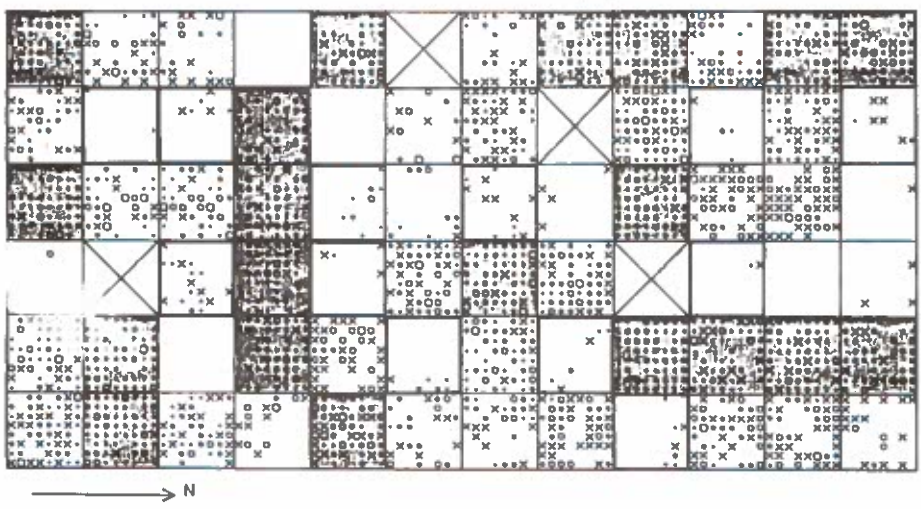

Figure 9. Trees infected by Gremmeniella abietina at Tärendö in August 1985.

V. Resistance and control measures

SOMACLONAL VARIATION USED TO DEVELOP CONIFER RESISTANCE TO GREMMENIELLA ABIETINA

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Introduction

The ultimate goal of forest pathologists and forest geneticists is to develop trees that are resistant to tree diseases. Genetic resistance in most cases is the only economically feasible method of disease control under most forest management situations. Applying protective fungicides is usually too expensive except in certain areas such as nurseries where the high value of the product warrants the cost of repeated fungicide applications. In most other situations, however, the forest manager must depend on genetic resistance to protect the trees from pathogens. The objective of this study is to develop disease resistance in forest trees using the somaclonal variation technique as an alternative to conventional tree selection and breeding.

During the last 50 years a large amount of time and money has been directed into programs for producing trees resistant to white pine blister rust, Dutch elm disease, and chestnut blight. Although these programs have achieved some success, in most cases the resulting trees have not exhibited the desirable resistant characteristics even after decades of research.

The long time required for each tree generation makes it difficult to produce multiple back crosses within the life span of a tree breeder Few managers of research institutions are willing to allow their scientists to spend 30 or more years working on a breeding program that may or may not produce a useful final product.

Screening trees from different seed sources

As an alternative to classical breeding programs some of us have spent many years screening large numbers of seed sources seeking that particular one that will yield trees with the desired disease resistance. The North Central Forest Experiment Station has been screening seed sources of many native conifers for the last 10 years looking for resistance to Scleroderris canker within the existing gene pool. This screening program has had some success with jack pine, Pinus banksiana; we have found a few seed sources that appear to be resistant to the North American strain of Gremmeniella abietina. However, the

program has not been successful with the other Pinus species. We see some variation in resistance within Scots pine (P. sylvestris), but red pine (P. resinosa), lodgepole pine (P. contorta), and ponderosa pine (P. ponderosa) have all shown extreme susceptibility to G. abietina under field inoculation conditions. With these species some type of genetic change probably will be needed to give us the desired resistance to G. abietina.

Somaclonal variation

One system that may produce the desired genetic change without the long time span and resulting high costs is somaclonal variation. It has the potential to incorporate one or more desirable traits into forest trees without breeding cycles of 20-30 years. The term somaclonal variation was proposed to describe variation exhibited by plantlets obtained from aseptic plant cultures (Larkin and Scowcroft 1981). With somaclonal variation it is now possible to take many old plant lines and create dozens of new lines in a a few months.

Many plants produced in asceptic cultures are not true to type. Rather than being clones of the parent plant, the regenerated plants may show variation in one or more traits. This variation in some cases may exceed that of plants produced by standard breeding practices (Larkin and Scowcroft 1981, Shepard et al. 1980).

Somaclonal variation was not recognized as a true genetic process until recently but this variation has been utilized in agriculture for many years. An example of natural genetic variation is the "Russet Burbank" potato, which is a sport or natural mutant (somaclone) from the Burbank potato. Examples of beneficial somaclones are becoming common. One of the earliest examples was produced in Australia. Larkin and Scowcroft (1981) produced lines of sugar cane that were resistant to eyespot disease caused by the fungus Heliminthosporium sacchari. This disease limited the usefulness of the new high-yielding cane types because of their extreme susceptibility to the Heliminthosporium fungus. Larkin and Scowcroft introduced a toxin produced by the eyespot fungus into the tissue culture medium. Plantlets grown in this toxin supplement medium were later screened for disease resistance. The amount of variation present in these plants was amazing. Most plants were more resistant than the parent line, and 70 percent maintained stable resistance through five vegetative generations. When six of these somaclones were given a second 6-month tissue culture cycle, 60 percent had similar or enhanced toxin tolerance relative to the primary somaclone (Scowcroft et al. 1983). It appears that characters can be "stacked", i.e., we can screen for modification of a second characteristic following a second culture cycle and expect that some will retain the first characteristic (Shepard et al. 1980).

The exact cause of somaclonal variation is not known. Larkin and Scowcroft have suggested that it is due to gene amplification, an increase in the number of specific genes, so that their combined product

has an enhanced effect. Other possible causes are gene or whole chromosome deletion involving the loss of a gene function or transposable elements and special DNA sequences that can move from one position in a cell's chromosome to another or even to a different chromosome. This may alter the activity of certain genes.

Apparently, somaclonal variation is a frequent phenomenon within many asceptic tissue culture systems. The frequency depends on the plant species grown in culture and the duration of the culture cycle. The longer the plant remains in tissue culture, the greater the variation.

The discovery of somaclonal variation in agronomic crops offers exciting possibilities in forest tree improvement. It is now possible to produce forest trees with specific characteristics in about the same time it takes for corn and wheat breeders to develop new lines.

The Populus somaclone study

The North Central Forest Experiment Station's Biotechnology Program is part of the USDA Forest Service's national research effort for forest tree improvement. We began our research into the development of disease resistance in forest trees utilizing somaclonal variation about 2½ years ago. To the best of our knowledge this was the first project using tissue culture techniques to promote the identification of variation in a forest tree species for disease resistance. We selected the genus Populus as the best candidate for our model system because it is important worldwide as a source of fiber and energy. In the United States, however, biomass yields from hybrid poplar plantations are limited by the foliar and canker pathogen Septoria musiva. Previously, the genus Populus has been grown in tissue culture, and plantlets from culture have shown variation from the parent plant (Lester and Berbee 1977).

The Populus study is a cooperative venture between the North Central Forest Experiment Station and the Department of Horticulture of the University of Minnesota. We have two principal objectives (1) to develop tissue culture systems that will regenerate plantlets at different levels of tissue organization, and (2) to develop screening systems that will allow us to identify and recover somatic variants that show increased resistance to S. musiva.

The hybrid poplar clone NE 299 (Populus nigra cv. betulifolia x P. trichocarpa) is the main research material. This clone was selected because it has good growth potential, but its usefulness in plantations is limited because of its high susceptibility to Septoria leaf spot and canker. Other clones with some resistance to Septoria are used for comparison. S. musiva isolates have been collected from poplar plantations throughout the United States. Using a medium with various auxin-cytokinin combinations we have developed methods to rapidly produce new Populus plantlets. These somaclones are screened for

Septoria resistance using a leaf disc bioassay. This assay has already identified more than 500 Populus somaclones with increased resistance to the Septoria fungus. These somaclones have been outplanted and are currently being rated for Septoria resistance under field conditions.

Although this project is only 2½ years old, the ability to produce disease-resistant plantlets in the laboratory is a strong indication of the possible value of the somaclonal variation system for forest trees

Somaclonal variation in Larix

Our second model system involves Larix and the fungus Gremmeniella abietina, the causal organism for Scleroderris canker. Although conifers are much more difficult to manipulate in vitro, the opportunity to obtain Larix plantlets resistant to Scleroderris in a short time offers a valuable tool for the forest pathologist. This is a cooperative study between the North Central Forest Experiment Station's Forest Disease Project and the Michigan Technological University's BioSource Institute. The project is now 18 months old. The primary species being used is European larch, Larix decidua. We can now produce plantlet clones of this species of any quantity needed. In addition we have been able to produce plantlets from tissue culture callus as well as from buds of mature trees. The challenge system for Larix uses both conidia of G. abietina as well as fungal metabolites from liquid cultures of the fungus. Although this program is not as advanced as the Populus program, we have identified larch somaclones that show varied resistance to both conidia and fungal metabolites. This work will continue and we expect to have our first larch somaclones ready for field testing in the spring of 1987.

Our goal with the larch somaclonal system is to be able to grow larch callus in liquid culture along with fungal metabolites of G. abietina. With this process only callus cells that are resistant to the metabolites will survive. If we can produce plantlets from the surviving callus cells, it is likely that we will be able to produce large quantities of resistant larch plantlets.

We are also starting to work with other conifers, especially Scots pine and ponderosa pine. Both species are more difficult to manipulate in tissue culture than larch and we expect that it will take several years to perfect the proper tissue culture systems for these two species.

Although we are optimistic about the opportunities available to us with somaclonal variation, it is important to realize that testing for disease resistance in the laboratory is different from field testing. Until we have shown that laboratory resistance can be correlated with field resistance, we must be careful how we use these new somaclones. We also want to emphasize that somaclonal variation is not a replacement for standard genetic breeding. It does, however, offer the breeder an

additional tool that may help the breeding program advance at a much faster rate. Any system that will allow us to develop disease-resistant trees within our own lifetime is highly beneficial.

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ABSTRACT

Plant tissue culture techniques are being used to obtain disease resistant forest trees by identifying genetic variation in tissue cultures. The study objectives are to identify somaclonal variation and to obtain resistance to Septoria leaf spot and canker of Populus (caused by Septoria musiva) and Scleroderris canker of Larix (caused by Gremmeniella abietina). More than 500 Populus somaclones with resistance to Septoria have been identified and are being field tested. Larix somaclones with various resistance to G. abietina have been tentatively identified in laboratory tests.

KEYWORDS

Somaclones, forest pathology, diseases, tissue culture, Larix, Populus

ZUSAMMENFASSUNG

Somaklonale Variation zur Entwicklung von Resistenz bei Koniferen gegen Gremmeniella abietina

Pflanzengewebekulturtechniken wurden angewendet, um krankheitsresistente Forstbäume durch die Identifizierung genetischer Variation in Gewebekulturen zu erhalten. Ziel der Versuche war es, somaklonale Variation zu identifizieren und Resistenz gegen Septoria-Blattflecken und Krebs bei Populus (verursacht durch Septoria musiva) sowie Scleroderris-Krebs bei Larix (verursacht durch Gremmeniella abietina) zu finden. Über 500 Populus-Somaklone mit Resistenz gegen Septoria wurden gefunden und im Feldversuch geprüft. Larix-Somaklone mit verschiedener Resistenz gegen G. abietina wurden vorläufig nur in Laborversuchen identifiziert.

TRIAL TO CONTROL SCLERODERRIS CANKER IN RED PINE PLANTATIONS

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SUMMARY

In a trial to control scleroderris canker in a 10-year-old red pine plantation having 67% of trees infected with Gremmeniella abietina (Lagerb.) Morelet, lower branches were pruned on four whorls and left on the ground. The control is good when less than 20% of trees are infected. In a portion of the plantation where the rate of infection was 76% without mortality, it was possible to control the disease by clipping residual infected branches the following years. Where 86% of the trees were infected and mortality had been caused by G. abietina, most red pines had to be cut down.

INTRODUCTION

Only a few reports have been published on the control of scleroderris canker caused by Gremmeniella abietina (Lagerb.) Morelet in pine plantations (Gremmen,1972). In a plantation, Bergdahl and Ward (1984) reduced the rate of infection, the inoculum densities, and the periods of inoculum production by pruning all branches from the lower one-third of red pines (Pinus resinosa Ait.). These authors were dealing with the European race of G. abietina and pruned branches left on the ground did not increase the infection level when compared to

sites where branches were removed from the plantation. Our trial was based on Bergdahl and Ward's results with the idea of integrating these control practices to plantation maintenance.

MATERIALS AND METHODS

This trial took place in a plantation of 5,000 red pines located at Kazabazua, 80 km north of Ottawa, Canada. In 1982, this 10-year-old plantation was surrounded by a plantation of non-infected jack pine (Pinus banksiana Lamb.) of the same age.

Trees infected with G. abietina were counted prior to pruning and then every following year in 10 sample plots of 50 trees equally distributed over the plantation. In 1982, all branches of four whorls were systematically pruned and they were left on the ground. If necessary, further intervention was decided the second year after pruning. The European race of G. abietina was identified from isolates.

RESULTS

Based on the initial rate of infection, the plantation was divided in to three zones (Fig 1). Zone I had 20% or less of trees infected; zone II had more than 20% of trees infected with no mortality caused by scleroderris canker; and zone III had more than 20% of trees infected with mortality caused by scleroderris canker.

The general rate of infection dropped from 67 to 22% after pruning (Table 1). In 1983 in zone I, infection was not found; in zone II, the rate of infection was about 18%; in zone III, 33% were still infected. In 1984, the rate of infection had not changed except in zone III where tree mortality had decreased the number of infected trees. Dead trees were discarded from the sampling because wood borer insects also caused some mortality in the plantation in 1983. Late in the summer of 1984, the insect Pityophtorus puberulus Le Conte caused symptoms on shoots similar to those of scleroderris canker. The general rate of infection by both these organisms was about 58%. In the fall of 1984, we clipped all branches showing symptoms of the disease or the insect and

we cut trees with more than half of the residual whorls infected. The following year, 100 trees were sampled to calculate the proportion of scleroderris symptoms and the insect symptoms and then, the rate of infection was estimated in the regular sample plots. Because the infection rate was still at 11% in 1985, branches were clipped in late summer of that year. New shoots did not show symptoms of G. abietina in 1986. Few trees had cankers on their trunks and P. puberulus was still active in the plantation.

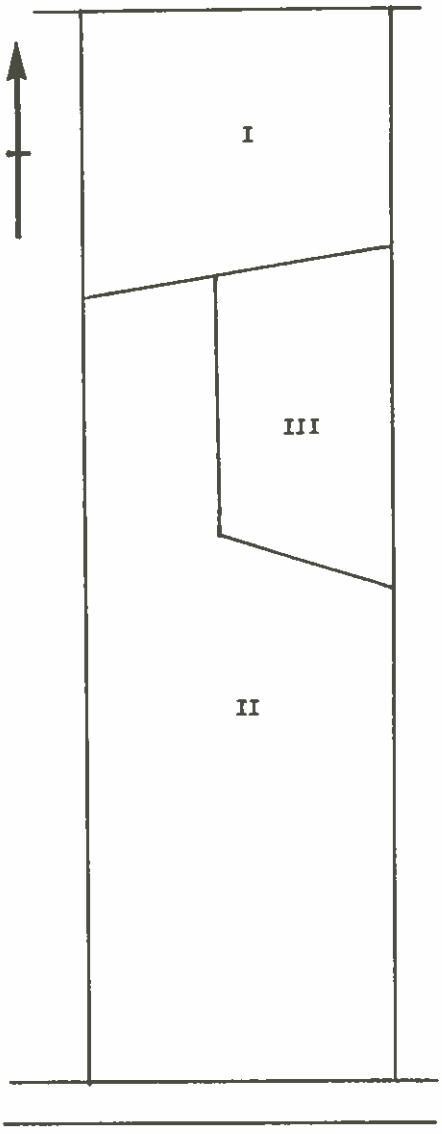

Figure 1. Zones of the red pine plantations based on the initial rate of infection by Gremmeniella abietina; I: 20% or less of trees infected; II: more than 20% of trees infected with no mortality; III: more than 20% of trees infected with mortality caused by G. abietina.

2. MATERIAL AND METHOD

Field observations were made in two mountains of S. Greece (Peloponese) and another one in N.W. Greece, for 4 successive years. Affected trees were examined in the field and the laboratory for symptoms. Samples of tissue from newly affected or dying trees were put on several media, including V-8 juice, asepticly or after surface sterilization in 10% sodium hypochloride for fungal isolation. Different levels of temperature were used for incubation.

3. RESULTS

After careful examination of the symptomatology of the disease, it appears that the bark of the trunk is the first to be affected. When the trees start to decline, the bark at the lower part of the trunk is brown longbefore, usually with bark beetle attack. It is not possible to detect when the bark begins to be affected, since the trees seem quite healthy, just before they start to become brown.

In many cases of recently dying trees, it can be detected the length of the affected bark of the trunk, which starts just above the ground and extends as up as 2 to 3 years growth of height, while the bark of the upper part of the trunk was not affected. The root system of these trees seems to be still unaffected, except for some necrotic streaks. In other cases trees which are still living near to the dead ones, have some necrotic streaks or patches of the bark on the trunk with bark beetles attack. These trees appear to be partly affected by the disease and they are living because the necrotic areas of the bark have not encircled the trunk. Very often these living trees show brown spots in the bark of the trunk which extend near to the cambium region and sometimes affect the wood. These symptoms show that it is a stem bark disease.

There were no fruit bodies of known pathogenic fungi on the bark of the diseased or dead trees. Also a lot of isolation work on several dates and media did not reveal any pathogenic fungi. The fungi which were isolated from the diseased bark were Sclerophoma pityophila (Corda) Höhn., Aureobasidium pullulans (de Bary) Arnaud, Botrytis cinerea, Alternaria sp., Penicillium sp., Ceratocystis sp. and some other moulds (see table below).

Transverse sections of the affected wood, just below the brown spot of the bark of living trees, did not reveal any fungal hyphae. In this case only the ray cells were abnormal and hypertrophied.

Table 1. Samples of dead or dying bark of diseased trees of P. nigra yielded fungi.

Tree status	No. of samples planted	Per cent of samples		Fungi isolated
		Sterile	Yielded fungi	
Living with brown spot in the bark	201	73.6	26.4	S.pityophila A.pullulans B.cinerea Alternaria sp.
Dead or dying	95	50.5	49.5	Penicillium sp. Ceratocystis sp. Several moulds

4. DISCUSSION

The disease appears mainly in stands after thinning or harvesting of the old trees. This implies a sudden change in the microenvironment of the stand. This change in the microenvironment may increase the transpiration of the trees, and so they lose much water for some period of time. Trees under water stress are usually predisposed to be attacked by a fungus, which under normal conditions is considered as saprophyte.

From the description of the symptomatology it was also concluded that it is a wilt disease. Wilt diseases are much less common in gymnospermous species of trees than in angiospermous. To date, only two wilt diseases of pines have been reported. One caused by blue stain fungi of the genus Ceratocystis (1) and the other caused by the nematode Bursaphelenchus xylophilus (2). Both the fungus and the nematode wilt diseases develop after attack of trees by insects. It has been reported also by MAMIYA (1983) that the nematode wilt disease in Japan is extended into uninfested areas by the introduction of nematode-infected pine logs. The fact that new groups of dead trees appear in stands where human activities have taken place before, suggests that nematode may be the causal organism. In the coming future we are going to examine this possibility.

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ZUSAMMENFASSUNG

Symptomatologie und Auftreten einer bisher nicht beschriebenen Rindenkrankheit bei Pinus nigra in Griechenland.

In natürlichen Schwarzkiefernwäldern zeigen sich sehr oft Gruppen toter Bäume in Beständen, in denen zuvor der Mensch gewirkt hat. Diese Gruppen toter Bäume treten gewöhnlich während des Sommers auf und dehnen sich im folgenden Jahr nicht weiter aus. Allerdings zeigen sich erneut tote Bäume in anderen Beständen. Die Rinde im unteren Teil des Hauptstammes zeigt zuerst Schäden, gefolgt von einem schnellen Absterben des Baumes. In der Regel werden junge Bäume im Alter von 8 bis 20 Jahren befallen. Bevor die Bäume anfangen braun zu werden, erscheinen keine anderen typischen Symptome. Isolierungen führten bisher zu keinem Krankheitserreger.

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