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Factors favouring pathogenesis of *Gremmeniella abietina*

Raija-Liisa Petäistö

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ABSTRACT

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Gremmeniella abietina (Lagerb.) Morelet is a pathogenic ascomycete causing damage to conifers especially in northern Europe, North America and Asia. It can damage, even kill, trees of all ages, being a problem both in forests and forest nurseries. Chemical control is routinely used in tree nurseries to prevent loss of the seedlings.

The growth phase of Scots pine (*Pinus sylvestris* (L.)) seedlings affects the success of *G. abietina* conidial inoculum. This was studied in two simulated and two natural growing seasons with second or third year seedlings. The inoculations made during the first half of the growing seasons resulted in higher disease occurrence than those made during the second half of the growing seasons. However, cold stress predisposed the seedlings to the disease in late summer. Drought stress in late summer increased cold hardiness but did not affect the susceptibility of the seedlings to the inoculation in the following autumn.

Evidence of the ability of *G. abietina* to further the development of the disease at low temperatures is provided by the fact that the conidia germinate on agar at the same rate at low temperatures, zero or plus five, as at higher temperatures, plus ten and twelve. However, at low temperature germination needed five to seven days more time to reach the same rate and the germ tubes grew much less. In pine seedlings, placing mycelium into the phloem caused necrosis in winter at low temperatures, even at two and four degrees below zero.

Pathogenic fungi can cause damage to their host by cell wall degrading enzymes and by toxins. *G. abietina* grew in media where pectin was the sole carbon source. Dry weight increased with culture time, measured at two, four and six weeks. Needle extract further increased the growth of the fungus but decreased induced polygalacturonase activity. The induced polygalacturonase activity was higher after four than after two weeks culturing in pectin-containing induction media. A long incubation time in the substrate in the activity assay is needed to reveal the activity of the enzyme because of enzyme-substrate complex. Xylan hydrolyzing activity was also detected from the culture media of *G. abietina* isolates. Needle extract added to xylan medium decreased the induced xylanase production by the fungus but increased the mycelia production.

For characterization of the races of *G. abietina*, the polygalacturonic acid hydrolyzing activity was compared *in vitro* between the North American and the European races of the fungus. For the reliable comparison of the races the variation among isolates of the same race and the variability of different components of the experimental and measurement errors were analyzed using variance component models. The European race induced more polygalacturonic acid hydrolyzing activity (in relation to mycelial dry weight) and produced less mycelial dry weight than the North American race in pure pectin media. No correlation was found between the activity and mycelial dry matter production within the races. A further study showed that the B-type of Finnish isolates produced more dry matter than the A-type, but no difference in the polygalacturonase activity was detected between these types. The results indicated metabolic differences between races and types. How these are reflected *in planta* requires further studies.

The protein pattern and immunoblot can be useful tools for detecting certain types of *G. abietina*. Characterization of the isolates electrophoretically and with immunoblotting was reliable when two-week-old cultures were used as samples, since protease activity is very low in young cultures. When using the developed sample preparation method no

protease activity was found. To make absolutely sure that no protease is present, protease inhibitors could be used in sample preparation for protein pattern analysis. In the separate protease studies, using sample incubation with divalent cations and enzyme inhibitors, calcium- and zinc-dependent metalloprotease activity was specifically detected, and also other protease activities may be present. Serine protease inhibitor phenylmethanesulfonyl fluoride showed only a minimal inhibitory effect.

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LIST OF PUBLICATIONS

This thesis is based on the following articles, which will be referred to in the text by their Roman numerals.

I PETÄISTÖ, R-L.; KURKELA, T., 1993: The susceptibility of Scots pine seedlings to *Gremmeniella abietina*: effect of growth phase, cold and drought stress. Eur. J. For. Path. 23, 385–399.

II PETÄISTÖ, R-L., 1993: Conidial germination and formation of necrosis in pine seedlings by *Gremmeniella abietina* at low temperatures. Eur. J. Forest Pathol. 23, 290–294.

III PETÄISTÖ, R-L; KAJANDER, E. O., 1993: The ability of *Gremmeniella abietina* to hydrolyze polygalacturonic acid. Eur. J. Forest Pathol. 23, 306–313.

IV PETÄISTÖ, R-L; TALVINEN, J.; KAJANDER, E. O., 1992: Detection of xylan hydrolyzing activity in culture extracts of *Gremmeniella abietina*. Eur. J. Forest Pathol. 22, 349–353.

V PETÄISTÖ, R-L; LAPPI, J., 1996: Capability of European and North American race of *Gremmeniella abietina* to hydrolyze polygalacturonic acid *in vitro*. Eur. J. Forest Pathol. 26, 000-000, in press.

VI PETÄISTÖ, R-L; RISSANEN, T.E.; HARVIMA, R. J.; KAJANDER, E. O., 1994: Analysis of the protein pattern of *Gremmeniella abietina* with special reference to protease activity. Mycologia 86, 242–249.

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LIST OF ABBREVIATIONS

EDTA ethylenediaminetetra acetate
LT lethal temperature
pCMB p-chloromercuribenzoate
PMSF phenylmethanesulfonyl fluoride
SDS-PAGE sodium dodecyl sulphate-polyacrylamide gel electrophoresis

1. INTRODUCTION

1.1 The Scleroderris canker: The causal agent and its occurrence

The ascomycete *Gremmeniella abietina* (Lagerb.) Morelet, the causal agent of Scleroderris canker, occurs in North America, Europe and Asia. *Pinus* species are the main hosts of *G. abietina*, but disease also occurs on *Picea, Abies, Larix, Tsuga* and *Pseudotsuga*. *G. abietina* damages the host at any age. In forest nurseries the disease has killed seedlings (KURKELA 1967, PUNTER 1967, TEICH 1972, BARKLUND 1989) and heavy damages have been found in plantations and older forests (KALLIO et al. 1985, SETLIFF et al. 1975, KOHH 1964).

In Finland the conidia of *G. abietina* disperse mainly in the spring and the ascospores later in the summer (NEVALAINEN 1985). The fungus commonly penetrates the living tissue of a shoot through the bud scale of the short shoot near the long shoot tip (PATTON et al. 1984), or it colonizes scales of the bud and the bract of short shoots in the bud and penetrates through these into living tissue of the shoot in late winter (LANG and SCHÜTT 1974, SIEPMANN 1976). It is not clear from these previous studies whether the bud scale and the shelter sheaths (surrounding the short shoot) play the same role in the infection.

The symptoms usually become visible in the spring following the infection. The bud is dying and resinous, the colour of the needles is usually greyish green and the bases of the needles are necrotic. The fungus also causes cankers on the branches and the main stem. Although the usual infection cycle lasts from the growing season to the next spring, when the symptoms appear, the fungus can stay latent for a longer time in the host (MAROSY et al. 1989).

1.2 Determinants of infection: Environmental factors

The presence of large amounts of humidity, free water and inoculum favour the outbreak of the disease (DORWORTH 1972), so the heaviest damages are in depressed areas (AALTO-KALLONEN and KURKELA 1985, SAIRANEN 1990). Cold stress (frost) during the growing season predisposes trees to the fungus (YOKOTA et al. 1975, PETÄISTÖ and REPO 1988), as well does shading (READ 1968, PETÄISTÖ and REPO1988, CAPRETTI 1990).

G. abietina mycelium *in vitro* grows optimally at about 18°C (SLETTEN 1971). The fungus can grow at low temperatures and growth has been found even below zero (ETTLINGER 1945, DORWORTH and KRYWIENCZYK 1975, SLETTEN 1971). Neither germination of spores nor mycelial growth has been found above +30°C (YOKOTA et al. 1974, DORWORTH and KRYWIENCZYK 1975), although SLETTEN (1971) found minimal growth at +31°C – +33°C. The survival of spores at high

temperature is, according to BLENIS et al. (1984), related more to average daily temperatures than to extreme daily temperatures. In their study the fungus survived daily exposure to +34°C, when the average temperature was +27°C, but did not survive a constant temperature of +32°C.

Time of inoculation, dormancy or growth phase of the host affect the occurrence of the disease. If the infection is induced by placing mycelium in a wound, inoculation during the rest period is the most successful (ROLL-HANSEN 1964, SLETTEN 1971, KURKELA and NOROKORPI 1979). Results of conidial infections performed at various phases of the growing season differ, the spring inoculation or autumn inoculation causing more disease (YOKOTA et al.1974, BAZZIGHER et al.1986, PETÄISTÖ and REPO 1988, AITKEN 1993, CAPRETTI 1990). The first-year container seedlings (seedling in its first season) have a different response to inoculation made at various phases of the annual development of the host than do older seedlings (HAMNEDE 1980).

1.3 Determinants of infection: Cell wall degrading enzymes

Microbes use enzymes to degrade plant cell components during penetration and destruction of the plant tissues. The middle lamella and primary cell wall of conifers contain a lot of pectic polysaccharides (O'NEILL et al. 1990). Pathogens and nonpathogens excrete several enzymes that can degrade pectin: for example, endo-polygalacturonase, pectin esterase, pectin lyase and pectate lyase. Some pectic enzymes are also known to activate plant defence mechanisms such as lignification, phytoalexin accumulation and necrosis (ROBERTSEN 1986, 1989, WALKER-SIMMONS et al.1984, CERVONE et al.1989).

Pectic enzymes can have a role in many diseases (COOPER 1983, BATEMAN and BASHAM 1976). For instance, it seems certain that polygalacturonase has an important role in the pathogenesis of *Cryphonectria parasitica*, causing browning of the inner bark of American chestnut (GAO and SHAIN 1995). The purified polygalacturonase (from culture filtrate of virulent isolate of *C. parasitica)* hydrolyzed cell wall material *in vitro*, and the activity was detected in infected chestnut bark. High levels of specific polygalacturonase inhibitor in the resistant host provide further evidence of the importance of this enzyme in the virulence of *C. parasitica*.

The role of endopolygalacturonase in diseases has also been studied using a gene mutant, and it has been found that this enzyme may not be a pathogenicity factor in the cases studied (e. g., SCOTT-CRAIG et al. 1990). A minus mutant of the pathogenic fungus *Cochliobolus carbonum* lacking a gene which encodes an extracellular endopolygalacturonase can also grow in medium where pectin is the sole carbon source, and the mutant could also infect maize as well as the wild type strain. However, it is possible that other pectin degrading enzymes than the endopolygalacturonase degrade enough pectin, or that the fungus does not need

pectin degradation in the colonization of the host tissue, or there may be other endopolygalacturonases that are induced only *in vivo* (SCOTT-CRAIG et al. 1990).

It is clear that xylanase is present in, for example, wood rot (BLANCHETTE 1989), as it is also in some diseases in *Graminea* (COOPER et al. 1988), since xylan exists abundantly in *Graminea* also in the primary cell wall. In *Gymnospermae* the xylan is mainly arabinoglucuronoxylan (SJÖSTRÖM 1981) and occurs especially in the secondary cell wall. Using a specific xylanase mutant, it has been found that one particular xylanase is not required for pathogenicity of *Cochliobolus carbonum* in maize (APEL et al. 1993), since the pathogenicity of the mutant is not distinguishable from the wild type. Xylanase can also have an other role in disease, inducing the defence system of the plant host (DEAN et al. 1989, LOTAN and FLUR 1990): induced ethylene production (DEAN et al. 1989), for example, is one of the first responses of plant cells to the attack by a pathogen.

1.4 Taxonomic position and races of *G. abietina*

The causal agent of Scleroderris canker, *G. abietina*, is an ascomycetous fungus and was first described by KARSTEN (1884). The disease on pine was first described by BRUNCHORST (1888). KARSTEN named the conidial form *Septoria* (*Rhabdospora*) *pinea*. Later, the imperfect state was named *Brunchorstia destruens* Erikss (ERIKSSON 1891) and then *Brunchorstia pinea* (Karst.) v. Höhn by HÖHNEL (1915). LAGERBERG (1913) named the perfect state *Crumenula abietina*, and thereafter the name was changed to *Scleroderris abietina* (Lgbg.) Gremmen (GREMMEN 1955) and to *Ascocalyx abietina* (Lagerb.) Schläpfer (SCHLÄPFER-BERNHARD 1968). MORELET (1980) separated the genera *Ascocalyx* and *Gremmeniella*, and came to the conclusion that the causal fungus of this disease was *Gremmeniella*, although MÜLLER and DORWORTH (1983) disagreed. Later, the name *Gremmeniella abietina* (Lagerb.) Morelet was accepted (PETRINI et al.1989, BERNIER et al. 1994).

G. abietina can be divided into the North American, European and Asian races using an immunological method (DORWORTH and KRYWIENCZYK 1975). The European race has been found not only in Europe but also in North America (DORWORTH et al. 1977). The races have different protein patterns (BENHAMOU et al. 1984, PETRINI et al. 1989, 1990), and also differences in pectinase isozyme patterns (LECOURS et al. 1994). Recently, differences between races were found by using random amplified polymorphic DNA markers (HAMELIN et al. 1993).

According to PETRINI et al. (1989, 1990), the species *G. abietina* can be divided into two varieties: *G. abietina* var. *abietina* (including North American, European and Asian serovars), and var. *balsamea*. UOTILA (1983, 1992, 1993) and HELLGREN and HÖGBERG (1995) have identified types (A- and B-type or large tree and small tree type) inside the European race in Scandinavia. Recently HAMELIN et al. (1996) found three distinct DNA amplification profiles in the European race of *G. abietina*

in Europe. Thus, within this species there seems to be taxonomic, serological and genetic variation but information on physiological and pathogenicity variation seems lacking or few.

2. THE AIMS OF THIS STUDY

Moisture is needed for dispersion of the conidia of *G. abietina*, which mainly occurs at the beginning of the summer. Ascospore dispersion occurs later in the summer and in the autumn. For control purposes and for predicting the disease occurrence, the infectivity of conidial inoculations made in different growth phases of the host during the growing season was studied. For the same reason, the possible effect of spring and autumn frost and autumn drought stress on pathogenesis were also studied (I).

Since the visible disease symptoms do not appear before the spring following infection, the conditions during winter may be important. That is why the effect of low temperature on the germination and germtube growth of *G. abietina* conidia was studied *in vitro*. Further the temperature effect on the amount of necrosis in the shoot of pine seedlings after mycelial inoculation during winter was examined (II).

The question of the mechanism by which the fungus causes the damage and exploits the condition of its host (growth phase, stress effect) was approached with enzyme studies. The exoenzymes, polygalacturonase and xylanase, degrading the cell wall components, pectin and xylan, were examined (III, IV).

There are obvious pathogenetic differences between the races and between the types of *G. abietina*. In order to further characterize the races and types, the differences in the polygalacturonase activity of the races and types were studied *in vitro*. The reliablity of different components of the experimental and measurement errors were analyzed so that a reliable comparison of this activity could be obtained (V). In order to identify the variation of *G. abietina,* the production of a reliable protein pattern of the soluble proteins of the fungus using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was studied. The protease activity of the fungus was studied in order to obtain reliable protein pattern. For immunological comparison, polyclonal antibodies were derived in rabbits against a mycelial homogenate of the fungus (VI).

3. MATERIAL AND METHODS

3.1 Infection: Environmental factors

3.1.1 Infection: Annual rhythm of the host

Whether the growth phase of the over one-year-old seedlings correlates with susceptibility to disease was studied in two experiments where the growing season was simulated, and in two experiments in the natural growing seasons (I). The light period during the simulated growing seasons was adjusted every 1–2 weeks to correspond to the natural day length. The hardening and dehardening processes of the seedlings were measured using an impedance method (REPO and PELKONEN 1986), and the growth of shoots and needles was used as criteria for verifying that the simulated growing season could be used in the study.

The seedlings were inoculated by applying a conidial suspension on the terminal bud of the seedlings, after which the seedlings were kept moist for two days using a water sprinkler. Separate groups of seedlings were inoculated at specified times from the beginning to the end of the seasons. In all four experiments, the conidia used were from at least two isolates. Conidia from both A- and B-type isolates were used in the simulated growing season experiments; in the first natural growing season experiment conidia from the B-type and from unclassified isolates were used, and in the last experiment all the conidia were from B-type isolates. The classified isolates were obtained from Dr. A. Uotila.

3.1.2 Infection: Cold and drought stress of the host

Two studies (one with a simulated and one with a natural growing season) were carried out with inoculation and controlled cold stress during the growing season in spring and in autumn (I). Using the impedance method (REPO and PELKONEN 1986), the cold hardiness of the seedlings was first tested and the temperature, chosen for cold stress (-4°C or -5°C), was so mild that it did not cause any visible damage to the seedlings. In the stress treatment, the temperature was gradually decreased to the stress temperature, and after reaching the lowest level it was raised, again gradually. The overall length of the treatment was 12–14 h (REPO and PELKONEN 1986).

Drought treatment (transplanting) was carried out at three different points of time in autumn in one simulated growing season experiment (I). The roots of the seedlings were washed, and the seedlings were put in a chamber for 15 min at +32°C. Thereafter, the seedlings were planted back in their containers. The seedlings were inoculated late in the autumn to test the predisposing effect of drought on the disease.

The effect on the cold hardiness was measured at the beginning of the following winter. The root growth of the seedlings was observed in the following spring.

3.1.3 Temperature dependence of conidial germination and of necrosis formation caused by mycelia

The germination of conidia and the growth of the germtubes at low temperatures were tested on water agar. The test temperatures were 0°C, +5°C, +10°C and +12.5°C. The germination rate was examined microscopically after 2, 3, 4, 7 and 9 days' incubation. To measure the germ tube growth, the surface of the agar media was photographed after inoculation every day. The length of the germ tubes was measured on photographs (II).

To examine the ability of this fungus to damage host tissues in winter (II), container pine seedlings were kept in winter in the dark at the test temperatures of +2°C, 0°C, -2°C and -4°C. They were inoculated with mycelium placed into the phloem, and the control treatments were inoculation with dead mycelium and wounding without any inoculum. After two and a half months, the length of the necrosis which had developed in the seedlings was measured.

3.2 Ability of the fungus to degrade pectin and xylan

3.2.1 Analysis of polygalacturonase activity

Culture filtrate was used as an enzyme preparation and polygalacturonic acid as a substrate in polygalacturonase activity analysis (III). The assay method has been described by MILLER (1959) and by BAILEY and PESSA (1990). We tested the pH dependence and the linearity of the activity. For this purpose, the fungus (B-type isolate Kai1.2 from Dr. A. Uotila) was cultured in 1 % apple pectin in Hoagland solution supplemented with vitamins and with 290 unit/ml penicilline-G (Sigma) and 108 unit/ml streptomycin sulphate (Fluka).

The inducibility of polygalacturonic acid hydrolyzing activity was studied by using different media:1 % pectin from apple and citrus fruits with and without a needle extract, 3 % glucose, and 1 % xylan in mineral medium. After culturing in these induction media, the mycelia were removed to a needle extract medium because the degradation products in the various induction media may have an inhibitory effect on activity (COOPER 1983) and because the amount of degradation products may be different in the different induction media. From the new medium, the induced polygalacturonic acid hydrolyzing activity was measured after four days. The activity was determined as mol galacturonic acid released per mg mycelial dry weight.

3.2.2. Analysis of xylanase activity

Xylan hydrolyzing activity (IV) was analysed (MILLER 1959, POUTANEN and PULS 1988) using culture filtrate as an enzyme preparation and xylan as a substrate. The pH dependence and the linearity of the enzyme activity was tested. For the tests the fungus was cultured in Hoagland solution with 20 % needle extract. Various induction media, 1 % xylan media with and without a needle extract and 1 % pectin were used to study the inducibility of the xylan hydrolyzing activity. Additionally, the effect of the needle extract on the xylan hydrolyzing activity was tested by diluting a crude enzyme preparation with autoclaved needle extract or buffer. The dilutions were incubated overnight at +20°C before the activity was measured.

3.3 Characterization of the fungus

3.3.1 Comparison of polygalacturonase activity in vitro *between isolates*

The ability of the European and North American races to degrade polygalacturonic acid was compared. In all,15 isolates of the European and 14 of the North American race (V) were cultured, each in four flasks, in 1 % citrus pectin medium. After two weeks, the mycelia were washed and moved to a new citrus pectin medium to avoid the possible increased amount of degradation products in the medium, as these products may have an inhibitory effect on activity. In the new medium, the mycelia were cultured for four days. The mycelial dry weight was measured and polygalacturonase activity was analysed from the culture fluids. In the same way, a smaller number of isolates, classified as A- and B- type, were tested for a possible difference in enzyme activity between the types. The purpose of the data analysis was to study the structure of the experimental errors, variability between isolates, differences between races and the correlation between polygalacturonase activity and mycelial dry weight. The data were analyzed using mixed linear models (SEARLE 1971). Computations were done with MIXED procedure of the SAS package (SAS Institute 1992). REML (Restricted Maximum Likelihood) was used as the estimation method (thus assuming normality of random effects). Different variance-covariance structures were compared using the χ^2-approximation for the log likelihood-ratio statistic G (e.g. SOKAL and ROHLF 1995). Fixed effects (race differences) were tested with F-tests.

3.3.2 Analysis of the protein pattern

The isolates for protein pattern analysis (VI) were grown in liquid V8 medium. The frozen mycelium was homogenized. The homogenate was centrifuged and the supernatant was used as samples for SDS-PAGE.

Protease excreted by the fungus was studied at pH 6 and pH 7.4 with the used and unused culture media as enzyme source, and ability to degrade the protein marker mixture was determined with SDS-PAGE. Mycelial endogenous protease activity was tested by incubating the supernatant of the mycelial homogenate for various times at 24°C, and possible degradation of the proteins was analyzed with SDS-PAGE.

The effect of protease inhibitors and divalent cations on endogenous protease activity in the supernatant fraction of the mycelial homogenate was studied by incubating the supernatant with and without these compounds. The samples were then run in SDS-PAGE. Additionally, the effects of phenylmethanesulfonyl fluoride (PMSF, serine protease inhibitor), of polyvinylpyrrolidone (adsorb phenol), and of thiourea (inhibit phenol oxidase) in the sample preparation on the protein pattern were tested.

Antibodies against the mycelium of four isolates were prepared in rabbits. Five boosters (mycelium homogenate) were injected at 1-week intervals into each animal. Serum was separated from the blood and used in immunoblotting.

3.4 Statistical analysis

The statistical package BMDP (1988) was used (II, III, IV) in variance and regression analysis. In V, the data were analyzed using mixed linear models (SEARLE 1971). Computations were done with the MIXED procedure of the SAS package (SAS Institute 1992). REML (Restricted Maximum Likelihood) was used as the estimation method (thus assuming normality of random effects). Different variance-covariance structures were compared using the χ^2-approximation for the log likelihood-ratio statistic G (e. g., SOKAL and ROHLF 1995), and fixed effects (race differences) were tested with F-tests.

4. RESULTS

4.1 Infection: Effect of environmental factors

4.1.1 Effect of growth phase of the host on infection

The inoculations made in the first half of the growing season were more successful than those made later in the season both in simulated (Exp. 1 and 2) and natural seasons (Exp. 3 and 4) (Table 1) (I). The inoculations made during the second half of the growing season were less successful the later the inoculation had been performed.

Table 1. Percentages of diseased (diseased and dead) seedlings, (A) inoculated during the first and (B) the second half of the growing season, and (C) percentage of diseased seedlings in controls in Experiments 1, 2, 3 and 4.

	Simulated growing season				Natural growing season			
	Exp 1		Exp 2		Exp 3		Exp 4	
	mean	S.E.M.	**mean**	S.E.M.	**mean**	S.E.M.	**mean**	S.E.M.
A	**51.2**	5.40	**66.4**	11.47	**88.7**	6.27	**75.4**	6.74
B	**0.4**	0.42	**17.3**	6.86	**13.8**	1.99	**27.0**	5.81
C	**0.0**		**8.3**		**7.8**		**16.2**	

Table 2. The effect of cold stress in spring (A) and in autumn (B) on the disease occurrence in the seedlings in Experiments 1 and 4. In the group 'inoculated, without stress', the figures in parentheses are the results of the inoculation made simultaneously with the stress, the figures without parentheses are the results of all the inoculations during the same half of the growing season (cf. Table 1).

	Experiment 1		Experiment 4	
	number of seedlings	% seedlings **diseased**	number of seedlings	% seedlings **diseased**
A (stress in spring)				
Not inoculated				
–without cold stress	60	**0**	74	**16.2**
–with cold stress	15	**0**	49	**18.4**
Inoculated				
–without cold stress	240 (15)	**52.8 (80.0)**	509 (44)	**74.5 (68.2)**
–before cold stress	15	**53.3**	49	**89.8**
–after cold stress	15	**46.6**	-	-
–in the autumn following the cold stress	15	**20.0**	47	**55.3**
B (stress in autumn)				
Not inoculated				
–without cold stress	60	**0.0**	74	**16.2**
–with cold stress	15	**6.7**	50	**20.0**
Inoculated				
–without cold stress	240 (15)	**0.4 (0.0)**	501 (50)	**27.2 (36.0)**
–before cold stress	15	**60.0**	49	**85.7**
–after cold stress	15	**26.7**	-	-
–in the spring preceding the cold stress	15	**80.0**	46	**82.6**

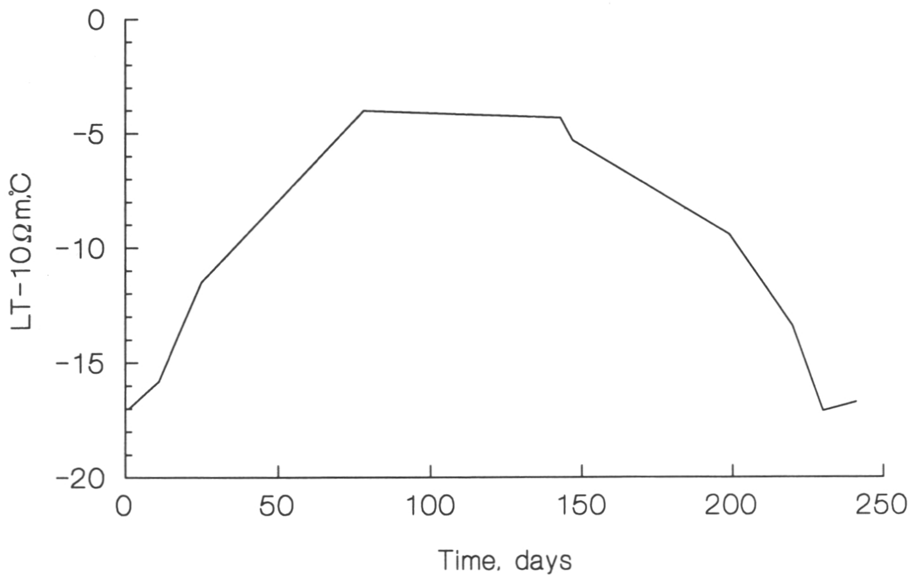

Fig. 1. The frost hardiness of the seedlings during the simulated growing season described as LTΩm °C. The x-axis shows the days from the beginning of the growing season.

During the first half of the growing season, the shoot and needles elongated and the cold hardiness decreased or was at its lowest stage. The timing of shoot and needle elongation under simulated growing seasons was comparable to that found during the natural growing seasons (I). The dehardening and hardening processes (Fig. 1) were also similar to those in the natural growing season (GLERUM 1973). The hardiness decreased with increasing temperature and photoperiod, and it increased when the temperature decreased and the photoperiod became shorter.

4.1.2 Effect of cold and drought stress of the host on infection

Frost increased the susceptibility of seedlings to the disease (Table 2), although the frost treatment was so mild that no injuries were found visually (I). Cold treatment performed in the autumn had a clear disease increasing effect when the inoculation was done in the autumn. The cold treatment in spring might also have had some effect on the success of the inoculation made the following autumn.

Our study showed that short drought stress in autumn had no effect on the disease condition in the next spring when the inoculation was done in late autumn (I). Although drought stress had no effect on disease outbreak, it affected the cold hardiness of the seedlings measured late in the same autumn. The earliest stress increased the hardiness less than the later ones. In addition, the stress retard formation of new roots in the following spring.

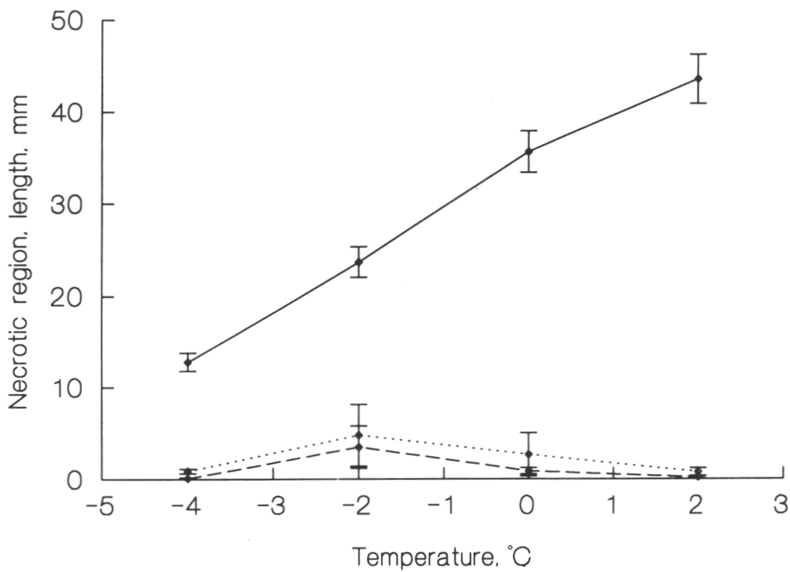

Fig. 2. The length of the necrosis in the shoots of pine seedlings 75 days after inoculation in winter. During this period the seedlings were kept in dark growth chambers. Wounds inoculated with *G. abietina* mycelium (—), control 1: the wound inoculated with killed mycelium (----), control 2: the wound without inoculum (····). The tested temperatures were -4°C, -2°C, 0°C and +2°C. Bars indicate standard error of the means.

4.1.3 Effect of low temperature on conidial germination and on ability of mycelium to cause necrosis in the host

The germination of conidia reached the same level at the tested temperatures (II), but at 0°C the germination required about 7 days and at +5°C about 3 days longer than that at +10°C and +12.5°C. The mean total length of the germtubes from one conidium was greater at higher temperature. The growth was about 25 times greater at +12.5°C than at 0°C.

The fungus caused clear necrosis in pine shoots even at -4°C. The length of necrosis was about 3.5 times shorter at -4°C than at +2°C. The length of the necrosis was clearly different from that in the controls (Fig. 2.).

4.2 Degradation of polygalacturonic acid and xylan

Study III showed that *G. abietina* grows well in liquid mineral media where pectin is the sole carbon source. Pure pectin media induced polygalacturonase activity more than the pectin media with needle extract, and glucose medium induced it least (Fig. 3.). Needle extract included in the pectin medium supported higher mycelial

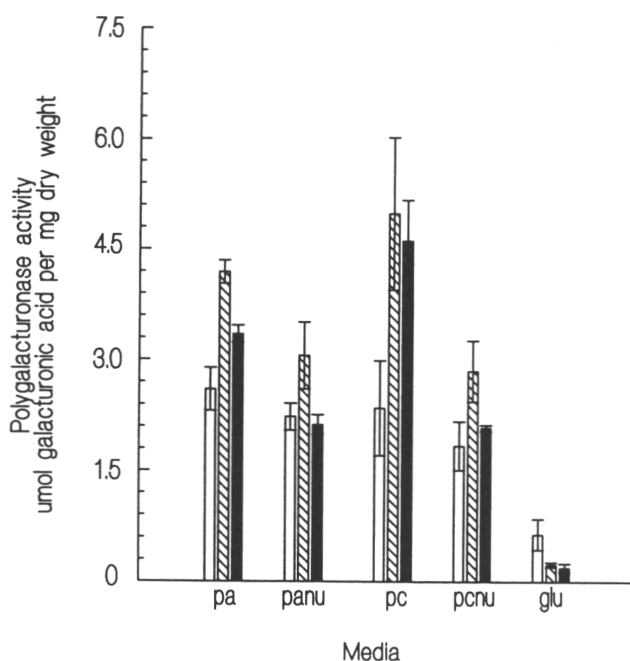

Fig. 3. Polygalacturonase activity in the needle extract medium after precultures in various pectin and glucose media. The cultures were started from conidia and cultured 2 (white), 4 (hatched) and 6 (black) weeks in media. Medium pa = 1% pectin from apple in Hoagland solution, pc = 1% pectin from citrus fruits in Hoagland solution, panu = pa medium supplemented with 20% needle extract, pcnu = pc medium supplemented with 20 % needle extract , glu = 3% glucose in Hoagland solution. The mycelia were transferred to 40 ml of a needle extract medium. Enzyme activities were measured after culture for 4 days from culture medium supernatants. The values are means of three to four triplicate assays. Bars indicate standard errors of the means.

dry matter production than the sole pectin media or the glucose medium.

The polygalacturonase activity was higher at pH 3.5 than at the pH values 4.5–8.5 (III). The activity decreased sharply between pH 4.5 and 5.4. A temperature of +38°C and a time of 2 h 30 min for the incubation proved to be suitable for the activity measurement.

G. abietina secretes xylanase and can grow in media where xylan is the sole carbon source (IV). Inducibility of xylanase activity was greater in pure xylan media than in media where needle extract had been added. The needle extract, however, increased the mycelial dry matter production. The pH value of 4.5 was optimum for xylanase activity. A temperature of +38°C and 3 hours were chosen for the activity measurement.

4.3 Characterization of the fungus

4.3.1 Differences in polygalacturonase activity between races

The rate of induction of polygalacturonase activity was higher for the European race than for the North American race of *G. abietina,* estimated as activity/mg mycelium (V). The estimated polygalacturonic acid hydrolyzing activity mean was higher (47.4 nmol/mg mycelial dry weight) in the European race than in the North American race (29.4 nmol/mg), $p<0.001$ when testing if this difference is zero. The dry weight production was smaller in the European race (86.9 mg) than in the North American race (115.1 mg) ($p<0.001$). The differences between races were of the same magnitude as the variation between isolates within races. Variance components related to experimental errors (variance of experiment, flask and measurement effects, see Fig. 2 in V) were quite large. The method used for activity analysis in known to be susceptible to variation (BAILEY et al. 1992), but because the differences were also found in dry weight, the variability also indicated sensitivity of fungal metabolism to some unidentified factors. There was no correlation between the amount of activity and dry matter production within the race. The interaction experiment x isolate occurred in dry weight but not in activity.
B-type had a higher mycelial dry matter yield (86.3 mg) than A-type (73.3 mg) ($p<0.039$) in liquid pectin media. There was no difference in the amount of secreted polygalacturonase activity between the A- and B-type (61 and 63 nmol/mg).

4.3.2 Reliability of protein pattern and immunoblotting

Endogenous protease activity of *G. abietina* was minimal (VI). No evidence of endogenous protease activity was detected in two-week-old cultures of two isolates at pH 6 and 7.4 after incubations. The two other isolates showed a low protease activity; after 18 h incubation, some bands in the protein pattern had lost intensity and some smaller molecular weight bands gained intensity. Endogenous protease activity was detected in old cultures: lots of small molecular weight proteins were present in SDS-PAGE, while proteins of large molecular weight were scarcely seen.

No exogenous protease activity was detected in this study. In the study of the protease activity, the marker proteins were not degraded, and not even the medium of a 42-day-old culture (one isolate) as enzyme preparation degraded the protein markers during incubation.

Mycelial protease activity was found in samples of one isolate after incubation. A somewhat different protein pattern was observed with the inhibitors used (pepstatin, PCMB, EDTA and o-phenanatriline) suggesting the presence of different proteolytic enzymes. The isolate used did not show clear evidence of serine protease (inhibited by PMSF), but did show evidence of metalloprotease (inhibited by EDTA and o-phenantriline) and small amounts of aspartate- and cysteine-class

proteases (inhibited by pepstatin and PCMB, respectively). The presence of metalloproteases was confirmed by the addition of divalent cations. Zinc and calcium markedly activated proteolysis.

In immunoblotting, polyclonal antibodies detected proteins in all isolates (VI). A 28 kD band was particularly strong in two of the isolates. With control serum (serum obtained before immunisation), there were no bands in immunoblotting. Perchloric acid precipitation had no effect on the sharpness of protein bands in SDS-PAGE, and the antigen pattern of treated samples was comparable to those of the untreated ones.

5. DISCUSSION AND CONCLUSIONS

5.1 Infection: Environmental factors

G. abietina inoculations performed in the first half of the growing season caused more disease than inoculation performed in the second half of the season (I). Obviously, plant tissues in the young and unhardened phase in the first half of the season are easier to colonize by the fungus, e. g., by the secreted fungal enzymes. Young tissues are less lignified than older ones (VANCE et al. 1980) and are perhaps therefore less resistant. There are conflicting results (BAZZIGHER et al. 1986, AITKEN 1993) on the effectiveness of the autumn inoculation of two-year-old or older seedlings, which might be due to the different environmental conditions, e. g., different moisture conditions, after the inoculation. In our studies, we ensured that the seedlings' surface remained moist for two days after inoculation, and the growing seasons in the growth chamber were planned to simulate a rainy and cool growing season, so the results are linked to these conditions.

The low disease-causing effect of the late inoculations might be partly due to the short time the inoculum had to affect the host before winter. However, the most likely reason for this is the decreasing susceptibility of the seedlings in late summer. For instance, inoculation in the autumn on cold-stressed seedlings (cold increased susceptibility, as will be discussed later) led to the same extent of disease as had spring inoculations on unstressed seedlings.

According to HAMNEDE (1980) and to the results of our unpublished studies, the relation of the disease occurrence to the inoculation time is different in first-year seedlings from that in older ones. The later inoculations seem to be more effective in first-year seedlings than in the older ones. The reason for this could be that the first-year seedlings remain in an active growing state until the autumn, and susceptible young tissues therefore exist also in late summer. The existence of buds could be one further reason, because it has been found in older seedlings that the bracts and bud scales are the first to be colonized by the fungus (PATTON et al. 1984,

LANG and SCHÜTT 1974, SIEPMANN 1976) and in first year seedlings, unlike in older seedlings, buds do not develop until late summer.

Cold stress performed in the autumn increased the effectiveness of autumn inoculations more than cold stress performed in the spring increased that of the spring inoculations (I, PETÄISTÖ and REPO 1988). This might be so because seedlings after autumn cold stress have a shorter time to recover from cold injuries than do the seedlings after spring cold stress, and healing is slow in autumn. In addition the lower effectiveness of spring cold treatments on the occurrence of the disease might be partly explained by the fact that inoculations in spring were so successful even in unstressed seedlings that they could not be more successful due to cold stress. Also, according to YOKOTA (1975), frost in September and October could predispose trees to *G. abietina* infection.

Drought stress (transplanting) in autumn had no effect on the success of the subsequent inoculation but increased the frost hardiness. While the seedlings in autumn were highly resistant to the fungal infection, the tested drought stress would have had to have a very strong predisposing effect in order to become evident. Additionally, the increased frost hardiness, due to stress, could decrease susceptibility to disease because characters of frost hardiness (early growth cessation and high level of dry matter contents) of pine have been found to correlate positively with resistance of pine to this disease (DIETRICHSON 1968). To determine the role of drought in this host-pathogen interaction, more data are needed, e.g., about the effect of drought at different times during the growing season and the effect of drought on the success of the infection the following year.

As far as temperature is concerned, *G. abietina* can grow during winter. *G. abietina* spores germinated at the same rate at 0°C as at higher temperatures, and the germtubes were able to grow at 0°C (II). The fungus grows even at temperatures below 0°C (ETTLINGER 1945, SLETTEN 1971).

In winter, a mycelial inoculum (II) caused clear necrosis in the seedlings at low temperatures (the tested temperatures were from -4°C to +2°C). Very heavy damages in the spring might be explained by that conditions during the summer had favoured the fungal progress so much that penetration to the living tissue of the host shoot had happened before winter, and the following mild winter or thick snow cover permitted the progress of the damage during the winter. The length of necrosis depends strictly on the temperature (II). We did not study whether the length of the necrosis was the same as the extent of the fungal growth. The exoenzymes of the fungus might have caused necrosis also further off the hypha.

It is much warmer under snow than above, and the temperature during winter can be close to 0°C most of the time (FRANSSILA 1949, I), and snow cover has been found to favour disease outbreak (YOKOTA 1975, DORWORTH 1972). Also, MAROSY et al. (1989) underlined the importance of winter temperatures for the success of infection by conidia: if the temperatures varied for more than 43 days within the range of -6°C to +5°C, the disease broke out in the following spring. The North

American race and the B-type in Scandinavia, which caused symptoms especially on the lower branches of the host tree or on seedlings, might need different winter temperatures or humidity (snow cover) compared to the European race in North America and the A-type in Scandinavia, which cause symptoms also on higher branches of the host.

The strong effect of inoculation performed in the first half of the growing season and the disease predisposing effect of frost are useful facts to know in the further experimental work concerning, e. g., the infection process and control of *G. abietina*. Further, it has been shown that the chemical control of this disease in the spring is very necessary for seedlings which are older than one season. In the autumn, care in control is especially needed if frost has occurred. In nurseries, the disease can be expected to occur more frequently after mild winters than after cold ones.

5.2 Infection: Cell wall degrading enzyme activities characterization of the fungus

5.2.1 Infection: Enzyme activities

Pectic enzymes may play a role in many plant diseases (COOPER 1983, BATEMAN and BASHAM 1976). Recent studies with enzyme-deficient mutants have, however, shown cases where pectinases are not important in pathogenicity, e. g., endopolygalacturonase may not be required for pathogenicity in the *Cochliobolus carbonum* fungus and maize interaction (SCOTT-CRAIG et al. 1990), and in the case of *Verticillium albo-atrum* in tomato plants the pectinases are not the determinants of pathogenicity, although they are virulence factors (DURRAND and COOPER 1988).

Pectin or xylan as the only carbon source supported essential growth of *G. abietina* (III, IV). Pectin degrading enzyme activity in *G. abietina* has also been documented earlier (HAANSUU 1992). Pectin induced the activity of polygalacturonase *in vitro* (IV). The pectin in plant tissue could induce *in vivo* the polygalacturonic acid hydrolyzing activity in *G. abietina*. In conifers, especially, the middle lamella and primary cell wall contain pectin. The developing new tissue contains relatively more pectin and it might be that new host tissue induces more polygalacturonase activity than older tissue.

There were similarities in the polygalacturonase and xylanase activities of *G. abietina*. The induction of polygalacturonase activity was stronger in mere pectin medium and of xylanase activity in mere xylan medium than in media where needle extract had been added. Pine needles may contain polygalacturonase/ xylanase enzyme activity inhibitory compounds, e. g., some of the phenols and resin acids (TOBOLSKI and ZINKEL 1982, HATCHER 1990). The needle extract seems to contain components that can be degraded by the fungus more easily than pectin or

xylan, since the dry matter production was higher in media with needle extract. The activity level of both polygalacturonase and xylanase was low, and in analysis a long incubation time is needed for *G. abietina* compared to many other fungi, e. g., in the xylanase activity of *Trichoderma* (POUTANEN and PULS 1988). The low enzyme activity of *G. abietina* fits the slow growth of this fungus and the slow progress of the disease.

The role of xylanase may be more essential in, for example, wood decay (BLANCHETTE et al. 1989) than in the disease caused by *G. abietina. G. abietina* caused damage to its host mainly under the periderm in the phelloderm and phloem, and not in the woody part rich in xylan. However, this does not prove that xylanase has no role in the pathogenesis of the fungus. Enzyme systems are complicated, and xylanase, for instance, has been found to induce ethylene biosynthesis in a plant (DEAN et al.1989). Xylanase excretion was induced by xylan *in vitro* and it is also possible that small amounts of xylan in the host cell wall induce xylanase production of *G. abietina in planta*.

The possibility of purifying the polygalacturonase and xylanase and to derive antibodies against them makes it possible to study the role of these enzymes in the disease using immunoenzymological methods. Indirect immunogold-labeling of lignin peroxidase and xylanase has been used to examine the role of the enzymes in the decay process of wood (BLANCHETTE et al. 1989). Forming enzyme deficient mutants of *G. abietina* would be useful in pathogenicity research.

5.2.2 Characterization of the fungus

The European race produced more polygalacturonase activity/mycelial dry weight than the North American race *in vitro* (V). The activity differences between the races were of the same order of magnitude as the variation between isolates within races. The variance components related to experimental errors were quite large. In North America, the European race was found to be more virulent (SKILLING 1977). It would be interesting to know whether the production of polygalacturonase activity is one reason for this. However, the results between races were from the *in vitro* experiments, and further research is needed to determine the situation in natural conditions.

Despite the lower amount in polygalacturonase activity, the highest mycelial dry matter production in pectin medium was found in the North American race. Obviously, the other pectin degrading enzymes (e. g. pectin methylesterases, pectin lyases (HAANSUU 1992)) supported more growth in the North American race. As there was experiment x isolate interaction in dry weight but not in activity, the other pectin degrading enzyme activities might be more sensitive than polygalacturonase activity to unidentified variation between experiments. The amount of dry weight did not explain the amount of activity announced/mg mycelial dry weight, since inside the races there was no correlation between activity and dry weight.

It has been found that pectic enzyme patterns distinguished the North American and European races on the basis of one cathodal polygalacturonase band present in the former but absent in the latter (LECOURS et al. 1994). The finding in the present study that there are differences in pectin degradation between the races corroborates the results of LECOURS et al.

The B-type grew better in pectin media than the A-type (V). According to UOTILA (1983), the A-type grows better (measured as colony diameter) *in vitro* in solid media at +15°C. This may suggest the existence of metabolic differences between the types, resulting in a better growth in the A- or B-type depending on the state or contents of the medium, or else the colony diameter did not represent the real growth, e. g., due to the different density of mycelia. We found no difference in the secretion of polygalacturonase activity *in vitro* between the A- and B-type isolates studied but as there was only a small number of isolates in this preliminary study, further studies are needed. LECOURS et al. (1994) also failed to find differences between the Finnish isolates (B-type) and other European isolates of *G. abietina var abietina* in pectic enzyme patterns, but they had only a small number of Finnish isolates in their studies.

The native PAGE was used in taxonomic studies of *G. abietina* (BENHAMOU et al. 1984, PETRINI et al. 1989, 1990). The protein pattern derived by SDS-PAGE could be one tool for comparing isolates of *G. abietina*. SDS-PAGE also permits the use of the Western blot method. The protease activity of the fungus must be prevented in order to acquire a reliable protein pattern comparison. When the developed sample preparation method and young, two-week-old cultures were used, the protease activity was lacking or minimal (VI). There are different intracellular proteases, e. g., metalloproteases that were activated by divalent cations zinc and calcium. The found metalloproteases can be inhibited by EDTA and o-phenanthroline.

No exocellular proteases were found (VI). GRIFFIN (1994) supposed that the reputed absence of exocellular proteases could many times be due to the fact that the carbon, nitrogen and sulphur sources in the culture medium repressed the protease synthesis. In our study in V8 medium, the added glucose (2%) or some other compounds might have decreased the exocellular protease synthesis. HAANSUU (1992) found extracellular proteases of *G. abietina* using substrate gel electrophoresis and concentrated culture media. In our study, the exocellular protease activity in the culture medium was obviously so low that it did not come out by the method we used. However, the medium used suits for the purpose of obtaining reliable protein patterns.

Protease activity was absent or very low in mycelial samples from two-week-old cultures made as describe above, but in older cultures protease was found to be present. Excreted protease was not found in used culture media not even in media of old cultures. Protease inhibitors were not necessary to obtain protein pattern from two-week-old cultures if they were processed with care, but to avoid the minimal

possibility of protease activity, inhibitors should be used.

The polyclonal antibodies produced against mycelial homogenate in rabbits can be used for further studies to compare isolates (VI). Depending on the use to be made of the polyclonal antobodies, it might be useful to test on larger scale the possible crossreactivity with proteins of other fungi. Earlier serological tests were used to identify the three races of *G. abietina*, the North American, European and Asian (DORWORTH and KRYWIENCZYK 1975). It is possible to identify the variation inside the races of *G. abietina* immunologically (PETÄISTÖ et al., submitted).

With the present information about the variation among *G. abietina* in North America and in Europe obtained with DNA markers (HAMELIN et al. 1993, 1996, HELLGREN and HÖGBERG 1995) there is need for other methods to identify the variation. The characterization of the types is needed to understand the different frequency of types in various environmental conditions etc. The studied protein pattern analysis with SDS-PAGE and immunoblotting has provided tools for identification. The study on the variation of polygalacturonase activity between races and types suggests further studies of *G. abietina* pathogenicity mechanism.

In conclusion, knowledge about the environmental factors that favor *G. abietina* infection has increased during the decades that the disease has been studied. This investigation has shown that the growth phase of the host and cold stress are important factors in infection. It is known that cell wall degrading enzymes and toxins excreted by pathogens can be important in the infection process. In this investigation, the xylanase and polygalacturonase production of the fungus was studied and the European and North American races and A- and B-types of *G. abietina* were characterized by comparing their ability to degrade pectin *in vitro*. The races and types are known to have differences in pathogenicity, which is one reason why methods for the identification of the races and types are needed. The reliable protein pattern together with immunoblotting is one tool which can be used for such identification.

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The susceptibility of Scots pine seedlings to Gremmeniella abietina: effect of growth phase, cold and drought stress

By RAIJA-LIISA PETÄISTÖ and T. KURKELA

Abstract

The effect of the growth phase of pine seedlings on the success of *Gremmeniella* infection was studied in both simulated and natural growing seasons. Also the effect of cold and drought stress on the success of the infection was studied.

Key words: *Gremmeniella – Pinus sylvestris* – Growth phase – Cold stress – Drought stress.

1 Introduction

Scleroderris canker caused by *Gremmeniella abietina* (Lagerb.) Morelet has damaged conifers both in forests and nurseries in northern Europe, Asia and North America (KOHH 1964; KURKELA 1967; TEICH 1972; KALLIO et al. 1985).

The conidia of *G. abietina* disseminate mainly in the spring and the ascospores later in the summer. The dissemination time of these two spore forms together cover the entire summer time to late autumn (SKILLING 1969; NEVALAINEN 1985).

There are many studies on environmental factors which favour disease occurrence (SLETTEN 1971; BLENIS et al. 1984; MAROSY et al. 1989). However, few studies deal with the annual rhythm of the host in connection with infection (BARKLUND and UNESTAM 1988; YOKOTA et al. 1974; KURKELA and NOROKORPI 1979). Usually the infection occurs during the growing season and the symptoms do not become visible until the beginning of the next growing season.

The aim of this study was to investigate infection on Scots pine caused by conidia of *G. abietina* during the physiologically different periods of the seedlings' annual development in simulated and natural growing season.

2 Material and methods

2.1 Conditions during simulated growing seasons

Experiment 1

One-year-old containerized Scots pine seedlings (of central Finnish origin) were taken into a dark storage room with the temperature +1 °C in the spring as snow was melting. When the frozen soil in the pots had melted, the seedlings were placed in a room where light was adjusted to 85 μmol $m^{-2}s^{-1}$. The daylength was adjusted to correspond to the natural daylength every 1–2 weeks (Fig. 1). The temperature was adjusted at different levels during the experiment (Fig. 1). The relative humidity was about 95 %. The temperature sum (threshold temperature 5 °C) reached 928 d.d. at the end of the growing season (duration 185 days). During the winter the test seedlings were kept at 0 °C to +1 °C and at

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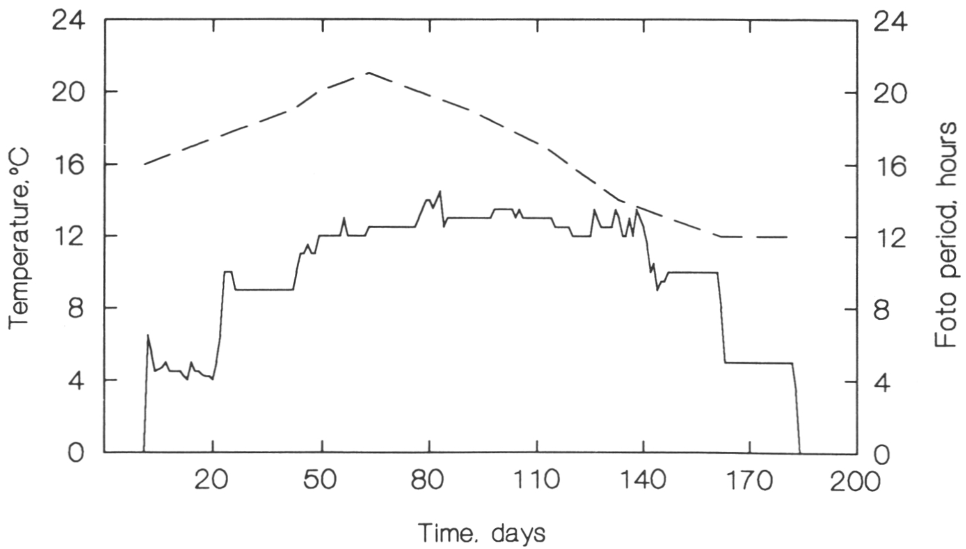

Fig. 1. The temperature (——) and the photoperiod (– – –) during the growing season in Experiment 1

a relative humidity of 95 %–100 %. The cold hardiness of the seedlings was tested fourteen times during the growing season and in the beginning winter using a method based on the difference in the impedance of the shoots before and after frost treatment. Ten seedlings were used in each six predetermined temperatures in each test (Repo and Pelkonen 1986; Petäistö and Repo 1988). The cold hardiness decreased during 80 days from the beginning of the experiment, when the temperature increased from 0 °C to about 12 °C and had remained about 12 °C for 30 days (Fig. 5). The sharpest increase took place when the day length was about 14 hours and the temperature began to drop. The length of the new shoot was measured from 8 seedlings weekly.

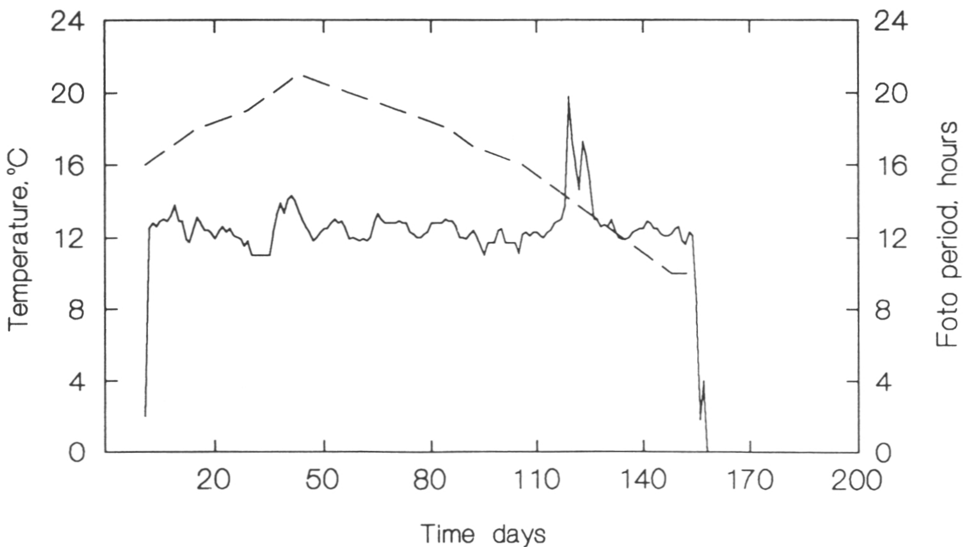

Fig. 2. The temperature (——) and the photoperiod (– – –) during the growing season in Experiment 2

Experiment 2

Two-year-old containerized Scots pine seedlings (of central Finnish origin) were taken into a dark room with the temperature 1 °C in the spring as snow was melting. When frozen soil in the pots had melted, the temperature was adjusted to 12.5 °C. The light period (intensity 90–100 μmol $m^{-2}s^{-1}$) was adjusted as in the previous experiment (Fig. 2). The temperature conditions during the experiment varied as shown in Figure 2. The temperature sum of the whole season was 1164 d.d. (duration 120 days). The cold hardiness was tested three times at the beginning of the growing season during two weeks and at the end four times during six weeks. The growth of the seedlings was measured weekly from 6 seedlings until growth ceased.

2.2 Conditions during outdoor experiments

Experiment 3 (1989) and 4 (1990)

One-year-old containerized Scots pine seedlings (of central Finnish origin) were brought to the experimental field at the end of April. The temperature conditions during the experiments are shown in Figure 3 and Figure 4. The temperature sums at the end of the experimental seasons reached 1230 d.d (Experiment 3) and 996 d.d. (Experiment 4), the duration of the growing seasons was correspondingly 155 and 140 days. During the following winters the lowest temperatures (measured under snow on the soil surface) were −15 °C and −20 °C, but most of the time the temperature varied between 0 °C and −5 °C. The growth of the new shoot of 15 seedlings and the growth of two needle pairs of 13 seedlings were measured weekly in Experiment 3. Similarly, in Experiment 4 the growth of the shoot of 50 seedlings and the growth of two needle pairs of 50 seedlings were measured.

In all the experiments the seedlings were fertilized ten times during the growing season. Each seedling was given total 16 mg N, 4.4 mg P, 16.5 mg K and micronutrients.

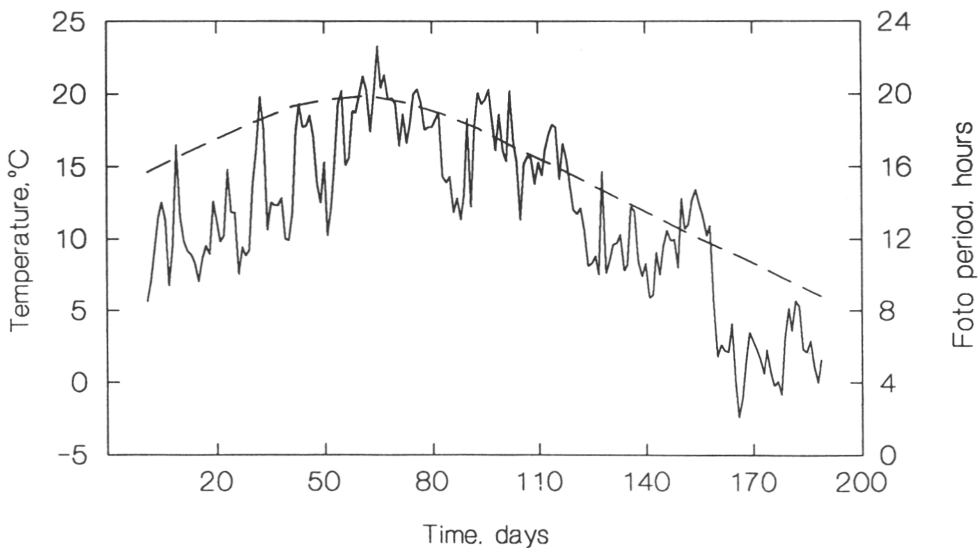

Fig. 3. The temperature (——) and the photoperiod (– – –) during the growing season in Experiment 3

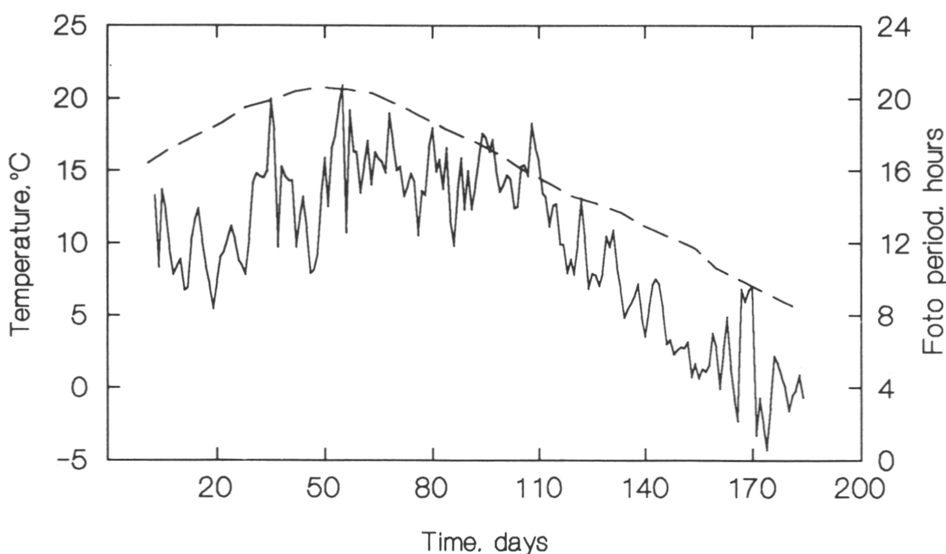

Fig. 4. The temperature (——) and the photoperiod (– – –) during the growing season in Experiment 4

2.3 Inoculation and examination of the seedlings

The seedlings were inoculated with conidia of *G. abietina*. Two (Experiment 2) to five (Experiment 1) isolates were used, which mainly originated from northern parts of Finland. In Experiment 3, one from the three isolates originated from southern Norway and one from central Finland. The conidia were produced on solid media of barley corn and Scots pine needle homogenate (10 g barley corn, 2.2 g needle homogenate, 20 ml H_2O, autoclaved at 120 °C for 20 min twice at one day interval). The amount of conidial suspension (3.2×10^6 conidia/ml) used was 0.27 ml/seedling ($= 0.86 \times 10^6$ conidia). The suspension was dropped on the terminal bud of the seedlings. After the inoculation the seedlings were kept moist for two days using a water sprinkler.

In Experiment 1, 15 seedlings were inoculated at each of the 32 inoculation times. For control we had 60 seedlings. In Experiment 2 and 3, 30 seedlings were inoculated at each inoculation time, in all 13 and 11 times correspondingly. For control we used 60 seedlings. In Experiment 4, 50 seedlings were inoculated at each of the 21 inoculation times. Eighty seedlings were used as controls.

After winter, when the new shoots had begun to grow, the seedlings were examined and classified to three classes: healthy, weak and diseased. The seedlings were classified 'diseased', if the base of the needles was brown and the bud was not elongated. The class 'weakened' meant seedlings whose colour was not fresh green and whose growth was poor. The seedlings were moistened after the examination and put into plastic bags and incubated at 16 °C. After two or three weeks the shoots were examined for the development of *Gremmeniella* conidial masses and mycelial tufts (cf. HUDLER et al. 1983). If conidial masses and mycelial tufts were found on the seedlings classified as another than the 'diseased'-class, they were included in the number of the diseased seedlings.

2.4 Cold and drought stress

The effect of cold stress on the development of the disease was studied in the Experiments 1 and 4. The cold treatment was applied in the spring when the number of degree days was about 70 and in the autumn when the number of degree days was 70 (Experiment 1) or 100 (Experiment 4) less than the total temperature sum of the season.

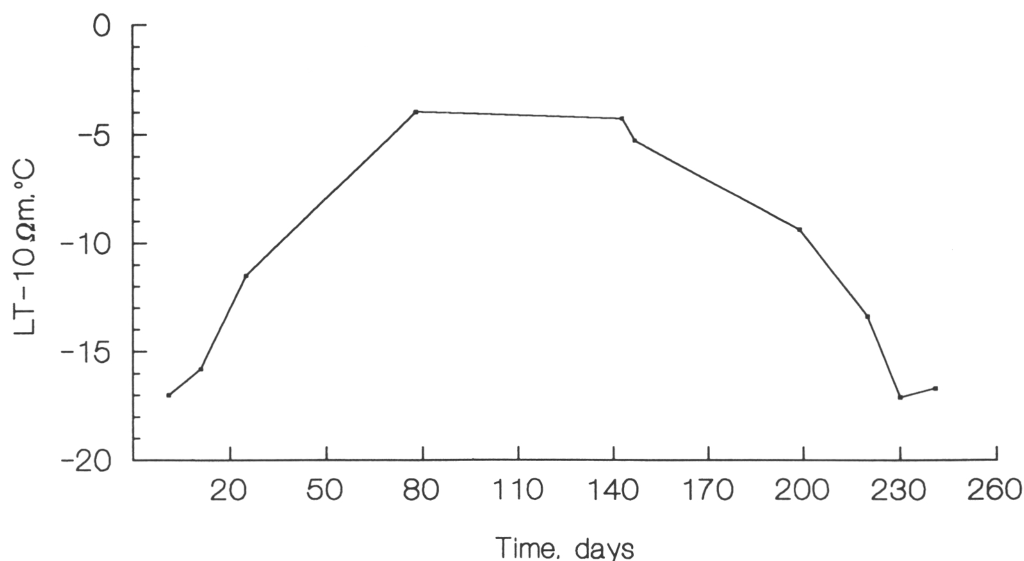

Fig. 5. The frost hardiness of the seedlings in Experiment 1 described as $LT_{-10\Omega m}$, °C. The x-axis is the same as in Figure 1

The cold hardiness of the seedlings was first estimated with the impedance method using seedlings reserved for this purpose. The temperature for estimation was chosen on the basis of the results of 6 different test temperatures, ten seedlings at each temperature (Repo and Pelkonen 1986). The chosen stress temperatures, in Experiment 1 in the spring −5 °C and in the autumn −5 °C, in Experiment 4 correspondingly −5 °C and −4 °C. The stresses were weak and the seedlings had no visible signs of stress. One group of seedlings was inoculated before and another after the cold stress. There was also a group of seedlings stressed but not inoculated and a group of seedlings inoculated but not stressed. In Experiment 1, each group consisted of 15 seedlings; in Experiment 4, of 50 seedlings.

The effect of drought on disease development and on cold hardiness was tested in Experiment 1. The seedlings were stressed at the end of the growing season, when the temperature was 10 °C, 5 °C and 0 °C (different seedlings every time). The seedlings were removed from the container and soil was quickly washed from the roots. The remaining water was wiped off with absorbent paper towels. Each time a new group of seedlings was weighed and laid for 15 min at 32 °C in the light. Then they were weighed again and transplanted in moist peat growth media. Fifteen out of a total of 30 seedlings were inoculated with conidia each time. There were also control seedlings withouth drought stress, either to be inoculated or not.

In the test for cold hardiness, seedlings with or without drought stress were used. In this experiment all inoculations were performed in the autumn, when the temperature had been kept at 0 °C for three weeks.

The beginning of root growth in the following spring was followed by observing the occurrence of white root tips. If the new growth was 0.5 cm or longer in four or more root tips, the root system was classified as 'good', if the new growth was shorter than 0.5 cm, or longer than 0.5 cm, but in fewer than 4 root tips, the root system was classified as 'weak'. The third class was 'no white root tips'.

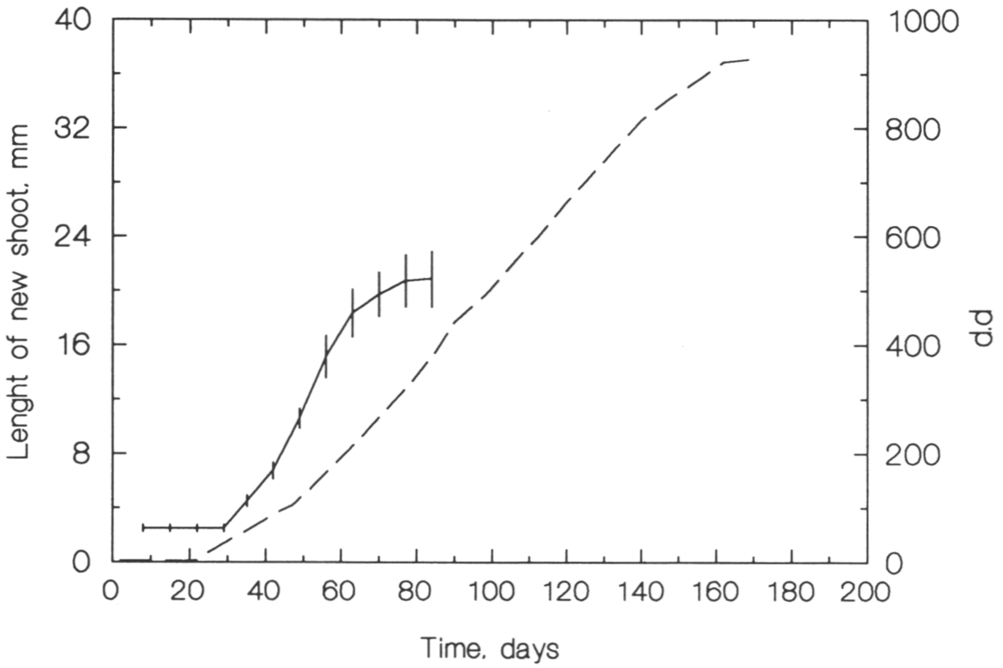

Fig. 6. The mean length of the new shoots (——) of the seedlings in Experiment 1 and the d.d. (– – –) of the growing season, x-axis is the same as in Figure 1. Bars indicate standard errors of the means

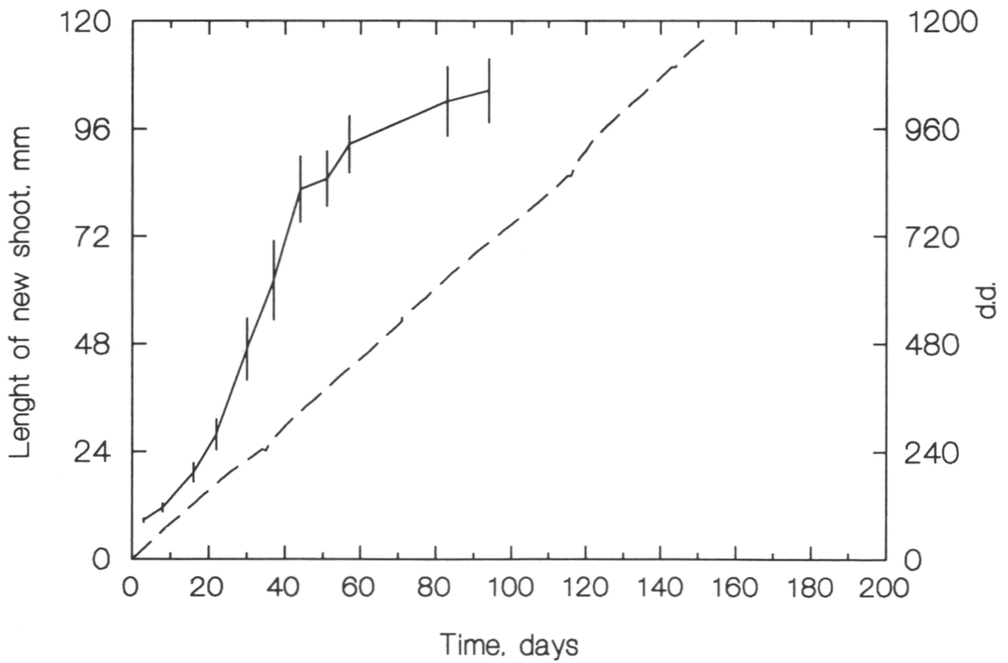

Fig. 7. The mean length of the new shoots (——) of the seedlings in Experiment 2 and the d.d. (– – –) and the time point of the growing season, x-axis is the same as in Figure 2. Bars indicate standard errors of the means

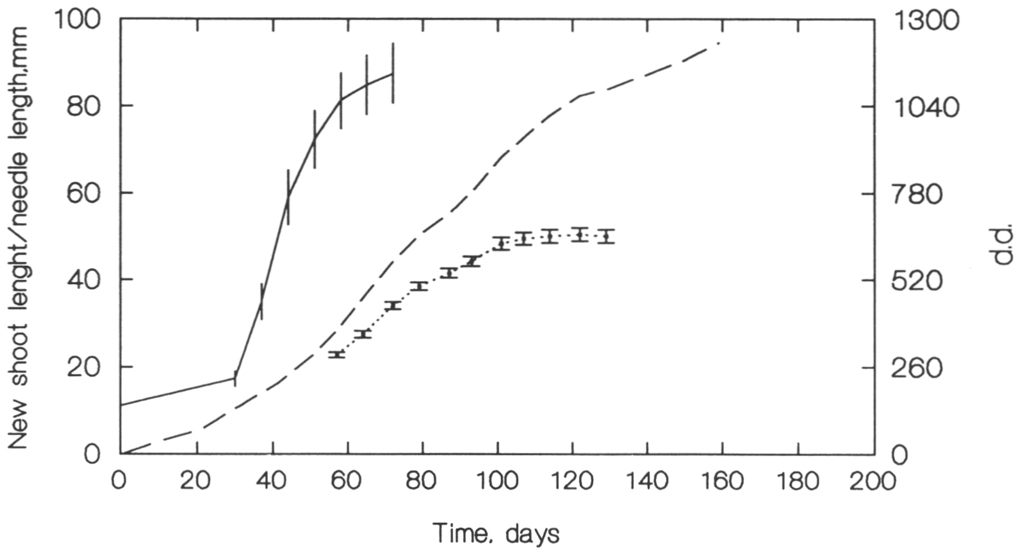

Fig. 8. The mean length of the new shoots (——) and needles (. . . .) of the seedlings in Experiment 3 and the d.d. (– – –) and the time point of the growing season, x-axis is the same as in Figure 3. Bars indicate standard errors of the means

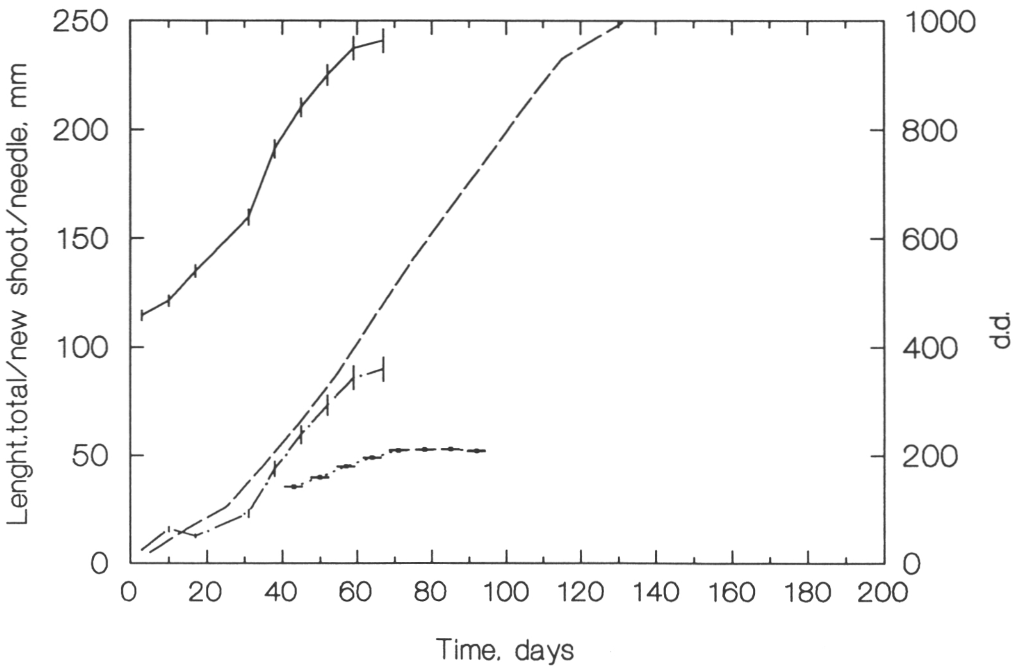

Fig. 9. The mean length of the seedlings (——) new shoots (–.–.–) and needles (. . . .) of the seedlings in Experiment 4 and the d.d. (– – –) and the time point of the growing season, x-axis is the same as in Figure 4. Bars indicate standard errors of the means

3 Results

The new shoot growth of the seedlings ended in Experiment 1 when the temperature sum was at about 350 d.d. (total 928 d.d.), in Experiment 2 at about 430 d.d. (total 1164 d.d.) and in Experiments 3 and 4 at about 550 d.d. (total 1230 d.d.) and 400 d.d. (total 996 d.d.) (Figs. 6, 7, 8, 9).

Both in simulated and natural growing seasons the number of diseased seedlings was higher when they were inoculated during the first half of the growing season (Figs. 10, 11, 12, 13; Table 1). In Experiment 1, the 16 inoculations in the spring caused successful infections: approximately 51.2% of the seedlings became diseased (Fig. 10; Table 1). The 16 inoculations in the autumn caused disease only in 0.4% of the seedlings. In Experiment 2 the inoculations during the first half of the growing season caused disease also more

Table 1. **Percentages of diseased (diseased and dead) seedlings**

	Experiment 1		Experiment 2		Experiment 3		Experiment 4	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
A	51.2	(5.40)	66.4	(11.47)	88.7	(6.27)	75.4	(6.74)
B	0.4	(0.42)	17.3	(6.86)	13.8	(1.99)	27.0	(5.81)
C	0.0		8.3		7.8		16.2	

(A) inoculated during first and (B) second half of the growing season and (C) percentage of diseased seedlings in controls in Experiments 1, 2, 3, and 4. Standard error of the means in parenthesis

Table 2. **The effect of cold stress in the spring (A) and in the fall (B) on the disease occurrence in the seedlings in the Experiment 1 and 4**

	Experiment 1		Experiment 4	
	Number of seedlings	% diseased	Number of seedlings	% diseased
A)				
Not inoculated				
– without cold stress	60	0	74	16.2
– with cold stress	15	0	49	18.4
Inoculated				
– without cold stress	240 (15)	52.8 (80.0)	509 (44)	74.5 (68.2)
– before cold stress	15	53.3	49	89.8
– after cold stress	15	46.6	–	–
– in the fall following the cold stress	15	20.0	47	55.3
B)				
Not inoculated				
– without cold stress	60	0.0	74	16.2
– with cold stress	15	6.7	50	20.0
Inoculated				
– without cold stress	240 (15)	0.4 (0.0)	501 (50)	27.2 (36.0)
– before cold stress	15	60.0	49	85.7
– after cold stress	15	26.7	–	–
– in the spring preceding the cold stress	15	80.0	46	82.6

In group 'inoculated, withouth stress' the numbers in parentheses are the result of the inoculation made exactly at the same time as the stress, numbers without parentheses are the result of all the inoculations in the same half of the growing season (see Table 1)

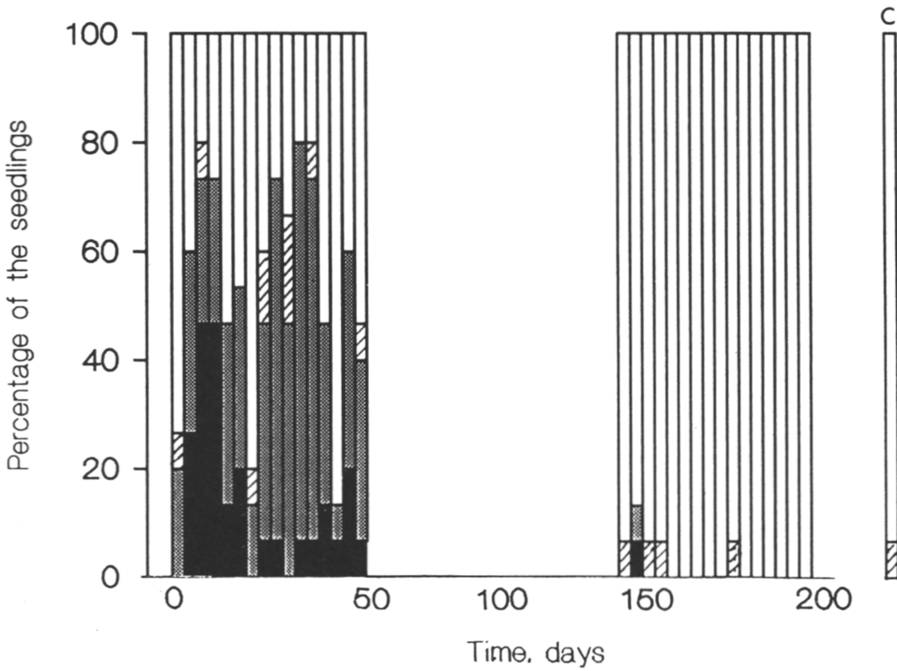

Fig. 10. Experiment 1. The condition of the inoculated and control seedlings in the spring following the experiment summer. The percent of dead ■, diseased ▩, weakened ▨ and healthy □ seedlings. The x-axis described the time point inoculation performed in the previous summer and is the same as in Figure 1. C = Control

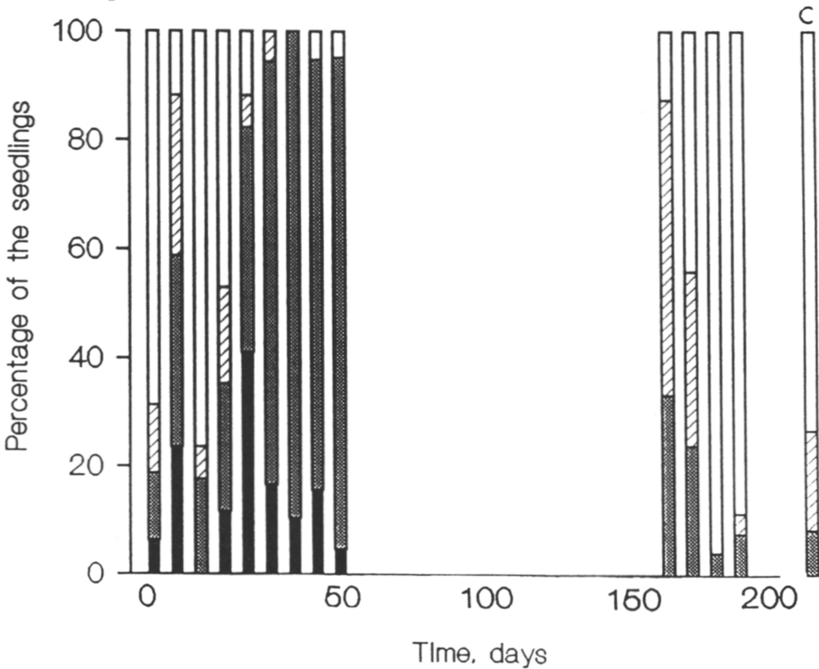

Fig. 11. Experiment 2. The condition of the inoculated and control seedlings in the spring following the experiment summer. The percent of dead ■, diseased ▩, weakened ▨ and healthy □ seedlings. The x-axis described the time point inoculation performed in the previous summer and is the same as in Figure 2. C = Control

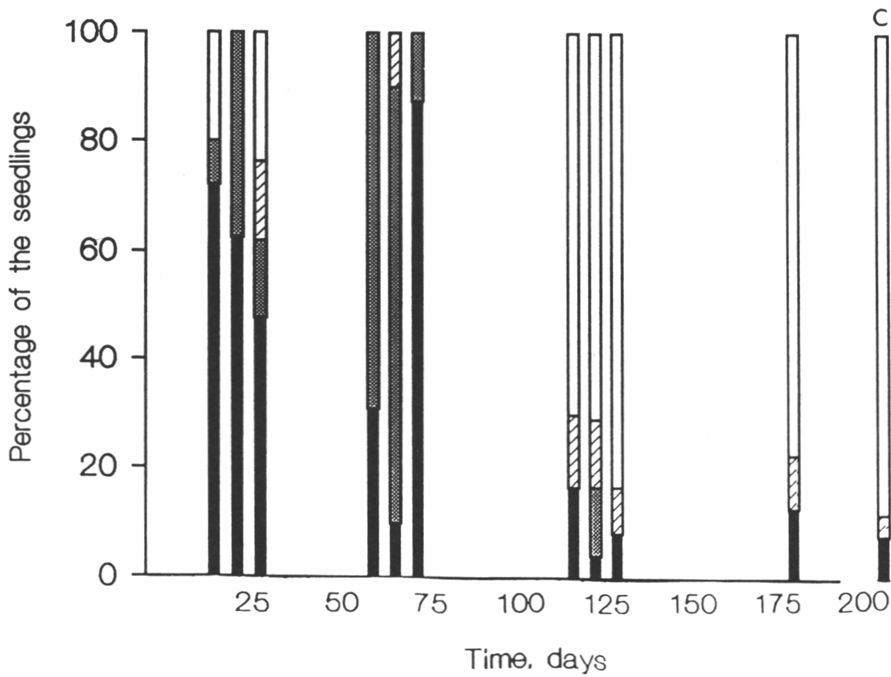

Fig. 12. Experiment 3. The condition of the inoculated and control seedlings in the spring following the experiment summer. The percent of dead ■, diseased ▩, weakened ▨ and healthy □ seedlings. The x-axis described the time point inoculation performed in the previous summer and is the same as in Figure 3. C = Control

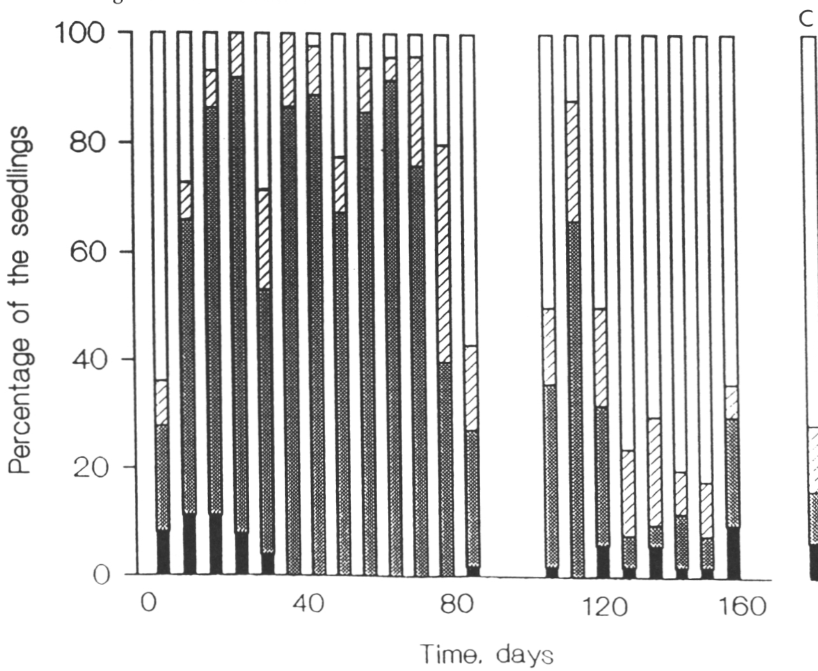

Fig. 13. Experiment 4. The condition of the inoculated and control seedlings in the spring following the experiment summer. The percent of dead ■, diseased ▩, weakened ▨ and healthy □ seedlings. The x-axis described the time point inoculation performed in the previous summer and is the same as in Figure 4. C = Control

(66.4 % of the seedlings) than the inoculations during the second half (Fig. 11; Table 1). The situation was the same in Experiments 3 and 4 (Figs. 12, 13 and Table 1).

According to the experiments the time of frost was important. Mild frost later in the summer had a clear effect. Inoculation combined with cold stress caused disease in Experiment 1, in 27–60 % of the seedlings and without stress in 0–0.4 %, while the cold stress in the spring did not increase the number of diseased seedlings when inoculation was performed in the beginning of summer. Inoculation with stress and without stress in the spring caused approximately the same amount of successful infections (Table 2). The situation was the same in Experiment 4: inoculation in the autumn combined with stress caused disease in about 86 % but without stress in 27–36 % of the seedlings.

In our experiment drought stress in the autumn (the weight loss of the seedlings was 16–18 %) did not increase the influence the *G. abietina*: when inoculations were made late in the autumn, there were no or extremely few seedlings diseased (Table 3). Thereafter stress increased the cold hardiness. The seedlings without drought stress had the cold hardiness −9.3 °C, but the seedlings that got stress earliest had cold hardiness of −11.5 °C and the seedlings that received the second and the third stress treatment had the cold hardiness of −15.2 °C and −15.0 °C. Drought stress markedly decreased root growth in the following spring, the first stress to a lesser extent than the others, new root formation was good in 80 % of the control seedlings, in 33.3 % of the seedlings with the earliest drought stress and in 6.7 % of the seedlings with the later drought stresses (Table 3).

Table 3. **Effect of drought stresses on root growth and shoot condition in the seedlings in the following spring**

	New root formation			Shoot condition		
	Good	Weak	None	Healthy	Weak	Diseased
	% of the seedlings					
No stress,						
– inoculated	86.7	13.3	0.0	100.0	0.0	0.0
– not inoculated	80.0	20.0	0.0	100.0	0.0	0.0
Stress, on the 152nd day						
– inoculated	13.3	13.3	73.3	66.7	33.3	0.0
– not inoculated	33.3	20.0	46.7	100.0	0.0	0.0
Stress, on the 171st day						
– inoculated	0.0	13.3	86.7	66.7	26.7	6.7
– not inoculated	6.7	26.7	66.7	73.3	20.0	6.7
Stress, on the 189th day						
– inoculated	0.0	20.0	80.0	80.0	20.0	0.0
– not inoculated	6.7	6.7	86.7	73.3	20.0	6.7

Stresses were conducted at different temperatures according to the growing season simulated in Experiment 1, on the 152nd, 171st and 189th day from the beginning of the experiment, but inoculated at the same time in the fall. Each percentage is calculated from 15 seedlings

4 Discussion

4.1 The experimental conditions

During the growing seasons simulated in growth chambers, the dehardening and hardening processes were like those in natural growing seasons (Glerum 1973; Aronsson and Eliasson 1970). According to Aronsson (1975) the dehardening process depends more on temperature than on the length of the photoperiod while hardening depends more on

the photoperiod. The number of degree days of the simulated growing seasons were similar to the average temperature sum of the area from where the seedlings originated (KOLKKI 1966).

The growth rhythm of the seedlings, timing of shoot elongation, was also comparable to that found in the natural growing seasons. According to KOSKI and SIEVÄNEN (1985) the number of degree days and night length together determined the shoot growth cessation of one-year-old pine seedlings but for two-year-old seedlings only the number of degree days. According to RAULO and LEIKOLA (1974) the growth cessation of 5–15-year-old Scots pines was at 510 d.d.

The temperature 0 °C during simulated winters corresponded to the temperature under the snow cover. The temperature 0 °C during simulated winters (Experiments 1 and 2) was somewhat warmer than the natural winters (Experiments 3 and 4), but also during the natural winters the temperature was most of the time between 0 °C and −5 °C.

The low light intensity in the simulated conditions, 85 μmol $m^{-2}s^{-1}$ and 90–100 μmol $m^{-2}s^{-1}$, made the shoot and needles more slender than those of the seedlings growing outside. The maximum outside light intensity for summer is about 800 Wm^{-2} (PELKONEN 1981), on cloudy days smaller (GATES 1980). This intensity could not be obtained in the growth chambers. The effect of lower light intensity for older seedlings during one growing season was perhaps not serious, because the growth of the seedlings is predominantly predetermined during the previous summer already in 5-year-old seedlings (MIKOLA 1950; LANNER 1976; KANNINEN 1990).

4.2 Results of the inoculations

Both in simulated and natural growing seasons the inoculation appeared to be more infectious when done during the first half of the growing season. This period included the shoot and needle growth phase of the seedlings and cold hardiness was decreasing or smallest. Dead seedlings, where *G. abietina* was not detected after moist incubation at the end of the experiments, were in all probability also diseased, because there were much fewer dead seedlings among the control seedlings.

In the earlier reported inoculation experiments (ROLL-HANSEN 1964; SLETTEN 1971; KURKELA and NOROKORPI 1979), where inoculation was performed by wounding and using mycelium as the inoculum, the host became diseased more often when inoculation was done during the rest period of the host. According to literature, inoculaton made with conidia in the beginning of summer has caused disease (GREMMEN 1968; SKILLING 1972; YOKOTA et al. 1974), but no comparison has been made to inoculations later in the growing season. Also in the autumn, conidial inoculation has caused disease (BAZZIGHER et al. 1986). Usually the reports lack exact data on environmental factors during the experiment. The environmental conditions here were registered and there were inoculations both in the spring and autumn in each of the four experiments.

A cold and frost prone growth site has been reported to make trees susceptible to the disease (YOKOTA et al. 1975; PETÄISTÖ and REPO 1988; READ 1967; SAIRANEN 1990). According to our experiments the time of frost is important. Mild cold stress made in the late summer appeared to increase the susceptibility of the seedlings to the fungus, if inoculation was performed at the same time. Cold stress in the beginning of summer did not increase or increased very little the susceptibility. One reason for the different effect might be that the seedlings have a shorter time for healing from cold damage before the winter after late frost damage.

Drought stress has been found to predispose the host plant to some pathogens (BACHI and PETERSSON 1985; CHOUN 1987). Drought stress in the autumn in our experiment did not make seedlings susceptible to the *G. abietina*. Inoculation was performed late in the autumn after drought stress. Thereafter stress increased cold hardiness. Drought stress

decreased markedly the root growth in the following spring, the first stress a little less than the others. The transplanting (which is partly drought stress) in the autumn affects the dry matter content and root growth of the seedlings depending on the transplanting time (PETÄISTÖ 1989). When these seedlings were inoculated (in the late autumn) disease developed, but at a small rate (PETÄISTÖ, unpublished data).

The dispersion time of *G. abietina* conidia in Finland is not well-known. However, there are results from two years, i.e. 1983 and 1984. In southern Finland NEVALAINEN (1985) observed conidia from the beginning of May to the middle of June or to the latter half of July, the peak being in May and in the beginning of June. Ascospores were observed from the middle of June to the end of October. The most successful inoculation in the experiments was made at the time when main conidial dispersion occurred.

The results of this study could prove useful in improving regimes of chemical control by taking into account the time of growing season and occurrence of frost.

Acknowledgements

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Summary

Susceptibility of Scots pine seedlings to *G. abietina* was studied during different simulated and natural growing seasons. The inoculations made with conidia during the first half of the growing seasons resulted in higher disease occurrence than those made during the second half of the seasons. Cold stress predisposed the seedlings to the disease in late summer. Drought stress in late summer increased cold hardiness but did not affect the susceptibility of the seedlings to the disease infection in the autumn.

Résumé

Sensibilité des semis de Pin sylvestris au Gremmeniella abietina: effet des périodes de croissance, du froid et du stress hydrique

La sensibilité des semis a été étudiée au cours de différentes saisons de végétation, simulées ou naturelles. Les inoculations effectuées avec des conidies pendant la première moitié de la saison de végétation ont abouti à une gravité de maladie plus forte que celles effectuées ultérieurement. Le stress par le froid a prédisposé les semis à la maladie en fin d'été. Le stress hydrique de fin d'été a augmenté la résistance au froid mais n'a pas affecté la sensibilité à l'infection en automne.

Zusammenfassung

Die Anfälligkeit von Pinus sylvestris-Sämlingen gegen Gremmeniella abietina: Einfluß von Wachstumsphase, Kälte- und Trockenstreß

Die Anfälligkeit von *P. sylvestris* gegen *G. abietina* wurde während verschiedener simulierter und natürlicher Wachstumszyklen untersucht. Inokulationen mit Konidien in der ersten Hälfte der Vegetationsperiode führten zu stärkerer Erkrankung als solche in der zweiten Hälfte der Vegetationsperiode. Durch Kältestreß wurden die Pflanzen für eine Infektion im Spätsommer prädisponiert. Trockenstreß im Spätsommer erhöhte zwar die Kälteresistenz, beeinflußte jedoch die Anfälligkeit der Pflanzen für eine Infektion im Herbst nicht.

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Conidial germination and formation of necrosis in pine seedlings by Gremmeniella abietina at low temperatures

By Raija-Liisa Petäistö

Abstract

The germination of conidia of *G. abietina* on water agar reached the same germination percentage at 0 °C as at 5 °C to 12.5 °C, but required more time. The fungus caused necrosis even at −4 °C in pine seedlings.

Key words: *Gremmeniella* – Germination – Necrosis – Temperature.

1 Introduction

Gremmeniella abietina (Lagerberg) Morelet can grow at low temperatures (Ettlinger 1945; Stephan 1970; Sletten 1971; Dorworth and Krywienczyk 1975; Uotila 1983). The symptoms of the disease become visible early in the spring. The fungus can also damage the host under snow cover (Yokota 1975; Yokota et al. 1975; Marosy et al. 1989).

Studies on the dependence of the mycelial growth of *Gremmeniella* on temperature have been carried out on agar plates. Ettlinger (1945) found growth at temperatures below 0°C, even at −5.8°C. Stephan (1970), Dorworth and Krywienczyk (1975) studied growth between 0°C and 25°C and Sletten (1971) reported results of growth between −2°C and 33°C. Yokota et al. (1974) studied the temperature dependence of conidia germination as well as the growth of germtubes. They examined the germination and germtubes on water agar after a 48 hr. incubation, and found approximately the same germination percentage between 0°C and 25°C. It is evident that there are no exact studies on the progress of damage in a living host at temperatures below zero.

In this paper the ability of *G. abietina* to cause damage on Scots pine (*Pinus sylvestris* L.) seedlings at low temperatures is reported. Conidial germination and the growth of germtubes at low temperatures on water agar were also studied.

2 Material and methods

2.1 The germination of conidia and the growth of germtubes at low temperatures

The germination of conidia was examined in Petri dishes on water agar. The suspension of the conidia (of three isolates: two from northern Finland and one from southern Norway, isolated by A. Uotila and M. Vuorinen) was spread on the agar, about 73 000 conidia per each Petri dish (approx. 1200 conidia/cm^2). The test temperatures were 0°C, 5°C, 10°C and 12.5°C. Six Petri dishes were kept at each temperature in the dark. At intervals of 2, 3, 4, 5, 7 and 9 days incubation, germination on 250–350 conidia (30 microscopic fields, 300× magnification) were counted at each temperature, always by using a new Petri dish. A conidium was considered germinated if the formation of a germtube could be seen. The counting was discontinued after 7 days at the temperatures of 10°C and 12.5°C.

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The growth of the germtubes from the conidia was also examined on water agar in Petri dishes. The conidial suspension was spread on the agar (approx. 600 conidia/cm^2). Conidia on the agar surface were photographed each day from a new Petri dish at each temperature. The germtubes were measured on photographs using a Derby Kurvenmesser. The length of all the germtubes from one conidium, on 40 conidia in total, was measured at each treatment.

2.2 The ability of G. abietina to cause necrosis in pine seedlings

Containerized Scots pine seedlings (originating from Central Finland) had been grown according to nursery practice, but in the second growing season without any fungicides, pesticides or herbicides. The following winter, on January 28th, the seedlings were lifted with snow, and kept at 8 °C until the snow and ice cover had melted. The test temperatures were −4 °C, −2 °C, 0 °C and 2 °C. Before exposing the seedlings to the above temperatures, those to be placed at 2 °C, 0 °C or −2 °C, were inoculated at −2 °C to ensure that they would not be exposed to a warmer temperature those tested. The seedlings to be placed at −4 °C were inoculated at −4 °C.

For inoculation a right-angled wound (5 mm × 5 mm) into the phloem was made 5 cm below the top of the shoot. A two-week-old mycelium of a Northern Finnish *Gremmeniella* isolate of B-type (Uotila, A., personal communication), Kai 1.2 (Uotila, A.), was used as inoculum. The isolate was grown at 16 °C in a V-8 liquid culture (20 % Campbell's V-8 juice and 80 % distilled water, filtered twice through Macheney-Nagel 640 W-filter paper; glucose was added to 2 % and the medium was autoclaved and inoculated with the conidia). The mycelium was cut into small pieces, the size of about 3 mm × 4 mm × 3 mm. One piece was placed under the flap of the wound which was covered with parafilm. Other treatments were two types of controls: 1) inoculation with mycelium killed by autoclaving, 2) wounding without inoculum.

For each test temperature, 20 seedlings were inoculated with mycelium, with 20 seedlings for controls 1 and 2. The seedlings were kept at the test temperatures in dark growth chambers on container trays. The trays were covered with plastic to avoid desiccation of the seedlings. After 75 days the total length of necrosis was measured from the wounds.

The analysis of variance and Tukey-test as well as the analysis of regression were used for statistical purposes.

3 Results

3.1 Conidial germination and the growth of germtubes

The germination rate at 10 °C and 12.5 °C reached about 90 % within two days, and increased up to 94 % and 96 % within three additional days (Fig. 1). The germination percentage at 5 °C was 37 after two days, and reached 90 within five days after the beginning of the experiment. At 0 °C the germination percentage after two days was only about 3 %, but within seven days after the beginning of the experiment 86 % were reached (Fig. 1).

The mean total length of the germtubes of one conidium at 12.5 °C after 5 days was about 0.6 mm and the respective length at 10 °C about 0.4 mm. In the same time span the growth at 5 °C and at 0 °C was about 0.1 mm and 0.04 mm, respectively (Fig. 2).

3.2 Development of the necrotic area

Both temperature and treatment affect the length of necrosis (Table 1). Because the length of necrosis in controls was not increasing with increasing temperature as in the treatment where living mycelium was as an inoculum, the effect of treatment was tested separately at each temperature. The results of analysis of variance at each temperature indicated that

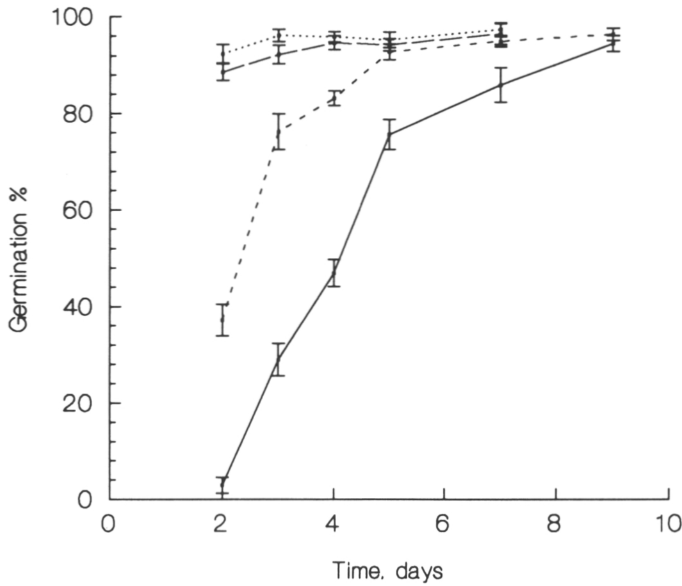

Fig. 1. Germination percentage of conidia of *Gremmeniella abietina* at different temperatures. 0 °C (——), 5 °C (- - - -), 10 °C (– – –), 12.5 °C (·····). Bars indicate standard error of the means

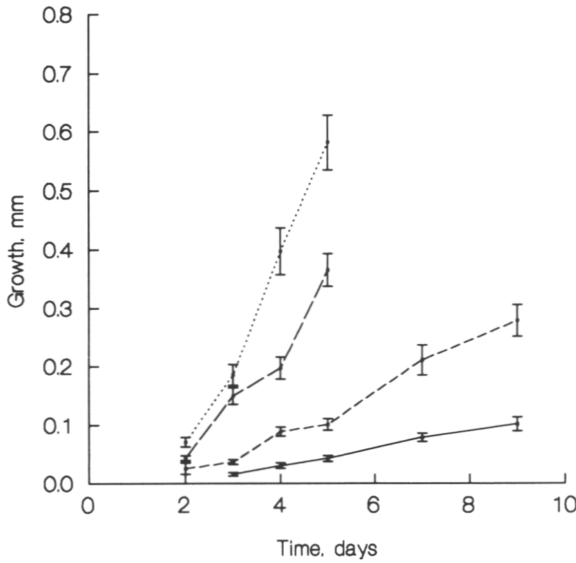

Fig. 2. The mean total length of the germtubes from one conidium, measured separately from 40 conidia on water agar at different temperatures during the study period. 0 °C (——), 5 °C (- - - -), 10 °C (– – –), 12.5 °C (·····). Bars indicate standard error of the means

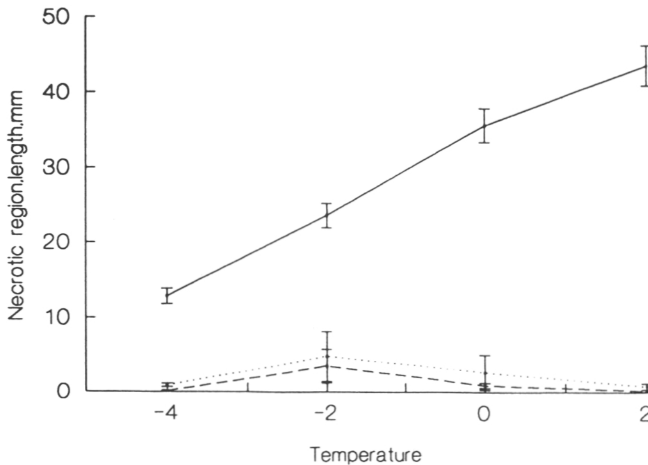

Fig. 3. The length of necrosis in pine seedlings after 75 days from inoculation in winter. The seedlings were during these days in dark growth chambers. Wounds inoculated with *G. abietina* mycelium (——), control 1: the wound inoculated with killed mycelium (- - - -), control 2: the wound without inoculum (·····). The tested temperatures: −4 °C, −2 °C, 0 °C and 2 °C. Bars indicate standard error of the means

treatment statistically significantly affected the length of the necrotic area. The necrotic area from the inoculated wounds in the seedlings was significantly ($p < 0.01$, Tukey-test) longer than that in the controls. The length did not differ between controls (Fig. 3). The necrosis was longest at 2 °C (mean = 43.5 mm, $\hat{y}=44.62$ mm) and 0 °C (mean = 35.7 mm, $\hat{y}=34.17.$ mm) differing statistically significantly from that at −2 °C (mean = 23.7 mm, $\hat{y}=23.72$ mm) and the shortest at −4 °C (mean = 12.8 mm, $\hat{y}=13.28$ mm). The length of the necrosis depended on temperature (between −4 °C and 2 °C) according to the equation $y = 2.8352 + 10.446x$ (y = length of the necrosis, $x=3+0.5$temperature:, $r = 0.812$, $p < 0.001$, $N = 76$).

Table 1. **Analysis of variance for the effect of temperature and treatments on the length of necrosis**

Source	d.f.	mean square	F	P
Temperature	3	1180.8518	19.95	0.0000
Treatment	2	19127.5435	323.11	0.0000
Interaction	6	1233.8077	20.84	0.0000
Error	222	59.1981		

4 Discussion

The temperature dependence of the conidial germination of *G. abietina* is not actually the same as that of the growth during the first days. The germination percentage at 0 °C was high, although the growth of the germtubes was minimal within the first 9 days (cf. also YOKOTA et al. 1974).

Rainy and cold growing seasons favour disease outbreak (e. g. PETÄISTÖ and REPO 1988). Winter conditions also play an important role in disease occurrence. MAROSY et al. (1989) found that if the temperature varies for more than 43 days within the range −6 °C to 5 °C the disease can break out.

This experiment proved that *G. abietina* can cause necrosis in the pine phloem at temperatures below 0 °C. Necrosis developed even at −4 °C. The length of the necrotic area depended linearly on temperature in the tested temperatures ranging from −4 °C to +2 °C (longer at 2 °C than at −4 °C). While soft snow is a good temperature insulate, under the snow the temperature is usually much higher than above (e. g. KERÄNEN 1920; DORWORTH 1972). In winter, the temperature may permit the activity of the fungus, and latent infections might easily break out e. g. in the nursery bed. The necrotic areas may be the bigger (i. e. the disease may be more serious) the longer the temperature is favourable for the formation of necrosis. During winter dormancy active defence reactions in the host plant do not prevent the progress of the damage (KURKELA and NOROKORPI 1979; MULLICK and JENSEN 1976).

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I wish to express my gratitude to EIJA LAPPALAINEN, laboratory technician, and MERVI AHONPÄÄ, laboratory assistant, for their help in the experiments and Drs. TIMO KURKELA, TARJA LEHTO and ANTTI UOTILA for their invaluable comments on the manuscript.

Summary

On water agar the conidia of *Gremmeniella abietina* reached the same germination rate at 0 °C as at 5 °C to 12 °C, but required more time. The germtubes grew much less at 0 °C and 5 °C than at 10 °C or at 12 °C during 3–5 days.

G. abietina caused necrosis in pine seedlings in winter at the tested temperatures −4 °C, −2 °C, 0 °C and 2 °C in dark growth chambers, when the seedlings were inoculated with mycelium into the phloem.

Zusammenfassung

Konidienkeimung von Gremmeniella abietina und durch diesen Pilz verursachte Nekrosenbildung auf Kiefernsämlingen bei tiefen Temperaturen

Bei 0 °C erreichten Konidien von *Gremmeniella abietina* gleich hohe Keimraten wie bei Temperaturen zwischen 5 °C und 12 °C, brauchten dafür aber länger. Die Keimschläuche wuchsen innerhalb von 3 bis 5 Tagen bei 0 °C und 5 °C viel weniger weit als bei 10 °C oder 12 °C. *G. abietina* verursachte bei −4 °C, −2 °C, 0 °C und +2 °C und Dunkelheit Nekrosen an Kiefernsetzlingen, deren Phloem mit Myzel des Pilzes beimpft wurde.

Résumé

Germination des conidies et formation de nécroses par Gremmeniella abietina chez des semis de pins à basses températures

Sur eau gélosée, les conidies de *G. abietina* atteignaient le même taux de germination à 0 °C qu'à 5–12 °C mais demandait plus de temps à 0 °C. Les tubes germinatifs poussaient beaucoup moins à 0 °C et 5 °C qu'à 10 °C ou 12 °C en 3 à 5 jours. *G. abietina* provoquait des nécroses chez les semis de pins en hiver aux températures testées de −4 °C, −2 °C, 0 °C et 2 °C dans des chambres de croissance à l'obscurité, quand les semis avaient été inoculés par du mycélium déposé sur le phloème.

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The ability of Gremmeniella abietina to hydrolyze polygalacturonic acid

By RAIJA-LIISA PETÄISTÖ and E. O. KAJANDER

Abstract

Gremmeniella abietina grew well in media with pectin as the sole carbon source. The fungus secreted at least one enzyme with polygalacturonase activity, and this was induced by addition of pectin to the culture medium.

Key words: *Gremmeniella abietina* – Enzyme activity – Polygalacturonase – Pathogenicity.

1 Introduction

The ascomycete *Gremmeniella abietina* (Lagerb.) Morelet is a pathogenic fungus which causes damage to conifers. The disease in conifer forests in North America, Northern Europe and Asia can be widespread and destructive (SKILLING 1981; KALLIO et al. 1985). The mechanisms behind the infection process, for instance, the enzyme production of this fungus, are not well known. One enzyme which could hydrolyze xylan has been previously detected and partially characterized (PETÄISTÖ et al. 1992). Excretion of hydrolytic enzymes acting on pectin, protein, cellulose and starch has also been recently observed (HAANSUU 1992). Pectin is the major component of primary cell wall in conifers. The main components of pectic polysaccharides are polyuronic acids made from D-galactosyluronic acids (O'NEILL et al. 1990). Pectin is also the main component of middle lamella and is involved in joining the components of cell walls together (calcium salts of pectic acid) (BACIC et al. 1988; TALMADGE et al. 1973).

There are several enzymes that can degrade pectin and these can be grouped as hydrolases and lyases. Pectin hydrolyzing enzymes may play a role as factors of virulence (COOPER 1983). Products from the decomposition of pectin may also act as elicitors that trigger the defence mechanisms of the host plant (ROBERTSEN 1987). The aim of this work was to study the ability of *G. abietina* to hydrolyze and utilize pectin as measured by its growth on pectin media and the excreted polygalacturonase activity.

2 Material and methods

2.1 Culture of G. abietina

Pectin from apple or from citrus fruits (Sigma No. P2157 and No. P 9135) was added at 1% final concentration to Hoagland solution (NIENHAUS 1969) supplemented with biotin, thiamine and pyridoxine at 0.3, 2.8 and 2.8 μM, respectively, and with the following minerals (as their final concentrations): 50 μM H_3BO_3, 10 μM $MnCl_2 \times 4H_2O$, 1 μM $ZnSO_4 \times 7H_2O$, 0.3 μM $Cu_4SO_4 \times 5H_2O$, 0.1 μM $H_2MoO_4 \times H_2O$. Scots pine needle extract was prepared by homogenizing 50 g of needles (1–2 years old) in 200 ml water with

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an Ultra Turrax (Janke & Kunkel KG, Staufen im Breisgau, Germany) followed by centrifugation at 3000 g for 15 min. The supernatant fraction was used as the needle extract and added at a 20 % final concentration to the above-mentioned media in the specified experiments as a natural source of nutrients.

In addition to the pectin media, 3 % glucose was used in mineral medium for culture in selected experiments. In some experiments, pure needle extract medium (20 % needle extract in Hoagland solution) was also used. All media were autoclaved at 120 °C for 25 min. The media were inoculated with conidia from the Kai isolate (from Northern Finland, a kind gift from Dr. ANTTI UOTILA). Cultures were incubated in the light on a shaker (30 horizontal cycles/min) at +16 °C.

2.2 pH and temperature dependence of polygalacturonic acid hydrolyzing activity

To measure polygalacturonic acid hydrolyzing activity (MILLER 1959; BAILEY and PESSA 1990), 0.4 % polygalacturonic acid in 0.1 M Sörensen's citrate buffer (routinely pH 3.5) served as substrate. 0.9 ml substrate solution plus 100 μl culture filtrate (apple pectin medium) as a crude enzyme preparate were incubated at 38 °C for 2.5 h. Then 1.5 ml dinitrosalicylic acid reagent was added, the mixture was boiled 10 min and 5 ml water was added. After centrifugation, absorbance at 540 mm was measured. Galacturonic acid was used as a standard. Enzyme activity is expressed as μmol galacturonic acid equivalents formed during 2.5 h.

The effect of pH on enzyme activity was studied with 0.1 M Sörensen's citrate buffer between pH range 3.5–6.5 or with 0.07 M Sörensen's phosphate buffer (pH 7.5 and 8.5). As an enzyme preparate, culture filtrate from a 2.5 month old reculture of 3 month old mycelium in apple pectin growth media was used. The linearity of the assay for polygalacturonic acid hydrolyzing activity was tested by determining the amount of released galacturonic acid after incubation for 15, 60, 120, 180 and 300 min at 25, 38 and 50 °C.

Also the linearity of enzyme activity according to added enzyme amount was tested. Samples of culture filtrate of one month old mycelium were diluted with water before triplicate activity measurements. The dilutions were 1 : 1, 4 : 5, 3 : 5 and 1 : 5.

2.3 Induction of polygalacturonic acid hydrolyzing activity

1 % pectin from apple or from citrus fruits in Hoagland solution with or without 20 % needle extract were used as so called 'enzyme activity induction media'. As a control, 3 % glucose medium was used. Media were inoculated with conidia from Kai isolate (about 12 000 conidia/ml). After 2, 4 and 6 weeks the mycelia (4 × 60 ml) were harvested by filtration, washed and transferred under aseptic conditions to 40 ml of pure needle extract medium (20 % needle extract in Hoagland solution). After 4 days of culture, the mycelia were harvested and their dry weights measured after drying for 20 h at + 80 °C. The polygalacturonic acid hydrolyzing activities from the culture supernatants are given as μmol galacturonic acid formed per mg mycelium dry weight and have been used as indicator of the enzyme inducing property of the tested culture media.

An experiment was also carried out to compare the level of excreted polygalacturonic acid and xylan hydrolyzing activities (PETÄISTÖ et al. 1992) by *G. abietina* as well as their induction by xylan and pectin. For this, the mycelia from two and four week old 1 % apple pectin cultures were subcultured in 1 % apple pectin or 1 % xylan (from oats spelts) media for 4 days. Correspondingly, the mycelia from 1 % xylan (from oats spelts) cultures were subcultured in 1 % apple pectin or 1 % xylan media for 4 days. Thereafter, the dry weights of the mycelia were measured and from the culture filtrates both polygalacturonic acid and xylan hydrolyzing activities were assayed as triplicate measurements of three cultures each. Values given are means ± standard errors of the mean (S. E. M.).

Statistical significance was evaluated with analysis of variance and the Tukey-test using the BMDP. statistical analysis program (BMDP Statistical Software Manual 1990).

3 Results and discussion

3.1 Detection of polygalacturonic acid hydrolyzing activity and its pH and temperature dependence

Cultures of *G. abietina* exhibited polygalacturonic acid hydrolyzing activity in the culture media tested. The activity was relatively high surpassing that of the other known secreted enzyme, xylanase, as compared from the following culture test: two and four weeks old mycelia first cultured in 1 % apple pectin medium and then transferred to new 1 % pectin medium, showed a polygalacturonase activity of 0.070 ± 0.009 and 0.046 ± 0.018, respectively, in the subculture media incubated for four days. The rate of the reaction was calculated as μmol galacturonic acid formed per mg mycelial dry weight. Correspondingly two and four weeks old mycelia cultured in 1 % xylan (from oat spelt) and then transferred into new 1 % xylan medium, showed after four days a xylanase activity of 0.003 ± 0.001 and 0.004 ± 0.001 (μmol reducing sugars formed per mg mycelial dry weight) in the medium. According to this result polygalacturonase activity was about 15 times higher than xylan hydrolyzing activity per mycelial dry weight (in 2.5 to 3 h incubation) in media considered to induce these enzymes (see later results in 3.3).

Within the tested pH-range the optimum enzyme activity was found at pH 3.5. Activity at this pH was higher than observed at pH 4.5 ($p < 0.05$) (Fig. 1). At pH 7.5 the activity was completely undetectable and at 8.5 minimal. The reported polygalacturonase enzyme activity of *Aspergillus nidulans* was also highest at or below pH 4 (Dean and Timberlake 1989) and that of *Aspergillus niger* and *Sclerotinia sclerotiorum* at pH 4.2 (Keon and Waksman 1990). Bailey and Pessa (1990) found that the pH optimum of the *A. niger* polygalacturonase was between pH 4.5–5.0. The pectate lyases have pH optima at ca. 8.5 (Collmer et al. 1988) and the minimal activity at pH 8.5 detected by us may have been caused by pectate lyase.

The incubation temperature and time affected the enzyme activity assay results (Table 1). The activity was almost linear at 25, 38 and 50 °C for about 3 hours (Fig. 2). At 15 min incubation the activity was a little higher at 50 °C than at 25 °C and 38 °C ($p < 0.1$, Tukey test) (d.f. 2, F 5.02, P 0.0523). Surprisingly the enzyme activity at 50 °C, although highest

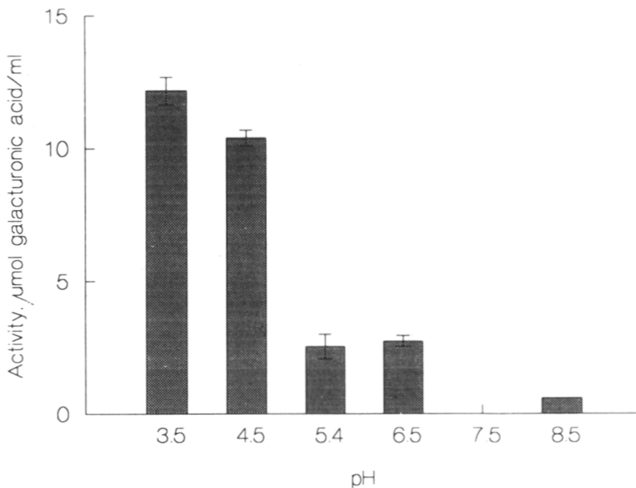

Fig. 1. pH dependence of polygalacturonic acid hydrolyzing activity of *G. abietina* (d. f. = 5, F = 198.97, P = 0.000). 0.4 % polygalacturonic acid was incubated with enzyme preparate from pectin medium at pH 3.5 to pH 8.5. Values are means of three replicates. Bars indicate standard errors of the means

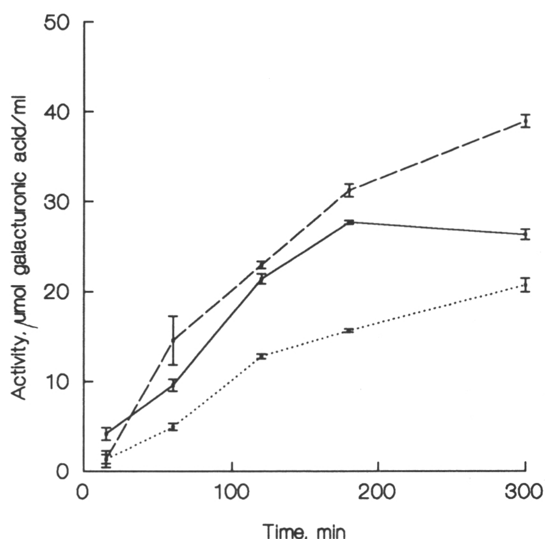

Fig. 2. Temperature dependence and linearity with time of polygalacturonic acid hydrolyzing activity of *G. abietina*. Enzyme preparate from cultures with pectin medium was incubated in 0.4% polygalacturonic acid for the time indicated at 25 °C (·····), 38 °C (– – –) and 50 °C (——). All values are means of three replicates. Bars indicate standard errors of the means

at 15 min, proceeded thereafter at an intermediate level. This may indicate the presence of two or more isoenzymes with different thermostabilities, as previously detected from *Fusarium oxysporum* (ARTES and TENA 1990). BAILEY and PESSA (1990) using an *Aspergillus niger* enzyme found that the temperature optimum was about 50 °C with an incubation time of 5 min but a longer incubation time resulted in a loss of activity. Here a temperature of 38 °C and incubation time of 2.5 h was routinely used in the activity assay. These assay conditions are very similar to those used by DEAN and TIMBERLAKE (1989) (37 °C and 1 h–3 h) in their assay studying polygalacturonase from *Aspergillus nidulans*.

The polygalacturonase activity was linear according to the added enzyme amount when tested with diluted samples. The dilutions were 1 : 1, 4 : 5, 3 : 5 and 1 : 5 and the corresponding activities were 45.6, 38.4, 27.7 and 9.8 μmol galacturonic acid formed/ml.

Table 1. **Analysis of variance for the effects of temperature and incubation time in the assay for polygalacturonic hydrolyzing activity**

Source	d. f.	Mean square	F	P
Temperature	2	441.6680	183.89	0.0000
Time	4	1061.0790	441.79	0.0000
Interaction	8	47.0344	19.58	0.0000
Error	30	2.4018		

3.2 Growth of G. abietina in the induction media

The production of mycelium, as assessed by dry weight, differed between media and time (Table 2, Fig. 3). After two weeks, there was no significant difference in dry weights between media (d.f. 4, F 0.81, P 0.5383). When comparing the media according to the dry weight after culture for 4 (d.f. 4, F 15.85, P 0.000) and 6 weeks (d.f. 4, F 64.18, P 0.0000), the dry weight obtained in pectin media with needle extract was higher ($p < 0.01$, Tukey test) than in pectin media without needle extract. The mycelial dry weights did not differ between citrus and apple pectin media. The mycelium production in glucose medium after 6 weeks was lower than in pectin media supplemented with needle extract ($p < 0.01$), but did not differ from the corresponding production in pure pectin media (Fig. 3). We can conclude that *G. abietina* could grow well on pectin without the presence of other

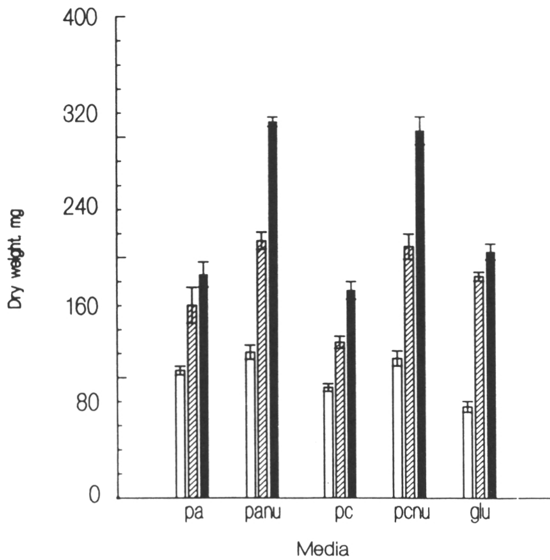

Fig. 3. Growth of *G. abietina* in pectin with or without needle extract and glucose media. Cultures were started by adding 12 000 conidia per ml and growth was measured after 2 (white), 4 (hatched) and 6 (black) weeks. Medium *pa* = 1 % pectin from apple in Hoagland solution, *pc* = 1 % pectin from citrus fruits in Hoagland solution, *panu* = pa medium supplemented with 20 % needle extract, *pcnu* = pc medium supplemented with 20 % needle extract, *glu* = 3 % glucose in Hoagland solution. Values are means of four cultures. Bars indicate standard errors of the means

Table 2. **Analysis of variance for the effects of time and media on the mycelial dry weight production of G. abietina**

Source	d. f.	Mean square	F	P
Time	2	90 939.8167	381.74	0.0000
Media	4	17 354.4750	72.85	0.0000
Interaction	8	3 593.9000	15.09	0.0000
Error	45	238.2222		

carbohydrates but needle extract still further increased the growth rate over pure pectin and glucose media. In all media, the dry weight of mycelium increased during the culture time indicating that the fungus utilized them for growth.

3.3 The activity of polygalacturonase excreted by the mycelia grown in various enzyme induction media

To see whether the polygalacturonase production depended on the type of pectin preparate and the effect of needle extract on the production, we cultured *G. abietina* in an induction medium containing pectin from apple or from citrus fruits with or without needle extract. Glucose medium served as a control. The mycelia grown for two, four and six weeks in pectin, pectin with needle extract or glucose media were transferred to the needle extract media for a further incubation of 4 days. The enzyme activity in these four day subcultures differed between previous induction media and time (Table 3, Fig. 4). The ac-

Table 3. **Analysis of variance for the effects of induction medium and age of the mycelium on the polygalacturonase activity**

Source	d. f.	Mean square	F	P
Age	2	6.4116	9.57	0.0003
Medium	4	22.9569	34.26	0.0000
Interaction	8	1.6776	2.50	0.0243
Error	45	0.6702		

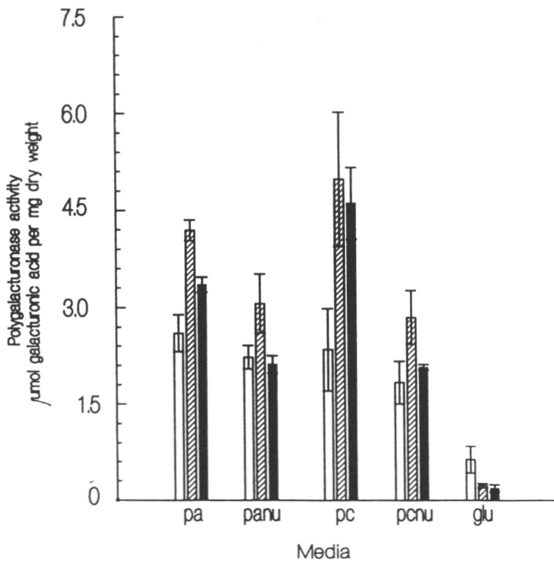

Fig. 4. Polygalacturonase activity in needle extract medium after precultures in various pectin and glucose media. As described in the methods, cultures were started from conidia and cultured for the time-points in the media described in Fig. 3. At the indicated time-points, the mycelia were washed and transferred to 40 ml needle extract medium. Enzyme activities were measured after culture for 4 days from the supernatants. Values are means of four triplicate assays. Bars indicate standard errors of the means

tivities were statistically tested separately in each medium with respect to culture time and correspondingly in each time with respect to culture medium. There were some differences in inducing activity between the culturing times in apple pectin (d.f. 2, F 15.45, P 0.0012), citrus pectin (d.f. 2, F 3.40, P 0.0792) and glucose medium (d.f. 2, F 2.70, P 0.0759). In pectin media with needle extract the differences were not so clear (apple pectin with needle extract: d.f. 2, F 3.07, P 0.0963, citrus pectin with needle extract d.f. 2, F 2.95, P 0.1032). After culturing for four weeks in apple and citrus pectin media, the induced polygalacturonase activity was higher than after two weeks' culturing ($p < 0.01$, $p < 0.1$) and in apple pectin media also only marginally higher than after six weeks' culturing ($p < 0.1$) (Fig. 4).

There were differences between media in their induction activity after two (d.f. 4, F 5.92, P 0.004), four (d.f. 4, F 13.55, P 0.0001) and six (d.f. 4, F 39.61, P 0.0000) weeks (Fig. 4). After two weeks of culturing, only glucose medium differed from the others, the activity being smallest ($p < 0.05$–$p < 0.01$). At the four weeks point, the situation was the same, but the differences were bigger and the citrus pectin media differed little from the corresponding media with needle extract ($p < 0.1$). After culturing for six weeks both apple and citrus pectin media induced enzyme activity more than the corresponding media with needle extract ($p < 0.05$, $p < 0.01$). Culturing in citrus pectin media the resulting induced activity that was higher than that obtained in apple pectin media ($p < 0.05$).

To test if the activity of polygalacturonase was really induced by pectin, we compared activities of polygalacturonase and xylanase excreted in the same culture media. An induction culture was made for two and four weeks in pectin media and mycelia were transferred for four days to pectin media. In that case the polygalacturonic acid hydrolyzing activity in the medium was 0.070 ± 0.009 and 0.046 ± 0.018 µmol galacturonic acid per mg dry weight and xylanase activity 0.015 ± 0.003 and 0.003 ± 0.001 µmol reducing sugars per mg dry weight (xylanase activities published previously by Petäistö et al. 1992). When subcultured in xylan media the corresponding polygalacturonase activity was 0.040 ± 0.002 and 0.032 ± 0.004 and xylanase activity 0.077 ± 0.006 and 0.018 ± 0.002 µmol per mg dry weight. The especially high xylanase activity (0.077) in this experiment was most likely caused by the good growth in the preculture in pectin medium. Polygalacturonase activity was higher in pectin subcultures and xylanase activity was higher in xylan subcultures. It can be concluded that the carbon source in the culture medium has an inducing

effect on the pattern of excreted enzymes involved in the metabolism of the carbon source. Since this fungus grows very slowly, it is likely that changes in the excreted enzyme pattern also take place slowly and the growth rate of the fungus may affect the results quite considerably. Thus a culture period longer than four days might bring about more marked changes in enzyme patterns.

It is remarkable that the dry weight production of the fungus was increased by addition of the needle extract although concomitantly polygalacturonase production was inhibited. Previously we have observed a similar finding with xylanase enzyme. It is apparent, that the fungus can better utilize nutrients of needle extract than pectin alone.

We have shown that *G. abietina* excretes an enzyme into its culture medium that can hydrolyse pectins and that the fungus grows very well with pectins as the source of carbon. Specifically we detected polygalacturonase activity and described its pH and temperature dependence and have shown that pectin induced the polygalacturonase activity.

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Summary

Gremmeniella abietina grew in media where pectin was the sole carbon source, the dry weight of the mycelium increased. Pectin from apple and pectin from citrus did not differ in their growth-supporting ability. Needle extract further increased the growth of the fungus but decreased the polygalacturonase activity. The induced polygalacturonase activity was high after culturing for four and six weeks in pectin growth media. The enzyme showed highest activity at pH 3.5 at 38 °C with long incubation times and the enzyme activity assay was linear for up to three hours.

Résumé

Aptitude de Gremmeniella abietina à hydrolyser l'acide polygalacturonique

G. abietina poussait dans des milieux où la pectine était la seule source de carbone et le poids sec de mycélium augmentait. La pectine de pomme et celle d'agrume ne différaient pas dans leur aptitude à permettre la croissance. L'extrait d'aiguille augmentait la croissance du champignon mais diminuait l'activité polygalacturonase. L'activité polygalacturonase était forte après 4 et 6 semaines de croissance sur milieux à la pectine. L'enzyme a montré un maximum d'activité à pH 3,5 à 38 °C avec un long temps d'incubation et une courbe linéaire d'activité pendant trois heures.

Zusammenfassung

Hydrolyse der Polygalakturonsäure durch Gremmeniella abietina

Gremmeniella abietina wuchs auf Medien mit Pektin als einziger Kohlenstoffquelle, das Trockengewicht des Myzels nahm zu. Pektine von Apfel und Citrus unterschieden sich in ihrer wachstumsfördernden Wirkung nicht voneinander. Durch Nadelextrakt wurde das Pilzwachstum zwar zusätzlich gefördert, die Polygalakturonaseaktivität wurde jedoch reduziert. Nach vier- und sechswöchiger Kultur auf Pektinmedien war eine hohe induzierte Polygalakturonaseaktivität vorhanden. Das Enzym zeigte seine höchste Aktivität bei pH 3,5 und 38 °C bei langer Inkubationszeit, und die Aktivitätskurve verlief für bis zu drei Stunden linear.

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Detection of xylan hydrolyzing activity in culture extracts of Gremmeniella abietina

By R.-L. Petäistö, J. Talvinen and E. O. Kajander

Abstract

Hemicellulose xylan is a major non-cellulose component of wood. Xylan hydrolyzing activity was detected in culture media of *G. abietina*. *G. abietina* grew well on xylan. Pine needle extract decreased xylanase activity.

Key words: *Gremmeniella abietina* – Xylanases.

1 Introduction

Gremmeniella abietina (Lagerb.) Morelet is a harmful pathogenic fungus causing damage to conifers especially in North America, Northern Europe and Asia, where it initiates canker formation and causes death of trees. The infection process has been studied (e. g. Patton et al. 1984; Sletten 1971; Siepmann 1976), but its mechanisms remain unresolved. As extracellular enzymes may play some role in the infection process, it is necessary to screen for various fungal enzymes, which are capable of hydrolyzing pine components. Plant cell walls contain mainly cellulose and hemicellulose, including xylan, a heteropolysaccharide hemicellulose. In softwood, there is mainly arabinoglucuronoxylan (7–10 % of wood), but glucuronoxylan is the main hemicellulose in hardwood (15–30 % of wood) (Sjöström 1981).

Xylan is decomposed by xylanases, which can be classified into β-xylosidases and xylanases (exo-β-xylanases and endo-β-xylanases). Synergism between xylanases and β-xylosidases in the hydrolysis of insoluble xylan has been found (Dekker 1983; Desphande et al. 1986). The purpose of this work was to study the possible secretion of xylanase by *G. abietina* as reflected in the ability of hydrolyze xylan.

2 Methods

2.1 Culture of G. abietina in xylan medium

Xylan from birch wood (Sigma, no. x0502) or from oat spelts (Sigma, no. x0627) was added to Hoagland solution (Nienhauss 1969) to give a final concentration of 1 % xylan. This medium was supplemented with biotin, thiamine and pyridoxine at 0.3, 2.8 and 2.8 μM and with the following minerals (as their final concentrations): 50 μM H_3BO_3, 10 μM $MnCl_2 \times 4H_2O$, 1 μM $ZnSO_4 \times 7H_2O$, 0.3 μM $Cu_4SO_4 \times 5H_2O$, 0.1 μM $H_2MoO_4 \times H_2O$. Pine needle extract was prepared by homogenizing 50 g of Scots pine needles (1–2 years old) in 200 ml water with Ultra Turrax (Jahnke & Kunkel KG, Staufen im Breisgau, Germany) followed by centrifugation at 3000 g for 15 min. The supernatant fraction was used as the needle extract and was added at 20 % final concentration to the above-mentioned media in the indicated experiments. Pure needle extract medium (20 % needle

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extract in Hoagland solution) was used in certain experiments (enzyme preparations for assaying pH and temperature dependence of xylan hydrolyzing activity and in xylanase induction experiment). Needle extract was used as a natural source of nutrients, because it is known that *G. abietina* can grow also in needles and even could develop pycnidia on the needles (READ 1968; STEPHAN 1970). All media were autoclaved at + 120 °C for 25 min. Vitamins were added after autoclaving.

The media including pectin were inoculated using conidia from the Finnish Toro isolate of *G. abietina* (a kind gift from Dr. Antti Uotila, University of Helsinki, Finland). About 100 000 conidia were added per ml medium. Cultures (twelve/each medium) were incubated in the dark on a shaker (30 horizontal cycles/min.) at + 16 °C. Mycelium dry weight was measured after drying washed mycelium for 20 h at + 80 °C. For statistical analysis the Student's t-test was used.

2.2 Measurement of xylan hydrolyzing activity

G. abietina was cultured starting from conidia for 1, 2 and 3 months in 1 % xylan media with and without needle extract supplementation. For each time point, quadruplicate cultures (each 30 ml) were treated separately. All the mycelia from each culture were harvested by filtration, washed and transferred using sterile technique to 10 ml of needle extract medium (20 % needle extract in Hoagland solution without xylan additions). After culture for 4 days, the mycelium was separated and the proteins in the medium were concentrated by acetone precipitation (two vol cold acetone plus one vol medium). After 2 h storage at − 20 °C, the precipitate was collected by centrifugation and then dissolved in deionized water so that the volume was 7.7-fold smaller than before concentration. Xylan hydrolyzing activity was measured by determining released reducing sugars using dinitrosalicylic acid reagent (MILLER 1959; POUTANEN 1988; POUTANEN and PULS 1988). As substrate, xylan either from oat spelts or birch wood was dissolved at 1 % concentration in 0.05 N sodium citrate buffer, pH 5.3. The incubation was started by adding 0.2 ml enzyme sample to 1.8 ml of the substrate. With this method the release of reducing sugars is measured, so the method is unspecific for any particular type of xylanase.

The effect of pH and temperature on enzyme activity was tested. 1 % xylan from oat spelts in 0.1 M Sörensen's citrate buffer (pH 3.5, 4.5, 5.4 or 6.5) or in 0.07 M Sörensen's phosphate buffer (pH 7.5 or 8.5) was incubated with concentrated needle extract mineral culture media of the fungus (50 fold concentration). The linearity of the xylan hydrolyzing activity was tested determining the released reducing sugars at incubation temperatures + 25 °C, + 38 °C and + 50 °C after 15 min, 60 min, 120 min, 180 min and 300 min.

The effect of the needle extract on the xylanase activity was tested. Xylan culture medium of *G. abietina* was acetone precipitated. This enzyme preparate was diluted with autoclaved needle extract or buffer. The pH of the enzyme preparate and buffer were the same. The dilutions were stored overnight at + 20 °C before the measurement of activity. Further the effect of acetone precipitation of needle extract on enzyme activity was assayed.

3 Results and discussion

3.1 Growth of G. abietina in xylan media

The ability of *G. abietina* to grow on xylan was tested in Hoagland solution with and without needle extract (Fig. 1). *G. abietina* grew on xylan as the only source of carbon. When needle extract was added to xylan media, the growth improved. Xylan from oat spelts supported the growth (dry weight) better than xylan from birch wood after one and two months ($p < 0.01$ and $p < 0.01$, respectively, Tukey test). At three months the difference was not statistically significant.

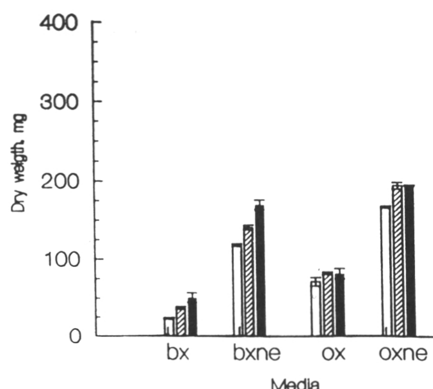

Fig. 1. Growth of *G. abietina* in xylan and glucose media. Cultures were started in various media by adding 100 000 conidia per ml and growth was measured as dry weight after 1 (white), 2 (hatch), and 3 (black) months. Medium bx: 1 % xylan from birch wood in Hoagland solution, bxne: 1 % xylan from birch wood in Hoagland solution supplemented with 20 % needle extract, ox: 1 % xylan from oat spelts in Hoagland solution, oxne: 1 % xylan from oat spelts in Hoagland solution supplemented with 20 % needle extract. Values are means of four cultures. Bars indicate standard error of mean

3.2 Xylanase assay

Xylanase activity produced by *G. abietina* was detected in the supernatant fraction of cultures though the activity was very low. The xylan hydrolyzing activity was high in the resuspended precipitate after acetone precipitation, thus indicating good stability of the enzyme. The linearity of xylan hydrolyzing activity was assayed for up to 5 h at various temperatures and the enzyme activity was temperature-dependent. At + 50 °C the increase in enzyme activity was not linear, indicative of enzyme denaturation. At + 38 °C enzyme activity increased linearly up to 5 h after beginning of the experiment. At + 25 °C enzyme activity increase linearly, but the activity was lower than at + 38 °C. Thereafter 3 h incubation at + 38 °C was routinely used for the assay. Enzyme activity was linear according to added protein when tested with diluted samples (sample dilutions 1:1, 4:5, 3:5 and 1:5, corresponding to enzyme activities 8.7, 7.3, 5.6 and 2.1 μmol/ml, respectively).

The pH-dependence of the enzyme activity was tested in the pH range 3.5 to 8.5. The highest activity was obtained at pH 4.5 and the lowest at pH 6.5–8.5 (Fig. 2).

Xylanase production of *G. abietina* differed from that of fungi studied previously. For instance, xylanase production in *Trichoderma* is very high with an activity optimum at around + 50 °C (POUTANEN and PULS 1988). *Trichoderma* is a fast growing saprophytic fungus whereas *G. abietina* is a slowly growing pathogenic fungus. The pH optimum of xylanase activity of *G. abietina* was comparable to that of *Trichoderma reesei* (POUTANEN 1988; POUTANEN and PULS 1988).

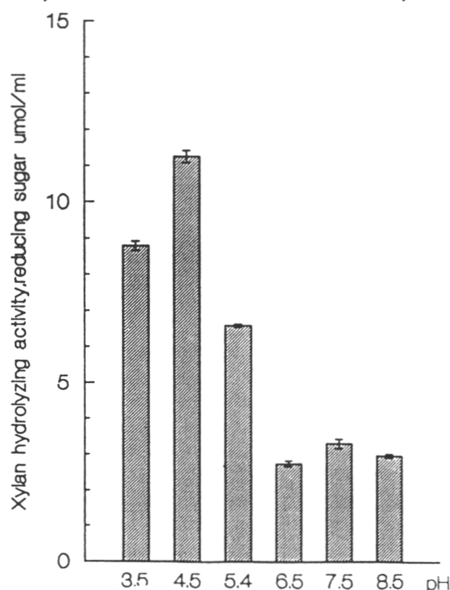

Fig. 2. pH dependence of xylan (from oat spelts) hydrolyzing activity of *G. abietina*. 1 % xylan from oat spelts was incubated with enzyme concentrate from needle extract medium at pH 3.5–pH 8.5. Values are means of three replicates. Bars indicate standard error of mean

3.3 Xylan hydrolyzing activity in G. abietina cultures

As shown in Figure 1, *G. abietina* grew better with xylan from oat spelts than with xylan from birch wood as the carbon source and growth was improved in both cultures by addition of needle extract. To see whether the xylanase production depended on the type of xylan preparate and the effect of needle extract on the production, we cultured *G. abietina* in an induction medium containing xylan either from birch wood or from oat spelts with or without needle extract. For reliable analysis of xylanase production, we then transferred all cultures to the same medium (Hoagland solution with 20% needle extract) for four days and activities were measured from this medium (Fig. 3). Thus, the measured activities represent xylanase production during 4 days after the end of induction culture. The highest activity was obtained with xylan from birch wood after one month of culture. This differed significantly ($p < 0.01$, Tukey test) from other one month cultures. Xylan from oat spelts induction culture resulted in less activity. Needle extract in the induction culture resulted in decreased xylanase production. Apparently the needle extract provided the fungus with a better utilizable nutrient than was available from xylan alone. This suggests that xylan induces xylanase production in *G. abietina*.

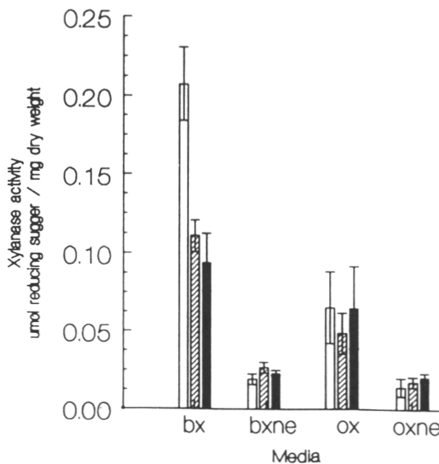

Fig. 3. Activity of excreted xylanase in needle extract medium after induction cultures in various xylan media. As described in the Methods, cultures were started from conidia and cultured for the time-points in media described in Figure 1. At the indicated time-point the mycelium was washed and transferred to 10 ml needle extract medium. Activities were measured after culture for 4 days. Values are means of four triplicate assays. Bars indicate standard error of mean

The unused xylan and needle extract culture media showed no enzyme activities. Needle extract, incubating with enzyme preparate (1:4) overnight at +20 °C before activity measurements, caused inhibition of xylanase activity in the enzyme assay (20–25%) but acetone precipitation removed inhibitory compounds (activities did not decrease compared to control). In this induction series, all samples were acetone precipitated. All samples were also in the same medium for the same time. Thus, the experiment measured enzyme activities under comparable conditions.

Our induction experiment lacked a negative control, i.e. a medium totally devoid of xylan. After these experiments, we cultured *G. abietina* in Hoagland solution supplemented with 1% pectin from apple (Sigma) for 2 and 4 weeks followed by a 4-day culture in a fresh medium supplemented with either apple pectin or xylan from oat spelt. Xylanase activity in the pectin and xylan media were, respectively, 0.015 ± 0.003 and 0.077 ± 0.006 after culture for 2 weeks and 0.003 ± 0.001 and 0.018 ± 0.002 after 4 weeks. The values are expressed as μmol reducing sugar produced per mg dry weight of fungus ± standard error of the mean and were each obtained from triplicate assays of three cultures. Thus xylan could bring about more than five-fold increase in xylanase production.

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Summary

Xylan hydrolyzing activity was detected from culture media of *G. abietina* isolate. Xylan could support growth of the fungus. After one month culture in two different xylan media, the dry weight gain was better with xylan from oat spelts, but xylanase activity was higher with xylan from birch wood. Needle extract added to xylan medium decreased the xylanase production by the fungus. Needle extract added to enzyme sample decreased the activity, but acetone precipitated needle extract had no decreasing effect. The pH optimum of xylanase was near 4.5.

Résumé

Détection de l'activité xylanase dans des extraits de culture de Gremmeniella abietina

L'activité a été détectée dans les milieux de culture de *G. abietina*. Le xylan permet la croissance du champignon. Après un mois de culture sur deux milieux au xylan, le gain de poids sec était plus grand en présence de xylan issu de grain d'avoine, mais l'activité xylanase était plus forte en présence de xylan issu de bois de bouleau. Un extrait d'aiguilles ajouté à l'échantillon enzymatique, réduisait l'activité; mais l'acétone qui précipite l'extrait ne diminuait pas l'effet. Le pH optimum de la xylanase était voisin de 4,5.

Zusammenfassung

Nachweis der hydrolytischen Wirkung von Kulturextrakten von Gremmeniella abietina auf Xylan

Die hydrolytische Wirkung von *Gremmeniella abietina* auf Xylan wurde vermutet, weil Zusatz von Xylan zu Kulturmedien das Wachstum des Pilzes unterstützte.

Die Trockengewichtszunahme in Kulturmedien mit Xylan von Hafer war stärker als in solchen mit Xylan von Birkenholz, dafür war die Xylanaseaktivität in Medien mit letzterem höher. Während die Zugabe von unbehandeltem Nadelextrakt zu den Xylanmedien die Xylanaseproduktion des Pilzes verringerte, hatte die Zugabe von zuvor mit Aceton ausgeschütteltem Nadelextrakt keine Wirkung. Das pH-Optimum der Xylanasen lag bei 4,5.

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Capability of the European and North American race of *Gremmeniella abietina* to hydrolyse polygalacturonic acid *in vitro*

By R-L. Petäisto and J. Lappi

Summary

Polygalacturonase was found to be one of the first enzymes secreted by a pathogen during infection. The polygalacturonic acid hydrolysing activity was compared between the North American and the European race of *Gremmeniella abietina in vitro.* Isolates were grown in pure pectin media from which the enzyme activity was analysed. Altogether, 29 isolates were tested in five experiments (experimental runs in a growing chamber). The data were analysed using variance component models that included fixed race effects and random experiment, isolate, flask and measurement effects. The European race secreted more polygalacturonic acid hydrolysing enzyme than the North American race and the mycelial dry weight produced was smaller for the European race. The differences between races were of the same order of magnitude as the variation between isolates within races; variance components relating to experimental errors were quite large. No correlation was found between the activity and mycelial dry-matter production within the races. Logarithmic transformation removed the apparent racial differences in the variability of the activity and mycelial dry weight. Results from the additionally tested A- and B-type of Finnish isolates indicated differences in dry-matter production.

1 Introduction

Pectic enzymes may play an important role in many diseases. Endopolygalacturonase in particular could be one of the key enzymes in the early phase of the disease process (e.g. Pagel and Heitefuss 1990). Pectin is an essential component of the middle lamella in conifers and also in the primary cell wall of conifers (O'Neill et al. 1990).

Gremmeniella abietina (Lagerb.) Morelet causes disease on many conifers. The fungus occurs in North America, Europe and Asia. *G. abietina* can be divided into European, North American and Asian races (Dorworth and Krywienczyk 1975; Petrini et al. 1989, 1990; Hamelin et al. 1993). The European race can infect its host in the upper part of the crown, whereas the North American race typically infects the lower branches (Dorworth 1979).

Variation between isolates within the races of *Gremmeniella* has been found morphologically, genetically and in protein pattern (Petrini et al. 1989, 1990; Hamelin et al. 1993). According to Uotila (1983, 1990, 1992, 1993) and Hellgren and Högberg (1995) two types (A- and B-type or large-tree and small-tree type) can be identified among northern European isolates. In inoculation experiments, the A-type caused higher rates of positive infections than the B-type (Uotila and Terho 1995) reflecting a possible difference in pathogenicity.

Gremmeniella abientina has been found to hydrolyse polygalactyronic acid *in vitro* and the activity is inducible by pectin (Petäistö and Kajander 1993). To characterize the European and North American race of *G. abietina*, the polygalacturonic acid hydrolysing activity produced *in vitro* (nmol galacturonic acid/mg mycelial dry weight) was compared between the European and North American race.

For a reliable comparison of the races, the variation among isolates of the same race and the variability of different components of the experimental and measurement errors were

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analysed using variance-component models. The activity was analysed from the culture media in which the fungus was grown. The production of mycelial dry weight in pectin media was also studied and the variability of different components was analysed, as was the correlation between the activity and dry weight. The material presented earlier in an IUFRO Working Party Meeting (PETÄISTÖ and LAPPI 1995) was extended in order to test the experiment × isolate interaction. Furthermore, a comparison between the A- and B-type within Finnish isolates was included.

2 Material and methods

2.1 Material

The *Gremmeniella* isolates used were obtained from D. SKILLING, G. LAFLAMME, A. UOTILA, S. NEVALAINEN and from our own culture collection (Table 1).

Pectin from citrus fruit as the sole carbon source (Sigma, No. P9135) was added at 1% final concentration to Hoagland solution (NIENHAUS 1969) supplemented with biotin,

Table 1. **The isolates used in the experiments 1–5**

Isolate	Host *Pinus*	Country	Collection year	Used in experiment no.
		European race		
Salla Naruska	*sylvestris*	Finland	1990	1
Karvia Alkia	*sylvestris*	Finland	1990	1
Kail.2	*sylvestris*	Finland	1989	1,3,4,5
Puu37	*sylvestris*	Finland	1990	1,5
Cembra	*cembra*	Finland	1990	2,5
Toro2.7	*sylvestris*	Finland	1989	2
Swei	*sylvestris*	Sweden	1991	2
Salla1.5	*sylvestris*	Finland	1992	2
Kankaanranta	*sylvestris*	Finland	1991	2
Sup1.1 × 1.8	*sylvestris*	Finland	1989	3
Joe 67	*cembra*	Finland	1986	4
CF-89-0002	*resinosa*	Canada	1989	3
CF-91-0033	*resinosa*	Canada	1991	3
CF-90-0003	*resinosa*	Canada	1990	4
CF-85-0108	*resinosa*	Canada	1985	4,5
		North American race		
Isolate	Host *Pinus*	Country	Collection year	Used in experiment no.
18-70	*resinosa*	MN, USA	1984	1
18-74	*resinosa*	MI, USA	1989	1,2
18-76	*resinosa*	WI, USA	1990	1
18-50	*resinosa*	MI, USA	1983	2
18-51	*resinosa*	MI, USA	1983	2
SC-60	*contorta*	Alberta, Canada	1975	1
CF-89-0010	*resinosa*	Canada	1989	3,5
CF-90-0037	*banksiana*	Canada	1990	3
CF-91-0032	*banksiana*	Canada	1991	3
CF-91-0066	*banksiana*	Canada	1991	3
CF-87-0010	*banksiana*	Canada	1987	4,5
CF-86-0031	*banksiana*	Canada	1986	4
CF-85-0157	*banksiana*	Canada	1985	4
CF-90-0009	*resinosa*	Canada	1990	4,5

thiamine and pyridoxine at 0.3, 2.8 and 2.8 μM, respectively, and with the following minerals (as their final concentrations): 50 μM H_3BO_3, 10 μM $MnCl_2 \times 4H_2O$, 1 μM $ZnSO_4 \times 7H_2O$, 0.3 μM $Cu_4SO_4 \times 5H_2O$, 0.1 μM $H_2MoO_4 \times H_2O$. Conidia for inoculum were produced on solid media of barley corn and Scots pine needle mass (PETÄISTÖ AND KURKELA 1993).

Five experiments (Exp. 1–5), each consisting of 3–5 isolates of each race, were carried out (Table 1). Four 100 ml flasks with 60 ml pectin medium were inoculated with conidia suspension (conidia from fresh conidia mass) per isolate so that the density was 12800 conidia/ml of medium. The inoculated flasks were incubated on a shaker at 16 °C in light (Osram, L36W/25, Weiss-Universal-White) intensity of about 5–7 μE/m²/s. After 14 days, the mycelium was washed with unused medium and transferred to 40 ml of fresh medium, and cultured for a further 4 days. The mycelial dry weight was measured after drying the mycelium for 20 h at 80 °C.

Polygalacturonase activity was analysed from the culture fluids as described by PETÄISTÖ and KAJANDER (1993), using polygalacturonic acid as substrate and galacturonic acid as the standard at a concentration of 5 μmol/ml–25 μmol/ml. The galacturonic acid produced was measured using the dinitrosalicylic acid (DNS) method (MILLER 1959; BAILEY and PESSA 1990). Triplicate measurements were made of each of the four culture fluids of each isolate. All samples belonging to the same experiment were analysed in one run. The activity is presented as nmol galacturonic acid per mg mycelial dry weight.

Polygalacturonase activity and production of mycelial biomass of the Finnish isolates classified to A- and B-type by A. UOTILA, were tested in two separate experiments (Table 2). The experimental procedure was as in the experiments above.

2.2 Variance stabilization

Preliminary results (PETÄISTÖ and LAPPI 1995) indicated that the variability of the polygalacturonase activity and of the mycelial dry weight within the North American race may be different from the variability within the European race. This study analysed whether these differences are directly related to differences in mean values. When standard deviations of measurements of different experiments were plotted against the mean values of the experiments, an approximately linear trend was found both for polygalacturonase activity and mycelial dry weight (Fig. 1). Because there are several random effects included in the hierarchical structure of the data (isolate, flask and measurement effects), sample standard deviation of measurements within each experiment did not directly correspond to the standard deviation of any single random variable, but can still be used as an approximate pooled indicator of variability.

If the standard deviation of a positive variable is proportional to the mean value in different experiments, then logarithmic transformation can be used to stabilize the variance across experiments. Because the activity is measured against the standard value, activity can also produce negative values. In the data in this study there was one negative value, −8.9 nmol/mg. Thus, the activity data were analysed using a logarithm of activity +20 as the dependent variable. The dry-weight data were analysed using log dry weight as the dependent variable. Note that it is not yet assumed that the logarithmic variables have equal variances for both races as this will be statistically tested. Differences and standard deviations in the logarithmic scale (multiplied by 100) can be roughly interpreted as percentages in relative comparisons (relative difference is the first-order Taylor series approximation for the log difference).

2.3 Statistical analysis

The purpose of the data analysis was to study the structure of the experimental errors, variability between isolates, differences between races and correlation between polygalacturonase activity and mycelial dry weight. The data were analysed using mixed linear

Table 2. **The Finnish isolates of A- and B-type from** *Pinus sylvestris* used in the additional experiments. Isolation and classification to types were made by Dr. Antti Uotila, University of Helsinki, Forest Field Station, Korkeakoski, Finland

		Exp. 6				Exp.7	
Isolate	Type	Collection year	Origin	Isolate	Type	Collection year	Origin
GA 17.6	A	1992	mycelium	MH1.1	A	1993	mono-ascospore
Ahvenlampi	A	1992	mycelium	MH1.4	A	1993	mono-ascospore
Hyytiälä	A	1992	conidium	Oulanka 1-kur	A	1993	conidium
Viheriäistenneva	A	1992	conidium				
Toro2.1	B	1989	mono-ascospore	Suo2.1	B	1993	mono-ascospore
Sup1.1 × 1.8	B	1989	pairing, uniascus monospores	PAT2.1	B	1993	mono-ascospore
Sup1.1 × 1.5	B	1991	pairing, uniascus monospores	PAT2.2	B	1993	mono-ascospore
Kai1.2	B	1989	mono-ascospore	PAT2.3	B	1993	mono-ascospore

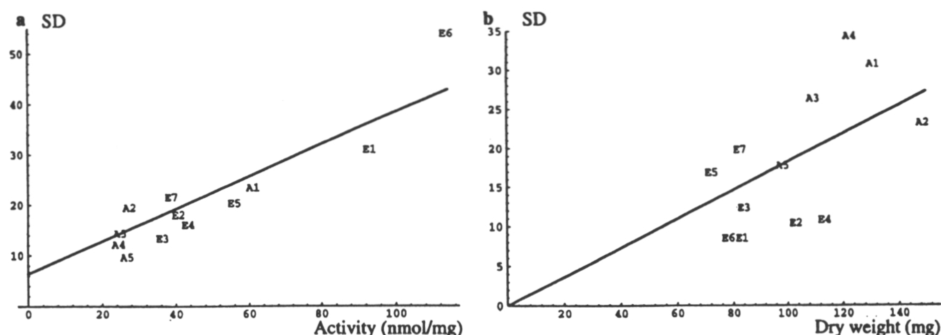

Fig. 1. The sample standard deviations within experiments with respect to the experiment means for the European race (E1–E7 = experiments 1–7) and for the American race (A1–A5 = experiments 1–5). Subfigure a is for the activity (nmol galacturonic acid/mg mycelial dry weight), and subfigure b for dry weight (mg/flask). The subfigure a contains the regression line obtained by regressing standard deviation of activity on mean+20 (SD = 0.31 (mean+20), $R^2 = 0.82$); subfigure b shows the regression line between SD of dry weight and mean (SD = 0.18 × mean, $R^2 = 0.38$)

models (SEARLE 1971). Computations were performed with the MIXED procedure of the SAS package (SAS Institute 1992). REML (Restricted Maximum Likelihood) was used as the estimation method (thus assuming normality of random effects).

Different variance–covariance structures were compared using the χ^2 approximation for the log-likelihood-ratio statistic G (e.g. SOKAL and ROHLF 1995). Fixed effects (race differences) were tested with F-tests.

It was assumed that isolates used in the experiments were a random sample from a population of isolates within both races (and within types A and B in the additional experiments). Isolate effects were thus assumed to be random effects. The results indicated differences between all the experiments. Because the growing conditions in the four experiments were the same, the experiment effects were also treated as random effects. By treating experiment and isolate effects as random, the tests for race differences obtained have a more general interpretation (they apply the 'broad inference space'; see SAS institute (1992)). If experiment and isolate effects were treated as fixed (which would be statistically valid as such), the tests would apply to the given values of the isolate and experiment effects present in the data.

The logarithm of activity + 20 (nmol/mg), denoted as y_a, was analysed using the following linear model:

$$y_{areifm} = \mu_a + \beta_{ar} + \gamma_{ae} + \lambda_{are} + v_{ari} + q_{arei} + u_{areif} + o_{areifm} \quad (1)$$

where y_{areifm} is the log(activity + 20) of measurement m of flask f of isolate i in experiment e for race (serovar) r, μ_a is the fixed overall mean, β_{ar} is the fixed race effect of race r, γ_{ae} is the random experiment effect for experiment e, λ_{are} is the random experiment × race interaction (allowing different experiment effects for both races), v_{ari} is the random isolate effect of isolate i of race r, q_{arei} is the random isolate × experiment interaction (allowing the same isolate to behave differently in different experiments), u_{areif} is the random flask effect, and o_{areifm} is the random error between measurements of the same flask. The variances of the random effects are assumed to be fixed constants within both races (e.g. variance of isolate effects is the same in all experiments). When model (1) is estimated separately for both races (combining race effect β with the race-specific μ and excluding experiment × race interaction), we get the most general model, which allows the variances of European race to be different from the corresponding variances of the American race.

The mycelial dry weight, y_w was analysed with a model of the same structure:

$$y_{wreif} = \mu_w + \beta_{wr} + \gamma_{we} + \lambda_{wre} + v_{wri} + q_{wrei} + u_{wreif} \quad (2)$$

where the terms have the same interpretation as in the activity model except that there is only one measurement for each flask, and thus the model contains no separate measurement term for each flask. Different random effects for the same dependent variable are assumed to be uncorrelated. But the random effects in the activity model may be correlated with the random effects of the same factor in the dry-weight model. For instance, the random isolate effect of the activity v_{ari} may be correlated with the isolate effect of the dry weight v_{wri}, and flask effects u_{areif} and u_{wreif} may be correlated.

Correlation between activity and dry weight was studied using flask averages of activity measurements. The correlation was studied using a simpler model where experiment effects are fixed and no experiment × isolate effects were included (the interactions are pooled into the flask component of the model). When estimating this multivariate model, activity and dry weight are defined as 'repeated measurements' in the MIXED-routine of SAS. When the activity and dry weight models are estimated separately, the sum of the log-likelihood values is the log-likelihood value for the model assuming no correlation (if there is no correlation, the probability of a two-dimensional (activity, dry-weight) observation is the product of activity probability and dry weight probability, thus leading to simple addition in the calculation of the log-likelihood value).

The difference between activity of A and B types of Finnish isolates was studied using a simpler model:

$$y_{ateifm} = \mu_a + \beta_{at} + \gamma_{ae} + v_{ati} + u_{ateif} + o_{ateifm} \quad (3)$$

where the dependent variable is log (activity + 20), t indicates the type, β_{at} is a fixed type effect and other terms are defined as in (1). The model for dry weight was similar, except it did not contain any measurement-error effect for flasks.

3 Results

Different models for the activity (using logarithm of activity+20 as dependent variable) were tested against the general model (1), where the variances were also assumed to be different in the two races. It was found that the most parsimonious description of the data is the model which assumes that the mean values of the races are different but the variability of both races is the same and that there is no experiment × isolate interaction or no experiment × race interaction. The p-value obtained for testing this model against the general model is 0.0938 (i.e. this model does not deviate significantly from the most detailed model for the data).

The estimated mean of the transformed activity was 0.31 larger for the European race than for the American race (3.90 for the American race and 4.21 for the European race). The standard error of this race difference was 0.06, and $p < 0.001$ when testing if this difference is zero. The difference between races and variability among isolates, even if statistically significant, are rather small in comparison to variability relating to the experimental errors (variances of experiment, flask and measurement effects; Fig. 2a). The estimated mean in the original activity scale is 47.4 nmol/mg for the European race and 29.4 nmol/mg for the American race.

The most parsimonious model for the logarithm of the dry weight included the fixed race effect and random experiment, isolate, experiment × isolate and flask effects, the variances of the random effects being equal for the two races. The overall p-value was 0.17 when testing if variances of races are different and if there is experiment × race interaction. The estimated mean of the European race was 0.28 (with standard error 0.08, $p < 0.001$) smaller than the mean of the American race. The estimated mean for the European race was 4.47 in the

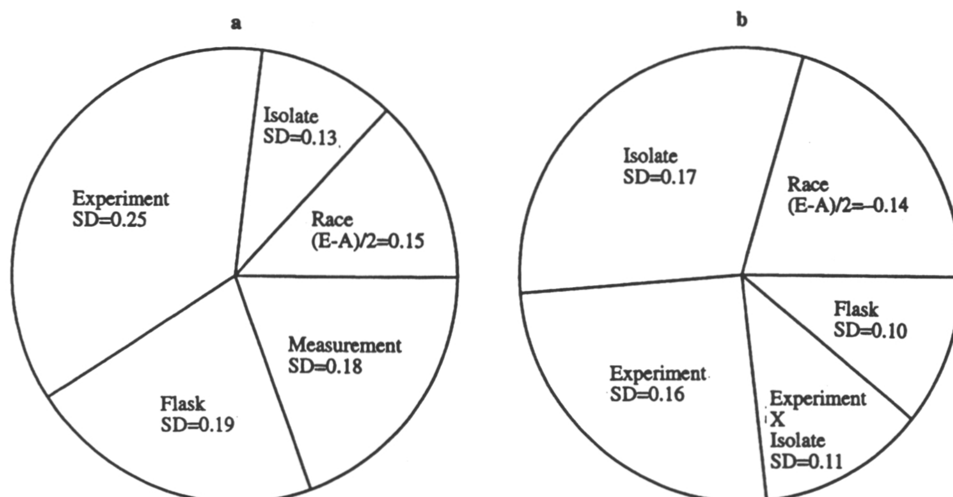

Fig. 2. Relative variances (proportional to the sector areas) of different effects of the log (activity+20); (a) and log (dry weight; b). The fixed-race effects were made comparable to the variances of the random effects by computing the variance of the random variable, which is equal to the mean of the European race with probability 1/2 and to the mean of the American race with probability 1/2 (i.e. 'variance of the race effect' is obtained by squaring the half of estimated difference between races). Within eaeh sector, the corresponding estimated standard deviation (half of the estimated race difference (E-A) for the race effect) is also shown. Standard deviations multiplied by 100 can be interpreted to be in a %-scale

logarithmic scale (86.9 mg in the arithmetic scale) and 4.75 for the American race (115.1 mg in the arithmetic scale).

The significant experiment × isolate effect ($p < 0.001$, the likelihood-ratio test is made using the accepted model as the full model) indicates that isolates did not behave quite consistently in different experiments, even if the estimated variance of the interaction term is rather small compared to the variance of the isolate effect and to the variance of the experiment effect (Fig. 2b). The difference between races and variability between isolates explain a larger part of the total variance in dry weight than the activity. The main reason for this is that there is no measurement variability within the flask as the whole mycelial amount in the flask is measured.

The likelihood-ratio test against the more general model, allowing correlations between random effects of activity and dry weight, indicated that there is no correlation between activity and dry weight ($p = 0.70$). Note that the sample correlation coefficient between activity and dry weight is -0.25 when computed directly from the raw data, but this apparent correlation is described by the fixed part of the model, i.e. by the fact that the European race has larger activity and smaller dry weight than the American race.

When studying the difference between A- and B-type (using experiments 6 and 7), the analysis was made both treating isolates derived from different ascospores of the same ascus as the same isolate (analysis 1) or separate isolates (analysis 2). The conclusions were the same using both definitions of the isolate. The activity of type A did not differ significantly ($p = 0.87$ in analysis 1 and $p = 0.64$ in analysis 2) from the activity of type B. The difference between experiments was significant ($p < 0.001$). The estimated average activity in the experiments was 61.0 nmol/mg for type A and 63.0 nmol/mg for type B.

For the logarithm of the dry weight, type A had 0.14 (indicating 14% relative difference) smaller estimated mean than type B. The standard error of the difference was 0.07 in analysis 1 ($p = 0.039$) and 0.06 in analysis 2 ($p = 0.029$). The estimated average dry-weight production

of type A was 73.3 mg, and 86.3 mg in type B. The estimated variances of the random effects were of the same magnitude as the previous results both for activity and dry weight.

4 Discussion

The results of the five *in vitro* experiments indicated that European race produced more polygalacturonase activity than the North American race. Furthermore, the mycelial dry-matter production of the North American race was greater in pectin media than that of the European race. The earlier analysis (Petäistö and Lappi 1995) indicated that, in addition to the differences in mean values of activity and dry weight, variances are also different in the two races. In this study, it was found that increased variance is so closely related to the increased mean value that logarithmic transformation removed racial differences in variances.

The variance-component model used to analyse deviations from the racial means serves three purposes. First, a proper account for different sources of variability is necessary for making valid tests for race differences, even if one would not be interested in the structure of variation as such. Second, the estimated variance of the random isolate effect helps us to compare the variability among isolates with differences in race means. It was found that, even if the race means of both mycelial activity and dry weight are different, the isolate distributions overlap. The standard deviation of isolate effects was about 50% of the race difference for both variables. Third, the variance components relating to experimental errors (experiment, flask, measurement) help us to get an idea of the possible problems and weaknesses of the experimental set-up used.

There is large variability from different sources (experiment, flask, measurement) in activity results. The activity analysis method used is known to be susceptible to variation (Bailey et al. 1992), and there may be therefore some differences between experiments. However, because the differences were also found in dry weight, the variability also indicated sensitivity of fungal metabolism to some unidentified factors. Differences exist although the conditions in all the experiments were adjusted to be the same; variability was found even between the flasks in each isolate in each experiment.

Experiment × isolate interaction found in dry weight indicate that the isolates respond differently to the small unidentified variations between experiments. No significant experiment × isolate interaction was found in polygalacturonase activity, but there should be more repeated isolates (isolates which are used in more than one experiment) before experiment × isolate interaction can be ruled out. While the interaction was found in dry weight, it might indicate that some other pectin-degrading enzyme also supporting the growth in pectin media, is more sensitive than the polygalacturonase analysed.

The two races grew differently in liquid cultures. Mycelia of the European race were more sparse than those of the North American race. The mycelia of the North American race grew tightly and secreted slimy material on their surface. The various growth characters were also documented on a solid agar medium by Dorworth and Krywienczyk (1975): the European race produced more aerial mycelium per mm^2 of colony area, whereas the North American isolates often had more appressed mycelia.

It would be interesting to know whether the higher polygalacturonase activity production of the European race found *in vitro* also reflects the amount of enzyme activity in nature. The European race was found to be more virulent (Dorworth et al. 1977; Skilling 1977) and there might be some connection excreted polygalacturonase activity.

No significant difference between the studied A- and B-type isolates in the secretion of polygalacturonase activity *in vitro* was found. Because the number of isolates and replicates was small in this study of the types, no conclusion concerning secreted activity and the A- and B-type can be made without further studies.

Between the types, difference was found in mycelial dry weight. The B-type showed higher mycelial dry-matter yield than the A-type in liquid pectin medium at 16 °C. Uotila

(1983) considered growth at 15 °C as one of the criteria for differentiation of the A-type from the B-type with Finnish isolates. The growth was measured as colony diameter *in vitro* in solid media and the A-type appeared to grow better. These different results could represent a pseudo-difference between the studies due to the different methods (mycelial dry matter/colonial diameter) of measuring the growth or indicate metabolic differences between the types, resulting in better growth in A- or B-types, depending on the state or contents of the medium.

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Resume

Aptitude des races européenne et nord américaine de Gremmeniella abietina *à hydrolyser* in vitro *l'acide polygalacturonique*

La polygalacturonase est une des premières enzymes sécrétées par les pathogènes durant l'infection. L'activité d'hydrolyse de l'acide polygalacturonique de la race nord américaine et de la race européenne de *Gremmeniella abietina*, ont été comparées *in vitro*. Les isolats ont été cultivés sur pectine pure où l'activité enzymatique a été analyssée. Au total 29 isolats one été testés dans cinq expériences (séries expérimentales an enceinte contrôlée). Les données ont été analysées suivant un modèle de décomposition de la variance, avec l'effet 'race' considéré comme fixe et les effets 'expérience', 'isolat', 'récipient' et 'mesure' comme aléatoires. La race européenne sécrétait plus d'enzyme que la race nord américaine et le poids sec de mycélium produit était plus faible chez la race européenne. Les différences entre races étaient du même ordre de grandeur qu'entre isolats de chaque race; la variance liée aux erreurs expérimentales était assez grande. Dans chaque race, aucune corrélation n'a été trouvée entre l'activité et la production de matière sèche mycélienne. La tranformation logarithmique a fait disparaître les apparentes différences raciales pour la variabilité de l'activité et du poids sec du mycélium. Les résultats obtenus avec des isolats finlandais de types A et B montraient des différences de production de matière sèche.

Zusammenfassung

Die Fähigkeit der europäischen und nordamerikanischen Rasse von Gremmeniella abietina, *Polygalacturonsäure* in vitro *zu hydrolysieren*

Polygalacturonase ist eines der ersten Enzyme, das von einem Pathogen während der Infektion abgegeben wird. Die europäische und die nordamerikanische Rasse von *Gremmeniella abietina* wurden hinsichtlich ihres Vermögens, *in vitro* Polygalacturonsäure zu hydrolysieren, verglichen. Die Isolate wurden in reinem Pektinmedium aufgezogen, um die Enzymaktivität zu messen. Es wurden insgesamt 29 Isolate in fünf Experimenten (Versuchseinheiten in einer Klimakammer) untersucht. Die Auswertung der Daten erfolgte mittels Varianzanalyse basierend auf einem 'variance component model'. Dabei wurden die Einflüsse des Experiments, des Isolats, der Kulturflasche und der Messung als zufällige Effekte sowie der Einfluß der Rasse als fixed Effekt betrachtet. Die europäische Rasse produzierte mehr Polygalacturonase als die nordamerikanische Rasse, dafür was das Trockengewicht des Myzels der europäischen Rasse geringer. Der Unterschied zwischen den Rassen lag jedoch in der gleichen Größenordnung wie derjenige zwischen den Isolaten der gleichen Rasse; der Anteil der experimentellen Fehler an der Gesamtvarianz was groß. Zwischen der Enzymaktivität und dem Myzeltrockengewicht konnte keine Korrelation gefunden werden. Eine Logarithmustransformation der Daten beseitigte die scheinbaren Unterschiede zwischen den Rassen bezüglich der Variabilität der Enzymaktivität und des Myzeltrockengewichts. Finnische A- und B-Isolate zeigten Unterschiede im Myzeltrockengewicht.

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Analysis of the protein pattern of *Gremmeniella abietina* with special reference to protease activity

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Abstract: *Gremmeniella abietina* is the causative agent in Scleroderris canker of conifers. This fungus grows very slowly and thus its protein pattern may be difficult to obtain, especially since fungal protease activities are unknown. We show that the protease activity is very low in young cultures grown in Campbell's V8 juice liquid medium but degraded some of the protein bands making it very difficult to reliably compare electrophoretic protein pattern from sample to sample. A calcium- and zinc-dependent metalloprotease activity was specifically detected, and it was inhibited by ethylenediaminetetraacetate and o-phenanthroline at a neutral pH. Other protease activities may be present since inhibition of the proteolytic activity by p-chloromercuribenzoate and pepstatin was also seen. Phenylmethanesulfonyl fluoride showed only minimal inhibitory effect. The fungal protein patterns were reproducible with only small intensity differences between different culture batches. The protein pattern resembled that of *Sirococcus strobilinus,* but immunoblotting with anti-*Gremmeniella* antibody showed no cross-reactivity and thus *Sirococcus* may not be closely related to *Gremmeniella.*

Key Words: *Ascocalyx, Gremmeniella,* immunotyping, protease, *Sirococcus*

INTRODUCTION

Scleroderris canker, *Gremmeniella abietina* (Lagerb.) Morelet [*Scleroderris lagerbergii* Gremmen, *Ascocalyx abietina* (Lagerb.) Schlaepher] is a serious problem in forestry in northern Europe, Asia and North America. Taxonomic studies (Petrini et al., 1989) have indicated that *Gremmeniella abietina* var. *abietina* includes North American, European and Asian serotypes. The following species and varieties have been separated: *Gremmeniella abietina* (Lagerb.) Morelet var. *balsamea* O. Petrini et al. *var. nov.*, and *Gremmeniella laricina* (L. Ettlinger) P. Petrini et al. The difference in virulence between the European and North American race is known (Skilling, 1977; Setliff et al., 1975). The mechanism and possible variation of pathogenesis within those races are unknown.

Many different methods have been used in the characterization of different *Gremmeniella* isolates and races. Conidial measurements (length, width, septation), growth rate and temperature dependence of growth have been used (Petrini et al., 1989; Dorworth and Krywienczyk, 1975; Stephan, 1979; Uotila, 1983). In addition, immunological methods, such as Ouchterlony double diffusion analysis (Dorworth and Krywienczyk, 1975; Skilling et al., 1984) or Western blots (Ouellette et al., 1988), have been used to separate the European and North American races. A comparison of the soluble proteins of isolates has been made by nondenaturing acrylamide gel electrophoresis (Petrini et al., 1989; Ouellette et al., 1988; Dorworth, 1974; Benhamou et al., 1984; Petrini et al., 1990). Separation of protein bands under native conditions is subject to variations due to subunit aggregation, disulfide bridging or modifications by carbohydrate content, phosphorylation, acetylation, phenolation, etc. Some of these modifications, such as cross-linking due to phenolic compounds, may take place during sample preparation (Loomis and Battaile, 1966). Furthermore, it is not known if such modifications vary according to the type of cultivation or age of the culture. Electrophoretic separation under denaturing conditions is generally less vulnerable with respect to modifications and results in reproducible patterns. We have now established a culture collection of *G. abietina* for the type analysis with respect to pathogenicity. In this report, sample preparation techniques were studied for reliable protein pattern comparison using SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) and Western blotting with special reference to fungal proteolytic activity. This study was important because this fungus grows very slowly, and thus the electrophoretic protein patterns of the fungus and the proteins secreted to the medium may be compromised by protease activity.

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MATERIAL AND METHODS

Culture of isolates studied.—Three of the 11 isolates used in this study were cultures obtained from a single ascospore which originated from northern Finland (Toro, Sup1.1, and Kima, isolated by Dr. Antti Uotila, Univ. of Helsinki, Finland). The Finnish isolate Cembra was cultured from a single conidium by R.-L. Petäistö. Other isolates were from Norway (Hedmark, initiated from conidia by Martti Vuorinen, Finnish Forest Research Institute, Finland). Japan (SJ-2 from Kuttchian, Hokkaido; SJ-3 from Kimobetsu, Hokkaido; both from single conidia provided by Shun-Ichi Yokota, Hokkaido) and North America (18-50 from Osteco Co., Michigan; 18-70 from Superior National Forest, Michigan; 18-76 from Chequamegon National Forest, Wisconsin and 19-132 from Newfoundland, Canada; all from single conidia provided by Dr. Darrol Skilling, Minnesota). The potato dextrose agar medium (PDA, Difco) was made according to the manufacturer's instructions. For V8-medium, one part of Campbell's V8-vegetable juice was mixed with four parts of water and filtered twice through MN (Macherey-Nagel) 640 W-filter paper. Glucose was added to a final concentration of 2% and the medium (pH 4.4) was autoclaved. Liquid cultures were initiated with conidia (final density about 25–30 × 10^3/ml). The conidia were produced on solid medium made of barley groats and Scots pine needle homogenate (10 g barley groats, 2.2 g needle homogenate, 20 ml distilled H_2O, autoclaved at 120 C for 20 min twice with 1 day interval) at 16 C in artificial light (Osram L36W/25, Swiss Universal White, Germany, 1 m above the cultures). Cultures were incubated at 16 C in the dark. *Sirococcus strobilinus* (G. Preuss) Petrak (*Discella strobilina* (Desm.) Died.), originated from Kviteseid in Norway, was obtained from the culture collection of Roll-Hansen and was also cultured in V8 medium. *Fusarium* sp. was isolated by us from Scots pine seedlings in Finland and cultured in V8 medium.

Preparation of mycelium for protein pattern.—Mycelium was separated from liquid cultures by filtering, washed with 20 ml of phosphate buffered saline (PBS) per g mycelium [PBS: NaCl 6.8 g, $Na_2HPO_4 \cdot 2H_2O$ 1.88 g, KH_2PO_4 0.2 g, deionized (Milli-Q, Millipore) H_2O to 1 L, pH 7.4], and then frozen. Routinely, homogenization was carried out by pressing 4–5 times at −20 C with X-press equipment (Biox, Järfälla, Sweden) followed by centrifugation at 14 000 × *g* for 10 min at 4 C. The supernatant fractions were stored frozen. In some experiments, the supernatant was precipitated with acetone as described in results. For SDS-PAGE, four parts of the supernatant were mixed with one part of sample buffer, pH 6.9 (0.325 M Tris-HCl, 10% SDS, 50% glycerol, 0.05% bromophenol blue, 20% mercaptoethanol). The samples were boiled at 100 C for 10 min and centrifuged as above, and the supernatant fraction was used for SDS-PAGE using 14% polyacrylamide gels that were prepared and run with a Bio-Rad (Richmond, California) Mini-Protean II dual slab cell following the manufacturer's instructions.

Mycelium was carefully taken from solid PDA media avoiding the agar. The mycelium was washed with PBS and pressed 5 times with the X-press. Sonication was also tested as a method for homogenization; a 0.5-ml sample was sonicated 5 times for 3 sec on ice at a low power level of 50 W (Labsonic 2000, B. Braun, Melsungen, Germany).

Studies on protease activity and its effects on SDS-PAGE.—To study the presence of excreted proteases and whether these fungal protease activities hamper protein pattern measurement, the medium from 17-day Hedmark V8-cultures was separated aseptically by filtering and used for testing possible excreted protease activity. Samples from the used and unused culture media (as a control) were divided into four portions and the pH was adjusted to 6 or 7.4. To half of the samples, the protein marker mixture was added [thyroglobulin (669 kD), ferritin (440 kD), catalase (232 kD), acetate dehydrogenase (140 kD) and bovine serum albumin (67 kD), from Kabi-Pharmacia, Uppsala, Sweden].

Mycelial protease activity was tested as follows: 1 g of washed mycelium was added to 1 ml deionized H_2O containing 5 mM mercaptoethanol and homogenized (as frozen) with X-press. The homogenate was divided into two parts and their pH was adjusted to 6 and 7.4, using concentrated phosphate buffer ($^1/_{10}$ of the volume, final concentration 20 mM phosphate buffer in 150 mM NaCl). Then the samples were centrifuged and supernatant fractions were filtered through a 0.22-μm filter.

All samples were incubated at 24 C for 0–18 h and stored at −80 C until analyzed with SDS-PAGE.

Supernatant fraction of mycelial homogenate from 14-day-old Sup1.1 was used to study the effect of general protease inhibitors and divalent cations on the detected protease activity. The tested inhibitors were 5 mM p-chloromercuribenzoate (pCMB), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/ml pepstatin, 10 mM ethylenediaminetetraacetate (EDTA) and 1 mM o-phenanthroline. Divalent cations were SeO_2, $ZnCl_2$, $MgCl_2$, $CaCl_2$, $FeSO_4$, all in 1 mM concentration. The inhibitors and cation salts were from Sigma (St. Louis, Missouri) or E. Merck (Darmstadt, Germany).

Effects of sample preparation techniques on the protein pattern.—The effects of the serine protease inhibitor PMSF, the phenol adsorbing polyvinylpyrrolidone and phenol oxidase inhibitor thiourea on the protein pat-

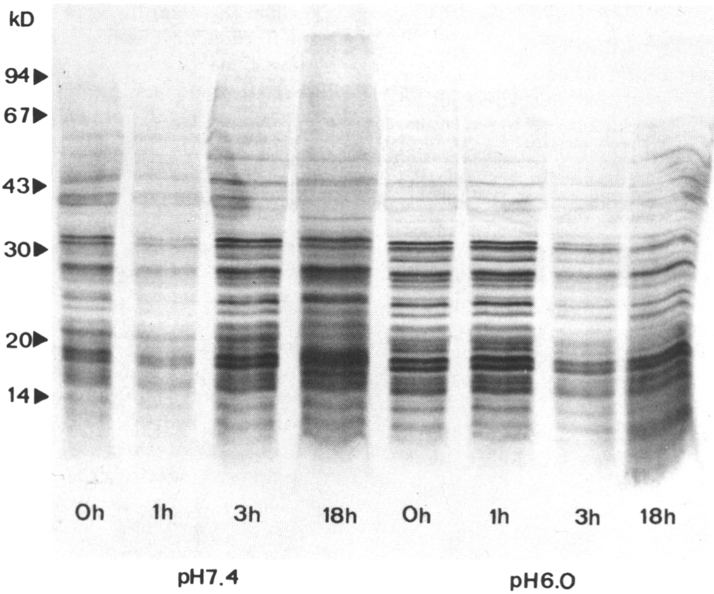

FIG. 1. Effect of pH and incubation time on endogenous protease activity of *G. abietina*. Supernatant fraction of mycelium from 14-day-old culture of Sup1.1 isolate incubated for 0–18 h at 24 C at pH 7.4 (four lanes on the left) and pH 6 (four lanes on the right). Samples (20 μg) were then subjected to SDS-PAGE on 14% gels. Numbers on the left side refer to the molecular weights of the standards in kD and at the bottom to incubation hours.

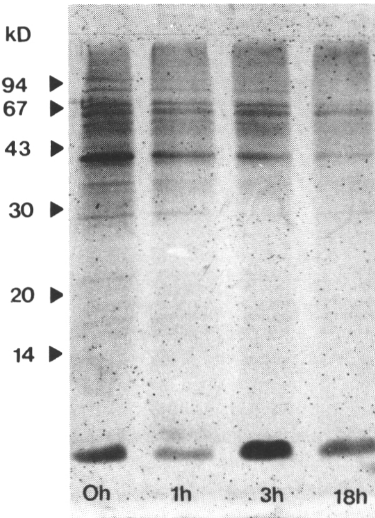

FIG. 2. SDS-PAGE of *G. abietina* and endogenous protease activity. Supernatant fraction of mycelium from 77-day-old culture of Toro isolate incubated for 0–18 h at 24 C at pH 7.4. Samples (20 μg) were then subjected to SDS-PAGE on 14% gels. Numbers on the left side refer to the molecular weights of the standards in kD and at the bottom to incubation hours.

tern (by SDS-PAGE) were tested using a Hedmark V8-culture. After culture for 14 days, one portion of mycelium was homogenized without inhibitors, another with 1 mM PMSF and the third with 1 mM PMSF, 10 mM thiourea and 25 mg/ml polyvinylpyrrolidone.

To test the effects of acetone or perchloric acid precipitation of the sample on the sharpness of the protein bands in SDS-PAGE and in Western blot, two volumes of acetone were added per one volume of supernatant from 2-wk-old Toro culture at 4 C, and kept at −20 C for at least 2 h followed by centrifugation at 14 000 × *g* for 10 min. The precipitate was dissolved with PBS. The control sample was prepared without acetone treatment. Perchloric acid was added at 10% concentration to homogenized mycelium and kept overnight at 20 C followed by centrifugation as above. The precipitate was dissolved with the original volume of 20 mM potassium phosphate buffer, pH 7.4, or SDS-PAGE buffer.

Immunization and immunoblotting.—Antibodies against the Toro, Sup1.1, Kima and Hedmark isolates were prepared in rabbits. Each animal received subcutaneously 1 ml of 2% (wet w/v) mycelium in 50% Freund's complete adjuvant (Sigma). About 300-μl boosters were injected without adjuvant into the ear vein after 2 wk followed by three more boosters subcutaneously at

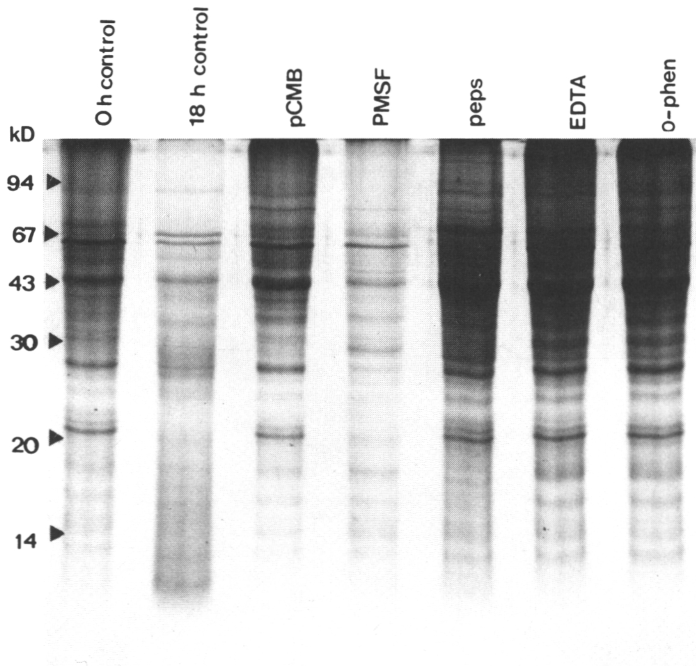

FIG. 3. Effect of inhibitors on endogenous protease activity. The effect of general protease inhibitors on the observed proteolytic activity of the supernatant fraction of mycelium from 14-day-old culture of Sup1.1 isolate incubated at pH 7.4 for 18 h together with 0 h and 18 h controls. Five millimolar p-chloromercuribenzoate (pCMB), 1 mM phenylmethanesulfonyl fluoride (PMSF), 10 μg/ml pepstatin (peps), 10 mM ethylenediaminetetraacetate (EDTA), and 1 mM o-phenanthroline (o-phen) were used.

1-wk intervals. The animals were sacrificed under anesthesia by cardiac puncture and serum was separated and stored at −20 C. For immunoblotting, fungus samples containing about 20 μg of protein were loaded on 14% SDS-PAGE gels. Proteins were transferred on nitrocellulose or Immobilon-P® (Millipore) PVDF-membrane which was then saturated with 2% fat-free powdered milk in PBS for 30 min, and washed with PBS, and antiserum was added (at a dilution of 1:100) in powdered milk-PBS-solution (Kajander et al., 1989). After 4 h at 20 C, the membrane was washed 3 times with PBS and incubated for 1 h with alkaline phosphatase-conjugated goat antirabbit immunoglobulins (1:500) from Zymed (South San Francisco, California). After five washes, the membranes were developed either with 1 mg/ml of 5-bromo-4-chloro-indol-3-yl phosphate or in combination with nitro blue tetrazolium (Harlow and Lane, 1988) in 0.15 M 2-methyl-2-aminopropyl-1-ol buffer.

RESULTS AND DISCUSSION

Culture of G. abietina.—Culturing for about 1–2 months is needed to produce conidia for establishing cultures from single spores followed by an additional 1–2 months to obtain the final culture. Thereafter, culture for at least 2 wk is needed to obtain samples for protein analysis. The protein patterns should be easily obtainable, reproducible and their variation with culture age should be known. Less than 1 mg of soluble protein could be obtained from 1 g of fungus with the methods used (X-press, sonication, SDS-boiling) from old agar cultures. This might have been due to difficulties in obtaining an agar-free sample (agar may interfere with X-press), or due to lack of soluble protein due to high protease activity, or due to covalent coupling of proteins, e.g., by enzymic reactions liberating reactive phenol compounds. However, PMSF, polyvinylpyrrolidone and thiourea did not improve the yield of soluble protein.

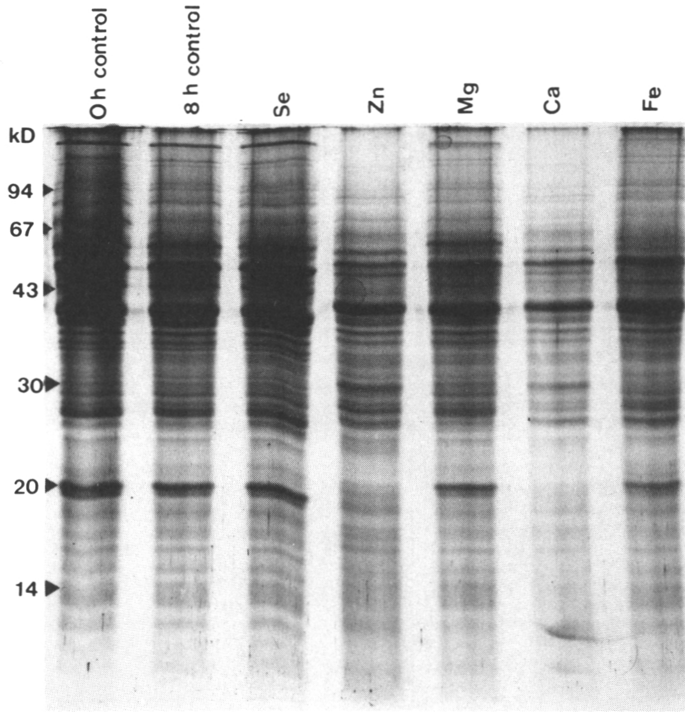

FIG. 4. Effect of divalent cations on endogenous protease activity. The effect of divalent cations on the observed proteolytic activity of the supernatant fraction of mycelium from 14-day-old culture of Sup1.1 isolate incubated at pH 7.4 for 8 h. Divalent cations were all 1-mM SeO_2, $ZnCl_2$, $MgCl_2$, $CaCl_2$, and $FeSO_4$.

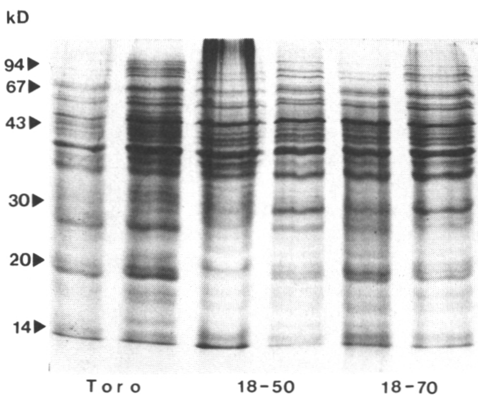

FIG. 5. Reproducibility of the protein pattern of *Gremmeniella abietina*. Supernatant fractions from two separate 14-day-old cultures of Toro, 18-50 and 18-70 subjected to SDS-PAGE using 14% gels.

Liquid cultures on V8 medium provided good protein yields of up to 40 mg soluble protein per gram of fungus. The protein content of V8 medium is very low after filtration and the medium can be easily removed by washing the mycelia. For these reasons, the protein patterns were studied using liquid cultures.

Effect of endogenous protease activity on the mycelial protein pattern in SDS-PAGE.—To prevent possible proteolysis, Benhamou et al. (1984) added PMSF to protein samples of *G. abietina*, although the effectiveness of PMSF was not documented. In this study, addition of 1 mM PMSF to the homogenization medium did not change the protein pattern obtained. To study further the possible presence of proteases, samples of the isolates Hedmark, Sup1.1 and Toro were homogenized with X-press followed by pH adjustment either to 6 or 7.4, and then the supernatant fractions were separated. Assay of acidic and neutral proteases was carried out by analyzing degradation of the soluble proteins. No evidence of protease activity was detected in 17-day Hedmark or 14-day Toro cultures at either pH

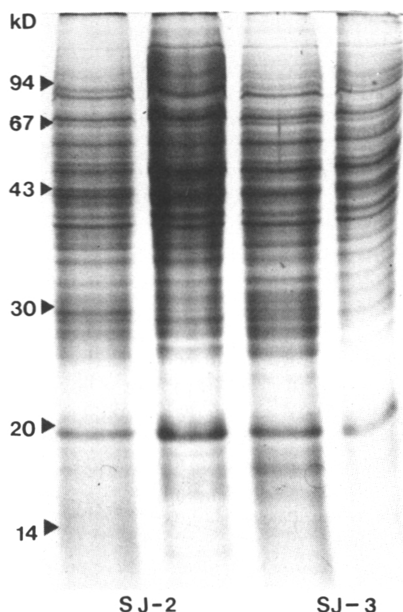

FIG. 6. Reproducibility of the protein pattern of *Gremmeniella abietina.* Supernatant fractions from two separate 14-day-old cultures of SJ-2 and SJ-3 subjected to SDS-PAGE using 14% gels.

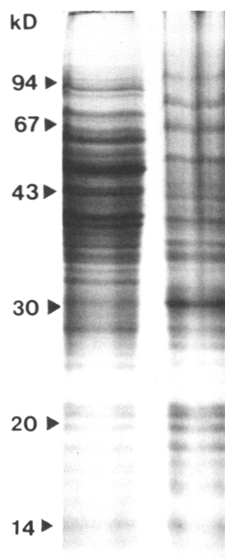

FIG. 7. The effect of duration of storage on protein pattern. After storing for about 6 months including repeated freeze-thawings, the protein pattern of 18-50 isolate in the right lane has markedly deteriorated as compared to the fresh sample of the same isolate.

6 or 7.4. However, FIG. 1 shows Sup1.1 patterns (14-day culture) in a similar experiment in which the double band of the molecular weight of 40–42 kD was reduced and a 38 kD band was increased in intensity at pH 7.4 after 18 h incubation. In addition, 42-day-old Hedmark culture showed proteolysis of a 19 kD peptide after 18 h incubation at both pHs. A sample from a 77-day-old culture of Toro showed widely degraded protein bands and a 40–42 kD band was reduced prominently after 18 h incubation at pH 7.4 (FIG. 2). The protein pattern obtained differed considerably from that seen in young Toro cultures (see FIG. 5).

In conclusion, protease activity was absent or very low in mycelium samples from 2-wk-old cultures. The SDS-PAGE pattern of soluble proteins was stable for several hours when no protease inhibitors were present at 24 C. Protease activity was detectable in old cultures. As seen in FIG. 2, protein pattern in old Toro culture was very different from that in young Toro culture (FIG. 5), probably due to proteolytic autodegradation in old cultures. This is suggested also by the presence of small molecular weight peptides in SDS-PAGE.

Effect of inhibitors and divalent cations on mycelial protease activity.—Pepstatin, pCMB, EDTA and o-phenanthroline inhibited mycelial protease activity (FIG. 3). Differences in proteolytic degradation could especially be seen for proteins of molecular weights of 70, 60, 50, 40–42, 36–38, 33 and about 21 kD. A somewhat different protein pattern was observed with the inhibitors used, suggesting the presence of several different proteolytic enzymes. However, PMSF did not markedly prevent proteolysis. These results are clear evidence for the presence of metalloprotease(s), which are in general inhibited by EDTA and o-phenanthroline. Results also indicate the presence of small amounts of aspartate- and cysteine-class proteases, since inhibition by pepstatin and pCMB was observed, respectively. Mycelium of Sup1.1 showed little or no serine protease activity, since PMSF had no inhibitory effect.

The presence of metalloproteases was further confirmed by the addition of divalent cations (FIG. 4). Zinc and calcium markedly activated the proteolysis. Iron was a weaker activator, whereas selenium and magnesium did not have any effect under the experimental conditions. The activated proteolysis by zinc and calcium was prominent for the proteins of the molecular weights of over 100, 60 and 21 kD. Iron activated the disappearance of over 100 and 60 kD proteins.

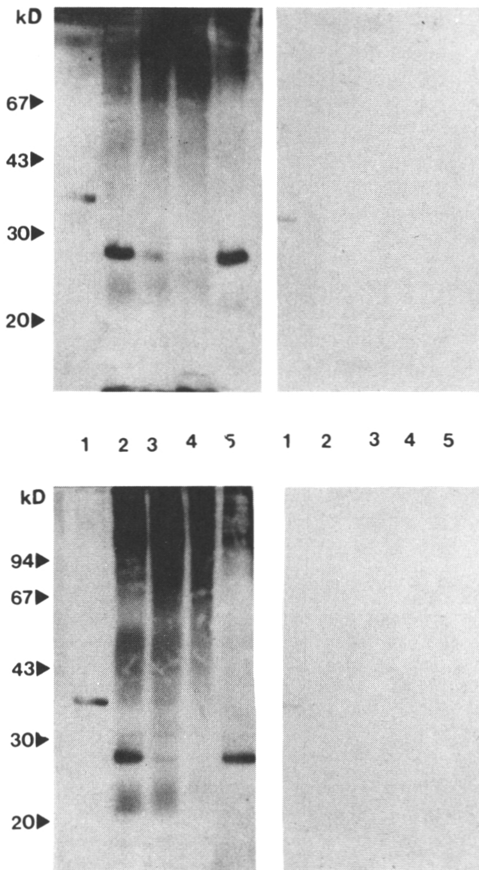

FIG. 8. Immunoblots of *Gremmeniella abietina* and *Sirococcus strobilinus*, and the effect of acid precipitation on the antigens. In all four immunoblots the antigen patterns are identical: lane 1, *Sirococcus strobilinus*; 2, *Gremmeniella* isolate Toro; 3, isolate Sup1.1; 4, isolate Hedmark; 5, isolate Kima. Each lane was obtained by subjecting 20 μg of soluble proteins to SDS-PAGE on 14% polyacrylamide gels and by transfer onto nitrocellulose membranes. Immunoblots on the left were developed with anti-immunoserum to Toro isolate as described in the methods, and immunoblots on the right were developed with preimmune control serum as the first antibody. Antigens in the two lower immunoblots were subjected to perchloric acid treatment to reduce their carbohydrate content before SDS-PAGE.

Protease activity in culture medium.—*Gremmeniella* secretes enzymes and possibly other proteins into culture medium at only very low amounts (Petäistö et al., 1992). In general, secreted protease activities may deteriorate the SDS-PAGE pattern of secreted proteins. To study this, five purified protein markers (thyroglobulin, ferritin, catalase, acetate dehydrogenase, bovine serum albumin) were added to the used and unused 2- to 6-wk-old culture media. The marker proteins were not degraded after 18 h at either pH 6 or 7.4 in media from 17- to 42-day-old Hedmark cultures or from unused V8-media incubated for an equivalent time (data not shown). An uncharacterized casein hydrolyzing activity has been previously detected in agar cultures of *G. abietina* by Petrini et al. (1989).

SDS-PAGE sample preparation and reproducibility.—Protease inhibitors were not necessary in order to obtain a good protein pattern from 2-wk-old cultures. The proteolytic activity of this fungus is very low and very long incubation times are needed to show a change in the protein pattern in SDS-PAGE. Addition of an inhibitor like EDTA can be used to reduce this marginal proteolytic activity. For total elimination of proteolysis, a mixture of pCMB, EDTA and pepstatin is likely to be needed. Bands sometimes were blurred and unclear. Addition of thiourea or polyvinylpyrrolidone did not improve separation, and thus phenol derivatives were not the cause of this phenomenon. Perchloric acid precipitation had no effect on the sharpness of protein bands in SDS-PAGE (FIG. 8). Long treatment with strong acid splits easily hydrolyzable carbohydrate. Thus, protein bands can be compared after acid precipitation of samples allowing simultaneous analysis of metabolites and other cellular constituents. Mycelium supernatant contains acetone soluble compounds; one such compound is a yellow green pigment present in the fungus supernatant (Ayer et al., 1989) that was extracted by acetone. However, acetone precipitation did not change the protein pattern in SDS-PAGE. Altogether, up to 40 bands could be separated.

SDS-PAGE of eight *G. abietina* isolates representing three different races showed some minor differences in protein patterns between the races. The SDS-PAGE protein patterns in 2-wk-old cultures were reproducible from cultures performed at different times for most of the isolates studied. As representative examples, protein patterns of European Toro and American 18-50 and 18-70 isolates (FIG. 5), and Asian SJ-2 and SJ-3 isolates (FIG. 6) are shown. Typically, American isolates contain a 27.5 kD protein band. Samples frozen after SDS-boiling and subjected to repeated freeze-thawing gave variable protein patterns (FIG. 7). Thus, freshly made samples for SDS-PAGE are necessary to obtain a reliable comparison. In conclusion, for reliable comparison of protein pattern by SDS-PAGE, freshly made samples preferable from 2-wk-old cultures are needed. Protease inhibitors should also be included in sample preparation steps, especially if older cultures are used. EDTA can be used to reduce the proteolytic activity of this fungus. For total elimination of the proteolysis, a mixture of pCMB, EDTA and pepstatin is likely to be needed.

Immunoblotting.—Polyclonal antibodies detected 70–100 kD proteins in all four isolates (FIG. 8) and a 28 kD band that was particularly strong in Toro and Kima isolates. The 28-kD band intensity was several times higher in Toro and Kima than in Sup1.1 and Hedmark in all immunoblots made using all four rabbit antisera. Similar results were obtained with immunosera against Sup1.1 or against Hedmark, although the two isolates had themselves only small amounts of this antigen. The antigen pattern in perchloric acid-treated samples was comparable to untreated samples (FIG. 8).

The specificity of antibodies against *G. abietina* was high (FIG. 8). No immunospecific bands could be detected in *Sirococcus strobilinus* protein samples using Toro antiserum. The 40-kD band (FIG. 8, lane 1 on the left) was an artifact caused by the commercial antirabbit antibody preparation, because it could be seen in the control immunoblot without anti-*Gremmeniella* antibody (FIG. 8, lane 1 on the right). Only a few very weak bands were obtained from *Fusarium* (data not shown). The absence of immunoreactive sites in *Sirococcus* proteins was remarkable because this fungus is a pathogen in conifers with many similarities to Scleroderris canker. Even its metabolites are partially the same (see Ayer et al., 1989), suggesting close relatedness. Furthermore, its protein pattern in SDS-PAGE was almost identical with that of *G. abietina*: only one out of 12 major bands had slightly different mobility (data not shown). However, the lack of immunologic cross-reactivity with polyclonal anti-*G. abietina* serum argues against any close genetic relationship to *G. abietina*. Identification of new serotypes of *G. abietina* could possibly be utilized in the search for markers of pathogenicity of this fungus.

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