Forest pathology research in the Nordic and Baltic countries 2005

Proceedings from the SNS meeting in Forest Pathology at Skogbrukets Kursinstitutt, Biri, Norway, 28–31. August 2005

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Preface

In 1972 a Nordic Cooperative Group on Forest Pathology was established on a request from the recently established Nordic Forestry Research Cooperation Committee (SNS) under the Council of Nordic Ministers (NMR). Since then a meeting for Nordic forest pathologists has been held every second year, the organising circulating between the Nordic countries. During the 1990s the Baltic countries were invited to participate, and in 2000 the first SNS-meeting for forest pathologists was held in a Baltic country, Estonia.

The present meeting was organized by Halvor Solheim with help from Isabella Børja and Knut J. Huse. Halvor Solheim was also responsible for the excursion, which included a visit to forests near the timber line, and hiking up to the mountain Ormtjernkampen in the recently established Ormtjernkampen National Park. In autumn 1938 a forest officer was in the area marking timber, and he realized there were no old stumps indicating human activity, which resulted in a process to prevent the forest. The area was protected in 1956, and in 1968 it was assigned the status of national park. The name Ormtjernkampen comes from three words: orm (= worm), tjern (= a small lake), and kampen (one of many different Norwegian words for a mountain).

In a sunny weather we passed Lillehammer, drove through the valley Gausdal where the national poet Bjørnstjerne Bjørnsson lived part of his life, and finally stopped in a mountain forest dominated by Norway spruce near Kittelbu in Gausdal municipality. Here we looked at different butt rots on stumps and logs in a stand where timber harvesting was ongoing. More information about these various rot types can be obtained from the SNS-meeting paper prepared by Halvor Solheim. In Ormtjernkampen National Park we first looked at Norway spruce trees severely attacked by the rust fungus *Chrysomyxa abietis* in 2004. In August 2005 the infected needles had shed, and we could observe a strong needle loss on some Norway spruce trees. Along the path to the top of mountain Ormtjernkampen we saw only minor pathological items such as fruitbodies of *Stereum sanguinolentum* and *Climacocystis borealis*, but the main focus with this field trip was to have a relaxing time when climbing the mountain. The weather was sunny, but windy so on the top of Ormtjernkampen we could hardly stand on our feet. However, the view was beautiful with valleys, hills, rivers and lakes and with mountain massifs in the background, Rondane in north and Jotunheimen in west-northwest. Maybe we also had a glimpse of Dovrefjell in north-northwest.

Altogether 38 forest pathologists and students were participating the SNS-meeting held at Skogbrukets Kurstinstitutt, Biri, Norway, during 28.-31. August. It was a great pleasure that as many as six participants from the Baltic countries were able to attend the meeting: Rein Drenkhan and Märt Hanso from Estonia, Talis Gaitnieks from Latvia, and Remigijus Bakys, Vaidotas Lygis and Rimvis Vasiliauskas from Lithuania. Rimvis is now working in Sweden and was actually part of a large Swedish group with Jan Stenlid as the leader. The other participants from Sweden were Johan Allmér, Jenny Arnerup, Pia Barklund, Mattias Berglund, Mårten Lind, Karl Lundén, Mikael Nordahl, Åke Olsson, Nicklas Samils, Elna Stenström and Johanna Witzell. Another large group arrived from Finland with Jarkko Hantula, Juha Kaitera, Risto Kasanen, Arja Lilja, Michael Müller, Seppo Nevalainen, Tuula Piri, Mikko Söderling, Antti Uotila and Martti Vuorinen. We had also the pleasure to have Halldór Sverrisson from Iceland with us and from the hosting country Isabella Børja, Carl Gunnar Fossdal, Ari Hietala, Svein Solberg, Halvor Solheim and Volkmar Timmermann participated.

Students, post doc students and researchers in forest pathology from other part of the world are often visiting the Nordic countries and this time we had the pleasure to have with us Joha Groebbelar and Berhard Slippers from South-Africa and Nenad Kea from Serbia.

For this meeting no special topic was chosen, so the 24 talks and 4 posters represented various topics within forest pathology. However, two of the main tree pathogens in northern Europe, *Heterobasidion* and *Gremmeniella* were frequently on the focus. The program was rather strict, but with so many interesting talks and posters it was easy to follow the schedule. Thank you all for the good talks, nice posters and for just being there with your friendly manner.

Sponsor of this meeting was as usual SNS (www.nordiskskogforskning.org/sns/), and this time also Norwegian Forest Research Institute contributed. The next meeting will be in Finland at Hyytiälä forestry station. It will be part of the new PATHCAR (Centre of Advanced Research in Forest Pathology) program from SNS, which started this year. The leader of this PATHCAR is Jarkko Hantula from Metla, and more information will be given later in 2006.

Ås April 2006
Halvor Solheim and Ari M. Hietala

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White rot fungi in living Norway spruce trees at high elevation in southern Norway with notes on gross characteristics of the rot

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Abstract

Norway spruce suffers from serious root and butt rot problems from sea level up to the timber line in Norway. In this paper the most common fungi causing white rot is presented with special notes on gross characteristics of the rot. During the meeting we visited a stand near the timberline where logging was ongoing. Isolations were done from nearly hundred rotten logs and the results are presented.

Introduction

Norway spruce [*Picea abies* (L.) Karsten] suffers from serious root and butt rot problems that cause great economic losses also in the Nordic countries. Various wood-rot fungi are agents of this disease (Bendz-Hellgren *et al*. 1998). In 1992, a survey on the occurrence of butt rot on Norway spruce was undertaken in Norway (Huse *et al.* 1994); 5000 forest owners counted the rot on spruce stumps in newly-cut stands and identified roughly, according to instructions given by the Norwegian Forest Research Institute, the decay agent on the basis of rot type. The survey revealed that 27.8 % of the trees had butt rot, and that the dominating rot type was that caused by *Heterobasidion annosum* s.l. while *Armillaria* rot was less common. Both *Heterobasidion* and *Armillaria* are root rot fungi, while the most serious wound-rot fungus in Norway spruce is *Stereum sanguinolentum* (Roll-Hansen & Roll-Hansen 1980; Solheim & Selås 1986). Also other fungal species may cause butt rot of Norway spruce and be damaging in certain areas, particularly if final harvesting is delayed. This paper describes the most common white rot fungi in old Norway spruce at high elevation with notes about gross characteristics of the rot.

Heterobasidion parviporum Niemelä & Korhonen

Heterobasidion parviporum is the most common rot fungus in the natural distribution area of Norway spruce in Norway, whereas *H. annosum* (Fr.) Bref. *s.s*. seems to occur infrequently on Norway spruce in this area (Korhonen *et al*. 1998; Solheim, unpublished). Based on observations in Sweden and Finland, only *H. parviporum* would be expected to occur at high altitudes in Norway (Korhonen *et al*. 1998). At the west coast, where Norway spruce does not occur naturally, *H. annosum* is the only *Heterobasidion* species found in spruce plantations. (Solheim 1996; Heggertveit & Solheim 1999). The two species of *Heterobasidion* behave similarly in Norway spruce, but the decay caused by *H. parviporum* tends to rise higher up in the stem (Vasiliauskas & Stenlid 1998).

Heterobasidion infects wounds and freshly cut stumps. Further spread takes place along roots and from tree to tree via root contacts or grafts. Stumps have been mentioned as the main entrance of infection in stands, but in Norwegian studies also summer-time wounds on the lower part of stem are rather frequently infested by *Heterobasidion*. Roll-Hansen & Roll-Hansen (1980) found that 12 out of 72 Norway spruce trees wounded in July (17 %) were infested by *Heterobasidion*, while none or only a few trees were infested after wounding in May, September or December.

The rot in its advanced stages is typical white pocket rot. Incipient rot is straw-coloured to light brown, and in more advanced stages it becomes darker. In the heartwood, the first sign of the presence of *Heterobasidion* rot is a violet-stained wood called aniline wood. This stain may be seen as a ring around the rot in the heartwood (Fig. 1) or as spots in the light-brown incipient rot. In advanced rot short black streaks or specks are seen, which are accumulations of manganese oxide; also other white rot fungi can accumulate it (Blanchette 1984). Also white specks often occur, and sometimes the black specks are surrounded by white ones. The black and white specks are easily seen in longitudinal or radial cuts (Fig. 2).

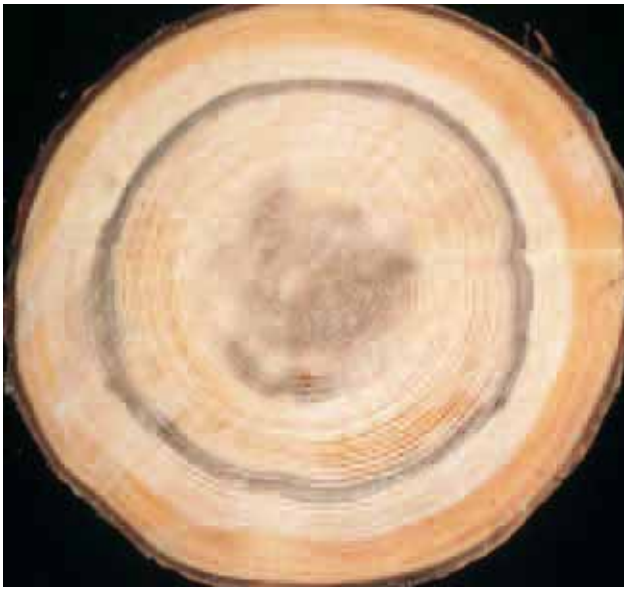

Fig. 1. A typical aniline wood ring surrounding the incipient Heterobasidion *rot in the heartwood of Norway spruce. Photo: H. Solheim*

Fig. 2. Black and white specks seen in a longitudinal cut of Norway spruce with Heterobasidion *rot. Photo: H. Solheim*

When the rot reaches the sapwood, the living cells react trying to stop further spreading of the fungus towards the cambium. This reaction zone is well described by Shain (1972). In fresh cuts it is nearly invisible, but there may be a weak light brownish colour. When oxidized it turns darker, greyish brown to olive brown, often with a greenish tint (Fig. 3). The rot column can rise high up in the stem, I have seen a 12-m-high column, but columns between 4 and 7 m are most common.

Fig. 3. A reaction zone surrounding Heterobasidion *rot in Norway spruce. Note the dry zone between the reaction zone and sapwood. Photo: H. Solheim*

Armillaria borealis Marxmüller & Korhonen

The *Armillaria* species are well-known saprophytes on all kinds of wooden material, but they can also act as pathogens on stressed trees, bushes etc. Young trees can be killed rather fast, while older trees may fight for many years. The crowns of attacked Norway spruce trees can become more and more yellow, while the shoots will be shorter and shorter until the trees die from the top. This occurs now and then in connection with summer drought in the southern part of Norway (Solberg *et al*. 1992).

Two species of *Armillaria* are common in Norway (Solheim & Keca, unpubl.). *Armillaria borealis* is the most common species and seems to be distributed all over Norway. *Armillaria cepistipes* Velenovsky is also common and has been found at least up to Trøndelag in the north. *Armillaria ostoyae* (Romagn.) Herink has for certain been found only once in Norway, but it is rather difficult to distinguish this species from *A. borealis*, and no one has looked for it in young pine stands where it locally occurs e.g. in Finland (Korhonen 1978). *Armillaria ostoyae* is usually darker, bigger, and has larger scales than *A. borealis* (Pegler 2000). Also genetically *A. borealis* and *A. ostoyae* are closely related (e.g. Sicoli *et al*. 2003). No comprehensive studies of the *Armillaria* species have been undertaken in Norway, but based on material in our herbaria and isolation studies at Skogforsk only *A. borealis* is found higher than 400 m a.s.l.

Armillaria species are agents of root and butt rot on various tree species and rather common on Norway spruce (Huse *et al*. 1994). In Norway, *A. borealis* is the most common *Armillaria* species associated to butt rot of spruce (Heggertveit & Solheim 1999, Solheim & Keca, unpubl.), and at high elevation it may be the only *Armillaria* species. However, there are no studies on this.

Armillaria species are not very aggressive pathogens of spruce, and the decay mostly keeps inside the heartwood. Incipient decay is grey to brown, often with a water-soaked appearance (Morrison *et al.* 1991). Yde-Andersen (1958) reported a yellowish colour in the early stage of decay, with caramel brown spots, and often short, dark cracks emanate from the medulla. Bacteria were often isolated from this stage. More advanced rot also often occurs as small spots (Fig. 4). Later on most of the heartwood may be decayed and rather soon totally destroyed. We call this «hullråte» («hollow rot») in Norwegian. Black sheets of hard fungal tissue (pseudoclerotial plates) are often observed in *Armillaria* rot (Greig *et al.* 1991). Other microorganisms may occur together with *Armillaria* rot, and often the colour is dark, nearly black (Roll-Hansen 1969). In Norwegian we call this «svartråte» («black rot») (Fig. 5). A combination rot with *Armillaria* and *Heterobasidion* is often observed. *Armillaria* rot usually reaches only a height of 1–2 m in the stem while *Heterobasidion* continues further up (Fig. 6).

Fig. 4. A small spot of Armillaria *rot on stump no. 5. Photo: H.Solheim*

Fig. 5. «Black rot» / «hollow rot» associated with Armillaria*. All the wood has disappeared in the centre, but the knots are left. Photo: H. Solheim*

Stereum sanguinolentum (Alb. & Schwein.) Fr.

This species is a wound specialist on Norway spruce, and it seems that every wound, from root to top, is vulnerable for infection. Usually the rot keeps inside the annual ring that is formed in the year of wounding. *Stereum* rot may be more common on Norway spruce than the stump investigations tell us. A small rot spot on stump may be an indication of root rot growing upwards, but it may also be a sign of *Stereum* rot growing downwards from a wound formed higher up on the stem (Fig. 7).

Fig. 6. A Norway spruce tree with a combination rot. Armillaria *has removed most of the wood up to the height of ca. 1 m, while* Heterobasidion *rot extends up to ca. 9 m. Photo: H. Solheim*

Fig. 7. A small spot of Stereum *rot on stump no. 3. Photo: H. Solheim*

S. sanguinolentum rot is typically a pale brown, stringy rot, but the colour may vary. Young rot is very homogenous and is separated from sound wood only by light brown or reddish brown colour. More advanced rot is also rather homogenous, but it may crack along the annual rings. A thin layer of whitish mycelium can be seen in the cracks. According to my observations the *S. sanguinolentum* rot it is always darker than *Heterobasidion* rot, sometimes the colour is almost chocolate brown. I have never seen white

pockets or black specks in association with *S. sanguinolentum* rot. However, according to Cartwright & Findlay (1958), *S. sanguinolentum* rot is like other *Stereum* rots: It starts as a reddish-brown rot, turns eventually into a white pocket rot, and ends as a white stringy rot. In the sapwood, and in cases where the rot is progressing from heartwood to sapwood, a similar zone can be observed as the reaction zone surrounding *Heterobasidion* rot (Fig. 8). The colour is greyish green or has a violet tone. In wounds infested by *S. sanguinolentum* the bleeding fruit bodies may be found.

Fig. 8. Decay caused by S. sanguinolentum *16 years after wounding. The rot is kept inside the wood created before the year of wounding. A reaction zone can be seen in the sapwood outside the rot. Photo: H. Solheim*

Important factors for infection are wound size and depth, but also the wounding season. The annual fruit bodies are produced in the autumn, and millions of spores are released into the air. *S. sanguinolentum* is a strong wound colonizer and may also infect older wounds. At least Vasiliauskas *et al* (1996) found a positive correlation between wound age and infection of *S. sanguinolentum*. In a survey of Norway spruce damaged by deer in Western Norway 16 % of the wounds were infested 5–7 years after wounding, while 39 % of the trees with 15 to 20-year-old wounds were infected with *S. sanguinolentum* (Veiberg & Solheim 2000).

Climacocystis borealis (Fr.) Kotl. & Pouzar

This species may cause root and butt rot in old forest at all altitudes. Fruit bodies are usually not seen before trees are dead, when hundreds of fruit bodies may be seen on the lower stem and on roots (Fig. 9). The fruitbodies are, when young and in humid weather, rather watery which has given the Norwegian name «vasskjuke» («water polypore»). The colour of young fruit bodies is whitish, while later the conks turn yellowish and rather hard.

The borealis rot is very characteristic white mottle rot. Incipient rot is light brown, later it may be more reddish-brown (Fig. 10). The rot is rather uneven. At a closer look, the rot is cubic with white mycelium in between (Fig. 11). The cubes are much finer (1–2 mm) than those of typical cubical brown rot. *Climacocystis borealis* has a strong reaction for laccase (Käärik 1965).

Fig. 9. Numerous fruitbodies of C. borealis *on a killed standing Norway spruce tree in Ormtjernkampen national park. Photo: H. Solheim*

Fig. 10. End of a log (no. 11 at Kittelbu) with C. borealis *rot. Note the zone surrounding the rot. Photo: H. Solheim*

Fig. 11. Characteristic rot caused by C. borealis *with small cubes and white mycelium. Photo: H. Solheim*

Infection takes place through wounds on roots and lower part of the trunk. The rot is typical heartwood rot and seldom reaches a height more than 2–3 m. Sometimes the sapwood is also attacked, and in places where the fungus reaches the cambium fruit bodies may be seen even on living trees. A greyish-green or greyish-violet zone may be seen surrounding the rot (Fig. 10).

Phellinus chrysoloma (Fr.) Donk

This fungus is common in old Norway spruce forests, and may be the most common cause of rot in some stands at high elevation, as reported by Juul & Jørstad (1939) from Dragås, Midtre Gauldal, Sør-Trøndelag. A brief survey in a Norway spruce stand in Lierne, Nord-Trøndelag, some years ago revealed that *P. chrysoloma* was as common as *Heterobasidion* (Solheim, unpubl.). Also in the spruce stand that we visited near Kittelbu (see below) this species was isolated from more logs than any other rot fungus. However, surveys have very seldom been undertaken in stands at high elevation, and hence we have no reliable data about the frequencies.

P. chrysoloma infests mostly through broken branches and tops, but also through wounds. The mostly perennial fruit bodies develop often at the point of original infection, on branch stubs or elsewhere on the trunk where the fungus has reached the cambium, but they are more frequent on stumps and fallen logs (Fig. 12). The fruit bodies are rather hard and vary much both in size and form. The pores are angular.

Fig. 12. Wind thrown Norway spruce with fruitbodies of P. chrysoloma*. Photo: H. Solheim*

The rot is a white pocket rot, but may be rather variable. White cellulose patches are typical; they appear in large numbers at a certain stage of rot (Fig. 13). Eventually they turn into holes that may grow together, this resulting in a honeycombed or long-fibred appearance at the ultimate stage of decay (Jørstad & Juul 1939). The white patches are similar to those observed in *H. parviporum* rot, but bigger and often more numerous. Also black specks are associated with *P. chrysoloma* rot. They are rather thin, more like lines (Fig. 14).

Fig. 13. A longitudinal cut of P. chrysoloma *rot with the characteristic white, rather large pockets. Photo: H. Solheim*

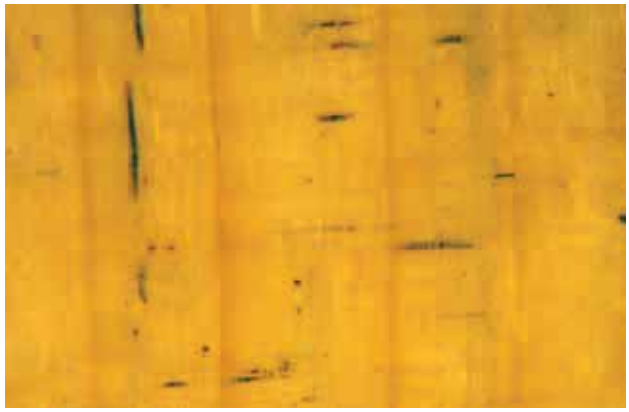

Fig. 14. Black lines in rot caused by P. chrysoloma*. Photo: H. Solheim*

At first the rot keeps in the heartwood, but rather soon it expands to the sapwood. Then a zone similar to the *Heterobasidion* reaction zone occurs. Its colour is dirty violet (Fig. 15), and in some places a dark brown zone is seen in the rotten area just inside the «reaction zone» (Fig. 16). The rot spreads easily in Norway spruce and may occupy most of the trunk. Jørstad & Juul (1939) refer to an 11-m-high tree where the rot had spread more than 8 m up. Korhonen (personal comm.) measured in southern Finland a 25-m-high Norway spruce tree where *P. chrysoloma* decay extended from the base up to the height of 22 m.

Only a 3-cm-thick layer of the outer sapwood was sound, but externally the spruce looked relatively healthy.

Fig. 15. Rot caused by P. chrysoloma *with a dirty violet zone surrounding it. Dark brown lines are separating different individuals of the fungus. Photo: H. Solheim*

Fig. 16. A cross section of a rotten area caused by P. chrysoloma *with the dark brown zone which may be seen now and then just inside the «reaction zone». Photo: H. Solheim*

Inonotus leporinus (Fr.) Gilb & Ryv.

Three closely related species of *Inonotus* are rare in Norway and red-listed (Direktoratet for naturforvaltning 1999). *Inonotus tomentosus* (Fr.) Teng has straight setae, and the fruitbodies are typically stipitate to substipitate and mostly found associated with root of conifers. The two others species have curved setae. *Inonotus triqueter* (Fr.) Karst. attacks Scots pine trees and has probably been found only once in Norway and, in addition, a few times in southern Finland and Sweden. It is more common further south in Europe (Ryvarden & Gilbertson 1993). *Inonotus leporinus* is red-listed both in Norway and Sweden (Larsson 1997) but seems to be more common in Finland (Kotiranta & Niemelä 1996). In Norway this species is the most common of the group and more than 100 specimens have been collected, two-third during the last ten years. Most of the samples in southern Norway is collected above 500 m asl. It causes a basal white pocket rot in Norway spruce. The rot occurs mostly in the roots, and extends seldom more than a few meters up. It may reach the cambium in big roots and at the lower part of the stem, where many of the annual fruitbodies may be seen (Fig. 17). I have seen only incipient rot, which is rather light brown. More advanced rot is very similar to *P. chrysoloma* according to Jørstad & Juul (1939), and sometimes also a dirty violet zone surrounding the rot has been observed.

Fig. 17. The author is looking at fruitbodies of I. leporinus *at the lower stem of a living Norway spruce. Photo: N. Keca*

Rot in an old Norway spruce stand near Kittelbu

During the SNS meeting for Nordic and Baltic forest pathologists we visited a stand belonging to Statsskog near Kittelbu, in Gausdal municipalty, Oppland county. The altitude was between 850 and 900 m asl, and the timber line in that area is around 1050 m asl. Logging in the stand was going on, and the cut timber was sorted in two piles, one with timber of good quality, and a smaller pile with timber of secondary quality, mostly affected by rot. The participants were walking around in the forest where some stumps had been marked, and they also visited the pile with

rotten logs (Fig. 18). A sheet of paper with pictures of the marked stumps and logs were handed out, and the participants were requested to discuss and «guess» the cause of rot in each occasion. However, it is not always easy to identify the rot type, especially based on horizontal cuts (stump surfaces or log ends). It may be easier if cuts can be made along the fibres. *Stereum*- like mycelium was isolated from stumps/logs no. 1, 3, 4 and 12. *Heterobasidion parviporum* was isolated from logs no. 10 and 13. *Climacocystis borealis* was isolated from logs no. 9 and 11. *Armillaria* mycelium was isolated from stump no. 7. A slow-growing mycelium with clamps was isolated from the log no. 8.

Fig. 18. Part of a pile with rotten log ends. C. borealis *was isolated from log no. 63;* H. parviporum *was isolated from logs no. 72 and 78;* P. chrysoloma *was isolated from logs no. 71, 74 and 82; S*. sanguinolentum *was isolated from log no. 66. Photo: H. Solheim*

After the SNS-meeting I visited the site again and I brought with me samples from nearly hundred logs. The most common rot agent was *P. chrysoloma* followed by *H. parviporum* and *S. sanguinolentum* (Table 1). As mentioned above, *P. chrysoloma* may be rather common in some stands at high elevation in Norway. Björkman *et al*. (1949) noted that this species could be the most common rot fungus in old and relatively intact spruce stands in the inner part of Norrland, Sweden.

In southern Norway the timber line is mostly between 1000 m and 1100 m asl. The same species of white rot fungi is found in the low land as near the timberline. However, some species seem to be more common at high elevation. The cause of that may partly be climatic. Important may also be that cuttings are more difficult and expensive at high elevation so we have more old growth forest at high elevation.

Table 1. Number of samples of each wood rotting fungus- from piles at Kittelbu (98 logs)

Wood rotting fungus	**Number**
Armillaria spp	12
Climacocystis borealis	13
Heterobasidion parviporum	25
Phellinus chrysoloma	36
Stereum sanguinolentum	24
Basidiomycetes spp.	13

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Pathogenicity in *Heterobasidion annosum* s.l.

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Distribution and speciation

Root rot caused by the basidiomycete *Heterobasidion annosum* s.l. is one of the most destructive diseases of conifers in the northern boreal and temperate regions of the world. Economic losses attributable to *Heterobasidion* infection in Europe are estimated at 800 million Euros annually (Woodward *et al* 1998). The fungus has been classified into three European intersterile subspecies P (*H. annosum*), S (*H. parviporum*) and F (*H. abietinum*) based on their main host preferences, pine, spruce, and fir, respectively. In North America, two intersterile groups are present, P and S/F, but these have not yet been given scientific names. Detailed interaction studies on this pathosystem have been complicated by the fact that there are no known avirulent strains of the fungus and no host genotype in *Pinaceae* with total resistance against the pathogen.

Although separated on different continents for a long period of time (Johannesson & Stenlid 2003), the North American and European P groups are morphologically indistinguishable (Korhonen & Stenlid, 1998) and fully interfertile (Stenlid & Karlsson, 1991). Furthermore, they also share similar broad host preferences and are thus probably best regarded as two subpopulations of the same species. An interesting observation of intercontinental introduction of the American P group into Italy was recently reported (Gonthier *et al* 2004). Based on distinctive mitochondrial markers, the authors concluded that the fungus was probably introduced with woody material to a military camp during the Second World War, thereby creating an opportunity for geneflow between the two P group populations.

The phylogenetic relationship between the S- and F groups was studied by comparing DNA sequences of four nuclear gene fragments; calmodulin, glyceraldehyde 3-phosphate dehydrogenase, heat stress protein 80−1 and elongation factor 1-α, and one anonymous locus, from 29 fungal isolates originating from Europe, Asia and North America (Johannesson & Stenlid 2003). The phylogeny of each separate gene locus as well as the combined dataset consisted of three main clades: European F group isolates, Euroasian S group isolates and North American S group isolates, suggesting them to be separated into phylogenetic species. The results also support the hypothesis of an early separation between the S- and F groups, indicating that their distribution have followed their host tree species for a considerable time period.

The taxonomic status of the North American S group is less clear, it is partly interfertile with both the S and F groups from Europe, but has a distinct evolutionary history and in contrast to its European relatives, has a broad host range.

The intersterility in *H.annosum* s.l. is controlled by a genetic system consisting of at least 5 loci; P, S,V1,V2, and V3 (Chase & Ullrich 1990). Similar + alleles at any of the loci allow for mating between two homokaryotic strains. This system opens up for hybridisation between the intersterility groups (Garbelotto *et al* 1996; Olson & Stenlid 2001; 2002). Hybrid mycelia has been detected in the field and laboratory tests show that heterokaryons carrying nuclei of the American P and S type express the pathogenicity representative of the parent cytoplasm (Olson & Stenlid 2001). Although the genetic background for interfertility between species in Europe has not been formally sorted out, an interesting study on higher degree of intersterility was reported between the S and F group populations growing in sympatry in northern Italy as compared to Italian F populations and Finnish S populations, (Korhonen *et al* 1992). It would be of interest to study whether selection against hybrids has driven the alpine *H. parviporum* and *H. abietinum* into more distinctive intersterility gene genotypes as compared with the allopatric Northern European *H. parviporum* vs *H. abietinum*.

In addition to fascinating possibilities for reticulate evolution, the hybridisation also allows for genetic analysis of pathogenicity traits. The first steps have been taken for Quantitative Trait Loci (QTL) analysis of pathogenicity by analysing progeny of such hybrids (Lind *et al* 2005).

Pathogenicity

In angiosperm systems, the expression of virulence by a pathogen initiates at the point of attachment whereupon host-parasite recognition is concomitant with the onset of defence reactions and often presumed to be a determinant of host plant specificity (Albersheim & Anderson-Prouty 1975; Jones 1994). Using non-suberized roots as an experimental model, spore adhesion has been documented within 2 hours following inoculation of primary roots of juvenile conifer seedlings with conidiospores of *H. annosum* (Asiegbu 2000). Adhesion occurred mainly on the mucilaginous regions of the root but rarely on non-slimy regions and adhesion was significantly reduced by treatment of spores with potassium hydroxide, di-ethyl ether, Pronase E or periodic acid (Asiegbu 2000). By contrast to observations with fine roots, pre-treatment of wood discs, with di-ethyl ether had no effect on spore germination. Removal of soluble compounds from the wood disc by pre-treatment with periodic acid or KOH considerably reduced the ability of the spores to germinate and become established on the host material. The effect of periodic acid and

KOH suggests that the adhesive component and part of the nutrient source for the spores was a sugar or carbohydrate.

The digestion of plant cell wall polymers provides nutrients and aids the penetration of cells, allowing survival and spread through woody tissues. However, few of the enzymes (amylase, catalase, cellulase, esterase, glucosidase, hemicellulase, manganese peroxidase, laccase, pectinase, phosphatase, proteases) secreted by *H. annosum* have been thoroughly studied (Johansson 1988; Karlson & Stenlid 1991; Korhonen & Stenlid 1998; Maijala *et al* 1995, 2003; Asiegbu *et al* 2004) and little is known about their role in pathogenesis. *H. annosum* s.l. secretes a range of polysaccharide-degrading enzymes. Cellulase, mannanase, xylanase, aryl-β-glucosidase and β-glucosidase have been identified although their role in pathogenesis is still not thoroughly investigated. Beta-glucosidase enables *H. annosum* s.l. to use the energy in the glucosidic bond of cellobiose, an enzyme system that appears to be rare in white-rot fungi. A higher number of polygalacturonase and pectin esterase isozymes are present in *H. annosum* s.s. than in *H. parviporum* (Karlsson & Stenlid 1991). Additionally, the total pectin-degrading capabilities of *H. annosum* s.s. are higher than in *H. parviporum*, which has been hypothesised to account for the greater host range of *H. annosum* s.s. (Johansson 1988).

Several low molecular weight toxins are secreted by *H. annosum*, including fomannoxin, fomannosin, fomannoxin acid, oosponol and oospoglycol (Basset *et al* 1967; Sonnenbichler *et al* 1989). Application of fomannosin to stem wounds provoked systemic response leading to accumulation of pinosylvin (Basset *et al* 1967). Another toxin produced by *H. annosum* s.l. is fomannoxin, which have a 100-fold greater toxicity to *Chlorella pyrenoidosa* than fomannosin (Hirotani 1977). This toxin has been isolated from *H. annosum* s.l. infected Sitka spruce stem wood (Heslin 1983). Uptake of fomannoxin by Sitka spruce seedlings resulted in rapid browning of the roots accompanied by chlorosis and progressive browning of needles. This, and the production of fomannoxin by actively growing hyphae, suggests a role for fomannoxin during pathogenesis.

One factor that has limited the research about *H. annosum* pathogenesis is the lack of coding sequence information. Therefore, a project on producing sequence data from *H. annosum* by generating ESTs was initiated (Karlsson *et al* 2003). The collection of sequence data will assist future research on *H. annosum* together with the high-density cDNA arrays that were also constructed in this work. It is interesting that 30 % of the genes identified did not have any similarity to any known proteins and 16 % had similarity only with proteins with unknown functions. This is a typical number of unknown unigenes for other fungal EST sequencing projects and highlights a lack of sequence information on fungi.

The next step was to identify individual genes that encode putative pathogenicity factors (Karlsson 2005). This was done by identifying genes that have high transcript levels during infection stages as compared to other treatments, and by studying sequence similarities with proteins that have a characterised role in pathogenesis in other systems. The transcriptional responses of several genes were studied with realtime-PCR during fungal infection of conifer material. Genes with a putative involvement in secondary metabolism, protection against oxidative stress and degradation of host material were shown to be differentially expressed. A cytochrome P450 gene displayed sequence similarities towards genes encoding proteins involved in toxin biosynthesis and was highly expressed during growth in Norway spruce bark. Transcript profiles of a superoxide dismutase gene and two glutathione-S-transferase genes suggest that oxidative stress is involved in the interaction. An arabinase gene was exclusively expressed during infection of Scots pine seedlings. An increase of the transcription rate of a laccase and a cellulase gene was detected during a time-coarse experiment of fungal infection of Norway spruce tissue cultures.

Recently, progress has been made in work on mapping the pathogenicity factors in *Heterobasidion* using a hybrid between North American P and S homokaryons. Based on AFLP markers, a genetic linkage map was established that allowed for mapping QTLs for pathogenic growth towards seedling roots and pine innerbark (Lind *et al* 2005). The next step underway is to verify the identity of candidate genes located within the established region of the genome. Future functional analysis of both QTL and EST-derived candidate genes should be aided by the recently established Agrobacterium-mediated transformation system in *Heterobasidion* (Samils *et al* 2006).

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Defence reactions in Norway spruce toward the pathogenic root-rot causing fungus *Heterobasidion annosum*

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Abstract

The root-rot causing fungus *Heterobasidion annosum* can attack both spruce and pine trees and is the economically most damaging pathogen in northern European forestry. We have monitored the *H. annosum* S-type (fairly recently named *H. parviporum*) colonization rate and expression of host chitinases and other host transcripts in Norway spruce material with differing resistances using quatitative real-time PCR. Transcript levels of three chitinases, representing classes I, II and IV, were monitored. Ramets of two 33 -year-old clones differing in resistance were employed as host material and inoculation and wounding was performed. clones in the area immediately adjacent to inoculation. Fourteen days after infection, pathogen colonization was restricted to the area immediately adjacent to the site of inoculation for the strong clone (589), but had progressed further into the host tissue in the weak clone (409). Transcript levels of the class II and IV chitinases increased following wounding or inoculation, while the transcript level of the class I chitinase declined following these treatments. Transcript levels of the class II and class IV chitinases were higher in areas immediately adjacent to the inoculation site in 589 than in similar sites in 409 three days after inoculation, suggesting that the clones differ in the rate of pathogen perception and host defense signal transduction. This an earlier experiments using mature spruce clones as substrate indicate that it is the speed of the host response and not maximum amplitude of the host response that is the most crucial component in an efficient defense in Norway spruce toward pathogenic fungi such as *H. annosum*.

Introduction

The root and butt rot fungus *Heterobasidion annosum* (Fr.) Bres. *s. lat.* can attack both spruce and pine trees and is economically the most damaging tree pathogen in northern Europe. Suberized bark tissues form a strong barrier to penetration by this pathogen (Lindberg & Johansson 1991). However, bark wounds caused by wind, animals, insects and timber extraction expose the trees to this pathogen, which is characterized by a high spore deposition rate and long spore viability in bark.

Norway spruce, among other conifers, has been screened with stem inoculations to identify clones that differ in resistance towards *H. annosum*. Based on lesion length and fungal isolations, considerable clonal variation in genetic resistance has been recorded for Norway spruce. However, the mechanisms contributing to variation in resistance against *H. annosum* remain unknown.

Chitinases, PR proteins produced particularly upon pathogen attack, hydrolyze the 1,4-*N*-acetyl-D-glucosamine (GlcNAc) linkages of chitin, a component of cell walls of higher fungi. Hydrolysis of chitin results in the swelling and lysis of the hyphal tips and the chitinolytic breakdown products generated can act as elicitors of further defense reactions in plants (Schlumbaum *et al.* 1986). The objectives of the present study were to monitor *H. annosum* colonization rate and expression of class I, II and IV host chitinases in Norway spruce upon infection by *H. annosum* (S-type) in order (i) to identify defense related chitinases, and (ii) to evaluate whether trees displaying variation in host resistance show differences in the expression of chitinases.

Material and methods

Ramets of two 33-year-old Norway spruce clones differing in resistance were employed as host material. Following bark inoculation with an agar plug containing pathogen mycelia, a rectangular strip containing phloem and cambium, with the inoculation site in the middle, was removed at the start and 3, 7 and 14 days after inoculation. Prior to sampling, the rhytidome and the periderm were removed. The tissue was then divided into 50mg sections (length, 2 mm; width, 5 mm; depth, approximately 3 mm), which were processed individually (Fig. 1).

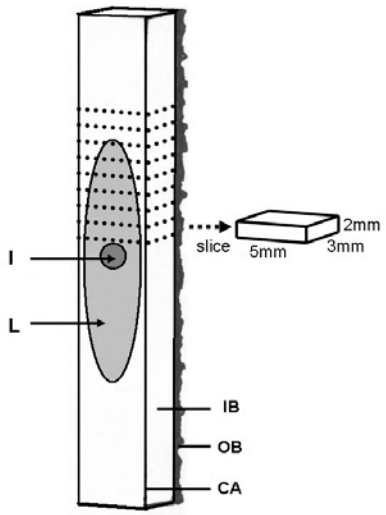

Fig. 1 Example of sampling from lesions. Inoculation point (I), lesion (L), outher bark (OB), Cambium (CA) and inner bark (IB) are marked. Two 33-year-old rametes of each clone were used in this inoculation experiment. DNA and RNA was extracted from the same section in each case to compare the colonization (genomic DNA of H. annosum *and Norway spruce) and the transcript level of the class I, II and IV chitinases.*

Chitinase expression levels were monitored with singleplex real-time PCR by using cDNA obtained from sampled sections and synthesised from total RNA as template (Hietala *et al*. 2004). Multiplex real-time PCR detection of host and pathogen DNA was performed on RNA prior to Dnase treatment (Hietala *et al*. 2003) in order to establish the colonization levels in each sampled section.

Results

Three days after inoculation, comparable colonization levels were observed in both clones in the area immediately adjacent to inoculation. Fourteen days after infection, pathogen colonization was restricted to the area immediately adjacent to the site of inoculation for clone 589, whereas it had progressed further into the host tissue in clone 409 (Fig. 2). Transcript levels of the class II and IV chitinases increased following wounding or inoculation, but the transcript level of the class I chitinase declined following these treatments. Transcript levels of the class II and class IV chitinases (Fig. 2) were higher in areas immediately adjacent to the inoculation site in clone 589 than in similar sites in clone 409 three days after inoculation. This difference was even more pronounced 2 to 6 mm away from the inoculation point, where no infection was yet established, and suggests that the clones differ in the rate of chitinase-related signal perception/transduction. Fourteen days after inoculation, these transcript levels were higher in clone 409 than in clone 589, suggesting that the massive upregulation of class II and IV chitinases (Fig. 2) after the establishment of infection comes too late to reduce or prevent pathogen colonization.

Discussion

On day 3 clone 589 had higher transcript levels of class II and IV chitinases than did clone 409 in areas adjacent to the inoculation site. This observation suggests that the time from signal perception and transduction to the induction of these genes was shorter in the more resistant clone. Chitinase enzyme activity and protein and transcript levels often are higher in resistant cultivars than in susceptible ones shortly after inoculation, when a lower level of chitinases may suffice to prevent or reduce hyphal penetration.

The higher class II and IV chitinase transcript levels in clone 589 during the early stages of infection also could result in earlier production of exogenous elicitors from the fungal cell wall, and an earlier triggering of other host defense reactions, *e.g.* increased lignification. To test the hypothesis that the rapidity of the overall response and the degree of coordination of the different defense strategies contribute to the level of resistance, studies of transcriptional activation of phenylalanine lyase and genes related to lignification at an early stage of *H. annosum* infection could be helpful. To allow an efficient screening of a larger amount of clones, sampling of bark inoculations could be restricted to the first 6 mm away from the inoculation point, an area where the clones now studied showed pronounced differences in chitinase expression.

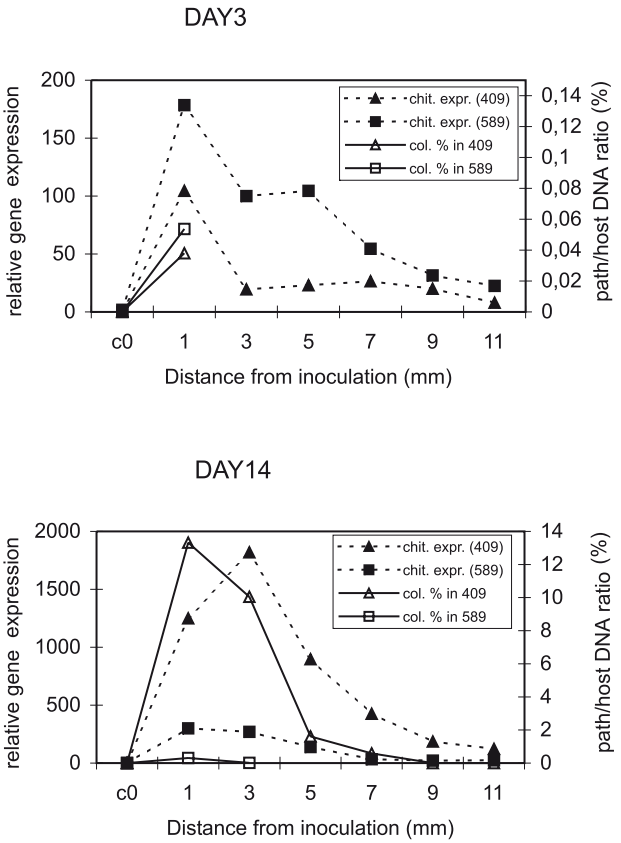

Fig. 2. Pathogen colonization levels and relative gene expression profiles of PaChi4, a class IV chitinase, in bark of two Norway spruce clones following inoculation with Heterobasidion annosum *(Hietala et al. 2004). The bark around the inoculation site was spatially sampled (see Fig. 1) 3 days (upper panel) and 14 days (lower panel) after inoculation. The basal transcript levels of the chitinase in clone 409 at the time of inoculation were used as a reference transcript level and defined as the 1x expression level, and the transcript levels of all the other samples are expressed as the fold change over this reference level. (Figure reproduced from Schmidt et al. 2005).*

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Spruce cull pieces left on cutting areas can increase aerial spread of *Heterobasidion* – preliminary results from field trials in southern Finland

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Abstract

The fruiting of *Heterobasidion* on cull pieces and stumps of Norway spruce on logging areas was investigated. Cull pieces showing butt rot were left on three clear-cut areas and on one thinning area. They were also transported to four mature unmanaged forest sites with a dense tree cover. During the succeeding 3–4 years the cull pieces were annually investigated for fruit bodies of *Heterobasidion*, and the actively sporulating area of the fruit bodies was determined. Root bases of spruce stumps in the logging areas were dug out and sporulating fruit bodies found on the stumps were also measured.

Immediately after cutting, *Heterobasidion* sp. was isolated from 76 % of the cull pieces; 85 % of the isolates were identified as *H. parviporum* and 15 % as *H. annosum s.s.* Fruit bodies developed on 395 cull pieces, i.e. 19 % of all 2077 initially rotten cull pieces. Fruit body formation was significantly affected by several characteristics of the cull pieces and various environmental factors. It was favoured by increasing cull piece diameter and advancement of decay but restricted by the presence of *Stereum sanguinolentum*-type rot. End-to-end soil contact of the cull piece also favoured fruit body formation compared to partial or no soil contact. The between-site differences were significant but could not be explained by differences of tree cover. At the end of the investigation period the average sporulating area of *Heterobasidion* per cull piece was higher than the average sporulating area per stump at three out of four managed sites. Hence, leaving cull pieces with butt rot in southern Finland can considerably increase local production of *Heterobasidion* spores.

Introduction

Present forestry guidelines in Finland recommend increasing the amount of decaying wood in managed forests in order to ensure biodiversity. In particular, the amount of high diameter decaying wood is deficient in managed forests. This deficiency could be met by leaving in the forest cull pieces of trees that are damaged by butt rot. As *Heterobasidion parviporum* Niemelä & Korhonen and *H. annosum* (Fr.) Bres. *s.s.* are the most common fungi causing butt rot of Norway spruce [*Picea abies* (L.) Karsten] in many parts of Europe, a large proportion of decayed cull pieces of spruce are inhabited by these fungi. Such logging residues can promote fruiting and spore production by *Heterobasidion*. Schütt and Schuck (1979) showed that *Heterobasidion* sporocarps can appear already one year after logging but their frequency is highest and size greatest generally 3–4 years after logging. However, it is not known whether the amount of sporocarps occurring on logging residues could significantly increase local spore production. Neither is it known whether *H. parviporum* and *H. annosum* show differences in sporocarp production on logging residues.

Our aim was to compare the spore production by *Heterobasidion* on cull pieces and stumps of Norway spruce in the same logging area, assuming that the quantity of spore production is related to the actively sporulating pore layers of the fruit bodies. Aerial spread of *Heterobasidion* is believed to take place mainly by basidiospores, conidia having probably a minor significance in contributing to the air spora of *Heterobasidion* (Redfern & Stenlid, 1998). Additionally, we investigated the effect of various factors on sporocarp production in a field trial lasting for 4 years at eight different locations. Here we publish preliminary results.

Material and Methods

Field sites

Two managed field sites are situated in Bromarv (southwestern Finland), one in Hausjärvi (southern Finland) and one in Vehkasalo (southeastern Finland). Norway spruce was the dominating tree species on all sites. The size of the managed sites varied between 2.8 and 5.6 hectares. Logging was performed in August 2000 (Vehkasalo and Bromarv A) or August 2001 (Bromarv B and Hausjärvi). As judged from the stumps, 30–41 % of the trees suffered from butt rot. The cull pieces were left by the harvester close to the stumps from which they originated and so their distribution on the logging areas conforms to the distribution of butt rot in the stand.

The unmanaged sites are in Siuntio, Mäntsälä, Sipoo (southern Finland) and Ylämaa (southeastern Finland). They are mature over 100 years old spruce stands with closed canopy. Cull pieces were transported to the unmanaged sites from Bromarv in December 2001 (one site) and April 2002 (three sites) and placed on a ca. one-hectare area at each site.

All sites include moderate slopes (<20 m). Healthy-looking cull pieces were left as controls on each experimental site. All the cull pieces were GPS-mapped and marked with a numbered label. Their dimensions (diameter, length), degree of ground contact (complete, one end, no contact), and bark condition (intact, partly removed, completely removed) were recorded. Altogether 2077 cull pieces with signs of decay and 441 healthy looking controls were included in the study.

All stumps on the managed sites were mapped, marked, and evaluated visually for the presence of butt rot.

Isolation and identification of Heterobasidion

At the beginning of the trials two discs, ca. 5 cm thick, were removed from one end of each cull piece. The first disc was discarded, the second was placed in a plastic bag, incubated at room temperature for 5–7 days, and thereafter stored up to one week at +4 °C until investigated under a dissecting microscope. Decay caused by *Heterobasidion* was identified from the discs on the basis of conidiophores. The fungus was isolated from conidia and the species was identified using mating tests (Mitchelson & Korhonen 1988). Other decays were visually classified into three types: *Stereum sanguinolentum* (Alb. & Schwein.) Fr. type, *Armillaria* type and unidentified type. The squared ratio between the average disc diameter and decay diameter was used as a measure of the degree of decay, i.e. proportional volume of decay in a cull piece.

Fruit body survey

In each September of the following 3–4 years after logging, randomly selected cull pieces ($^1/_3$ or $^1/_4$ of total) were investigated and actively sporulating (white) pore layers of *Heterobasidion* fruit bodies were drawn onto a transparent that was later scanned and subjected to image analysis in order to obtain the area counts. In order to estimate the background spore production (without cull pieces) on the managed sites, a random sample of spruce stumps showing butt rot ($^1/_3$ or $^1/_4$ of total per year) was also investigated for the presence of *Heterobasidion* fruit bodies. The root bases were dug out and active fruit bodies were measured as from cull pieces.

Statistical analyses

Statistical analyses were done using the SPSS 13.0 for Windows program (SPSS Inc. Chicago, USA).

Results and discussion

Immediately after cutting, *Heterobasidion* sp. was isolated from 76 % of the cull pieces; 85 % of the isolates were identified as *H. parviporum* and 15 % as *H. annosum.* In the course of 3–4 years after logging *Heterobasidion* fruit bodies were found on cull pieces on every experimental sites. Altogether they were found on 395 cull pieces, corresponding to 19 % of the total of 2077 cull pieces with butt rot.

During the first three years after cutting the active pore layer area of the fruit bodies increased. On two managed sites the logs were investigated during four successive years; on one site the pore layer area decreased in the fourth year from the maximum recorded in the third year, whereas on the other site the pore layer area increased also during the fourth year. Significant differences were observed between the pore layer area found on different sites. The tree cover on the sites could not explain these differences since high and low values were found both on clear-cut and unmanaged sites. In a logistic regression analysis the most significant variables explaining fruit body formation were the diameter of the cull piece and the proportional volume of decay at the time of cutting. The higher the diameter of the cull piece and the higher its decay volume, the higher was the probability of fruit body development. Also the soil contact of the cull piece and the presence of *S. sanguinolentum* type of decay were highly significant variables, but their effect was smaller than that of cull piece size and advancement of decay. Initial presence of *S. sanguinolentum* type of decay and absence of soil contact lowered the probability of fruit body development. Bark injuries on cull piece or *Heterobasidion* species causing decay did not affect the probability of fruit body formation on the cull pieces.

Fruit bodies of *Heterobasidion* were also found on seven of the initially healthy- looking control cull pieces, corresponding to 1.6 % of their total number. They have not necessarily emerged from new infections after cutting but may originate from incipient decay that was not observed during the initial investigation of the cull pieces. Hence, we consider that leaving healthy looking spruce cull pieces on cutting areas infested with *Heterobasidion* does not noteworthy support spore production by this fungus.

The sporulating fruit body area on cull pieces was highest in the last survey year 2004 on all but one of the eight experimental sites. On three out of the four managed sites the average pore layer area per cull piece exceeded that found in 2004 on stumps. At one site the average pore layer area per cull piece was half of that found on stumps in 2004. As it can be supposed that spore production is related to the actively sporulating area of fruit bodies, these data show that leaving decayed cull pieces can considerably increase local spore production by *Heterobasidion*. Hence, leaving decayed cull pieces of Norway spruce on logging sites infested by *Heterobasidion* can support the spreading of this pathogen to the next tree generation and to the surrounding forests.

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Resistance in hybrid aspen to pathogens

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Abstract

Wide-scale plantations of aspen *(Populus tremula)* and hybrid aspen *(P. tremula x Populus tremuloides*) have recently been established in Nordic and Baltic countries after the forest industry has become interested in aspen fibre. As the number of aspen stands increases, the fungal diseases will become economically and ecologically important. *Neofabraea populi* was recorded for the first time in Fennoscandia early in 1960's and subsequent observations of the disease were made later in 1970's. In 2000's, serious damage was observed in second generation of hybrid aspen in Finland. Since conditions in dense coppice stands are probably favourable for the spread of *N. populi*, the fungus could pose a potential threat for short-rotation coppices of hybrid aspen. To study the variation in the resistance of hybrid clones, artificial inoculations were made. The bark of a total of 100 trees (10 clones) was wounded and inocula were placed under the bark. The reactions of the trees and the advance of the cankers were recorded; resistance was considered to be expressed as healing of the cankers. In conclusion, hybrid aspen clones, despite of the fact that the original selection was based on only yield and fibre characteristics, show variability in resistance. A promising observation was made by combining the results from separate trials; the best-growing clone is one of the most resistant ones. Thus it seems likely that there are possibilities to select for both growth and resistance traits in breeding.

Introduction

European aspen (*Populus tremula* L.) is the most widespread poplar species and one of the most widely distributed tree species in the world. Aspen has been found in many diverse habitats throughout its distribution area. In Finland, aspen grows mostly in mixed stands dominated by conifers, and as such makes up only about 1.5 % of the total volume in Finnish forests (Finnish Statistical Yearbook of Forestry, 2001). Wide-scale plantations of aspen *(Populus tremula)* and hybrid aspen *(P. tremula x Populus tremuloides*) have recently been established in Nordic and Baltic countries after the forest industry has become interested in aspen fibre. As the number of aspen stands increases, the fungal diseases will get more important both economically and ecologically. Based on experience from agriculture and clonal forestry with poplars and willows, it is known that damages caused by the fungal diseases may increase as a result of the use of clonal monocultures. To ensure a sufficiently wide range of genetic variation, breeding populations with aspen and hybrid aspen are presently being established at Finnish Forest Research Institute (Metla).

Fig 1. Stem cankers two years after inoculation with N. populi*: A) suscpetible clone, B) resistant clone, C) control, which was inoculated with agar.*

Neofabraea populi Thompson (Thompson 1939) was observed in Norway in early 1960's only a decade after hybrid aspen was imported to Norway and the plantations were established (Semb & Hirvonen-Semb 1968, Roll-Hansen & Roll-Hansen 1969). In late 1960's family trials were surveyed and variation in disease incident was observed between hybrid aspen families (Langhammer 1971). Later in 1970's (Kurkela 1997) and in early in 2000's (Kasanen *et al*. 2002) observations on the same type of symptoms in several stands were recorded also in Finland. After molecular and morphological analyses, Kasanen *et al.* (2002) concluded that *N. populi* was the causal agent of canker disease. In this disease, 2nd generations of trees (root suckers) are seriously damaged; they bear cankers and dead bark. Infections appear as depressed areas in the bark. Later the bark in lesions splits longitudinally. Older cankers can be from 50 to 100 cm long, elliptical and girdling the stem for one-half or more of its circumference. The bark in the center of canker is slightly sunken and split vertically. Cankers can also appear as slightly sunken areas that completely encircle the stems without any callous formation. Since conditions in dense coppice stands are probably favourable for the spread of the cortical pathogen *N. populi*, the fungus could be a potential threat for hybrid aspen cultivation (Kasanen *et al.* 2002).

The breeding system used for aspen and hybrid aspen is time-consuming and expensive (large-scale field tests over the whole rotation period). Such large scale field trials are needed to fulfil the requirements of the EU regulations for marketing forest regeneration material that came into force from 1.1.2002. A method for pre-screening the material in the nursery for e.g. pathogen resistance, in order to exclude unsuitable clones before the field trials are established, would save a lot of costs. In an ongoing project at the Punkaharju Research Station (Metla) such a nursery testing for both family and clonal material of aspen and hybrid aspen is being developed. Both natural and artificial infection may be used to test for resistance in the nursery.

This paper describes the experimental set-up for testing the resistance in hybrid aspen to *N. populi*, briefly reports the preliminary results and finally combines the data from separate trials for growth measurements and resistance testing. The applicability of the results is discussed in relation to the possibility to select for both superior growth and resistance.

Materials and methods

Field trials

The field performance (height increment and viability) of numerous clones planted in late 1990's had been surveyed in 13 field trials, which in total include over 21000 seedlings. Ten hybrid aspen clones, which were in 1999 the most commonly used in forest regeneration, were subject to resistance testing.

The field trial for resistance testing was established in summer 2000 at Suonenjoki Research Station (Metla). A total of 1000 seedlings (10 clones) were planted in rows. Each row consisted of 10 repeats with 10 seedlings per repeat. The clones were placed in rows so that each row was started with a different clone, followed by others in numerical order. The experimental field located in poor sandy soil was fertilized prior to the experiment and occasional drought damages were excluded by watering.

Inoculations

A total of 110 inoculations were made in August 2003. In addition to ten fungal inoculations per clone, one control inoculation was made. Prior to inoculation, an L-shaped wounding (1 cm*2 cm) was cut with knife to the bark. The edge of the wounding was gently lifted and a 1cm*1 cm block of fungal culture (malt agar) was placed under the bark. The bark was closed and the wounding was sealed with parafilm. Control inoculations were made with sterile agar blocks. Prior to the experiment a pilot test was made in 2002 with similar methods. Only one fungal strain was used in the inoculation experiments.

Measurements

The dimensions of the canker (length, width) were measured one year after inoculation, and also diameter of the stem above the canker, breast-height diameter and height of each tree were measured.

Results and discussion

Five out of ten control seedlings, which were inoculated with agar only, were totally healed already one year after inoculation. Regarding seedlings inoculated with the pathogen, four out of ten trees of the most susceptible clone were girdled by the cankers (Fig 1). Although no statistical analysis was made in this preliminary analysis two conclusions can be made; i) the canker height was probably the best variable for describing variation in resistance (Fig 2) and ii) the differences in canker height are most likely statistically significant. As shown in Fig 3 the height increment was also highly variable between hybrid aspen and aspen clones and families.

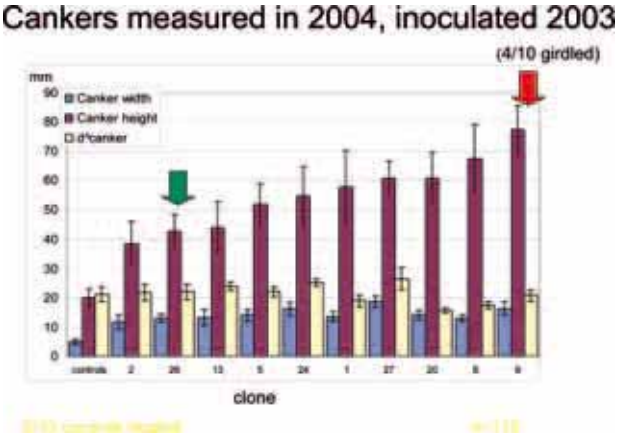

Fig. 2. Canker dimensions measured one year after inoculation.

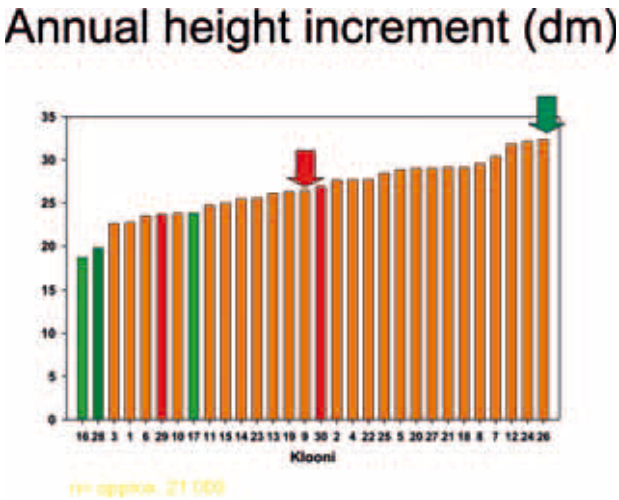

Fig. 3. The annual height increment of hybrid aspen clones (orange), aspen clones (green), hybrid aspen seed families (red) and aspen seed families (dark green). Red arrow points out the most susceptible clone (Fig 2), the clone with the highest disease resistance is shown with green arrow.

It is widely known that fungal strains have variance in virulence. In this study, only one fungal strain was used in inoculations. In our previous study (Kasanen *et al* 2002) we observed that all the isolates of *N. populi* were very similar according to the used markers since practically no variation was observed within ascospore isolates, canker isolates or reference isolates. Although it is known that no marker system can give ultimate resolution of genotypes, and the traits related to virulence are most likely not linked with the RAMS markers used, the absence of marker polymorphism suggests that the fungal isolates studied are very closely related. Thus we conclude that the use of only one fungal strain was justified by the absence of any detectable variation.

It can be concluded that the hybrid aspen clones, despite of the fact that the original selection was based on only yield and fibre characteristics, show variability in resistance. A promising observation was made by combining the results from separate trials; the best-growing clone is one of the most resistant ones. Thus it seems likely that there are possibilities to select for both growth and resistance traits in breeding. Since the occurrence and damages caused by shoot blight *Venturia tremulae* Aderh. were also surveyed on this field trial it will be interesting to see whether the resistance of the clones to several pathogens correlate.

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Fungal infections and chemical quality of subarctic *Vaccinium myrtillus* plants under elevated temperature and carbon dioxide

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Abstract

The environmental changes associated to the projected global climate change may alter the plant metabolism in a way that has consequences for plant resistance to natural enemies. Using open top chambers, we investigated the short-term effects of elevated temperature and carbon dioxide (CO_2) enrichment on the amino acids and phenolic secondary metabolites of subarctic *Vaccinium myrtillus* (L.) plants. The chemical data was correlated with severity of fungal infections on the plants, in order to find out whether the altered chemical quality could explain the abundance of fungal infections. The results demonstrated that the chemical quality of *V. myrtillus* leaves varies markedly during the growth season. Temperature elevation had the strongest capacity to alter the chemical quality and fungal infection patterns on *V. myrtillus*, whereas CO_2 enrichment had, at most, an additive effect. However, we did not find clear-cut and consistent relations between the measured plant metabolites and the severity of fungal infections. Thus, we conclude that the analyzed chemicals are not major determinants of the success of parasitic fungi on subarctic *V. myrtillus* plants under climatic perturbations.

Introduction

According to the climate models, the global average temperature and atmospheric accumulation of human-made greenhouse gases, such as carbon dioxide (CO_2) will continue to rise during the 21st century (IPCC 2001, Novak *et al*. 2004). These changes are expected to cause alterations in the biogeochemical cycles of carbon (C) and nitrogen (N) (Lee 1998). Since C and N are essential elements in the biological processes, the climate change is expected to have substantial effects on the physiology and ecology of plants. Such effects may be especially pronounced in high-latitude and high-altitude areas where the plants have adapted to low temperatures and limited availability of nutrients (Tamm 1991). The projected ecological effects of climate change include alterations in abundance of plant natural enemies, i.e., pathogens and herbivores that may be directly affected by the environmental changes (Ayres & Lombardero 2000, Bale *et al*. 2002, Mitchell *et al*. 2003). However, since the levels of different C-based and N-based metabolites may strongly determine the plant quality to consumers (e.g., Harborne 1993, Biere *et al*. 2004 and refs. within), the ecological consequences of climate change may also derive from the environmentally induced changes in plant chemical quality. Due to the complex web of interactions between different external factors and feedbacks between plant C and N metabolism (Rustad *et al*. 2001, Norby & Luo 2004, Novak *et al*. 2004, Volder *et al*. 2004), it is difficult to forecast the outcome of plant-parasite/pest interactions during the climate change. To increase the precision of climatic models and predictions, more information about plant responses to environmental manipulations is needed.

Although climate change associated changes in the growth and chemical quality of northern plants have been actively studied (e.g., Laine & Henttonen 1987, Hartley 1999, Richardsson *et al*. 2002), only few studies have considered both the C-and N-based metabolites or tested the ecological importance of the possible changes in plant chemistry to pathogen infections. Here, we addressed the questions of whether elevated temperature and CO_2 may cause alterations in the chemical quality of subarctic *Vaccinium myrtillus* (L.) plants, and whether these alterations could explain the possible changes in abundance of fungal infections in the same treatments. The study was carried out as a short-term experiment with open top chamber (OTC) CO_2 treatments and soil/air warming in the subarctic woodland of northern Sweden. During one growth season, we studied the fungal infection status on *V. myrtillus* plants subjected to elevated CO_2 and temperature (administered individually and in combination). In order to detect whether the possible treatment-induced changes in fungal infection patterns could be explained by altered chemical quality of the plants, we quantified the easily digestible amino acids, as well as low molecular weight phenolic metabolites with potential antifungal properties. The chemical analyses were conducted at three different time points of the growth season in order to address the seasonal variations in plant chemistry.

Material and methods

Study site

The study site is located in Stordalen, northern Sweden near the Abisko Scientific Research Station (68º35´ N 18º82´ E, 380 m above sea level). The experiment was carried out in the dwarf shrub understorey of an open birch (*Betula pubescens* Ehrh. ssp. *tortuosa* (Lebed.) Nyman) woodland. The understorey is dominated by evergreen (*Empetrum hermaphroditum* Hagerup and *V. vitis-idaea* L.) and deciduous (*V. myrtillus* and *V. uliginosum* L.) dwarf shrubs (Sonesson & Lundberg 1974). The mean temperature of July (1961–1990) in the region is 11ºC. Hence the climate of the area is subarctic, when the 10ºC -isotherm is used to define arctic zones (Andersson 1996).

Experimental design

The climate manipulation experiment was established in June 2000. The climate manipulation treatments were conducted on 0.5 m^2 plots that were surrounded by 30 cm high open-top chambers (OTC). The treatments were: 1. elevated temperature of the soil and air (control +5ºC; hereafter referred to as eTEMP), 2. elevated CO_2 (700 ppm; e CO_2) and 3. combination of these treatments (eTEMP + e CO_2). The soil warming was carried out with heated cables buried in the humic layer 5 cm below the soil surface (Hartley *et al*. 1999) and the air was simultaneously heated with infrared lamps. The CO_2 mixed with normal air was blown into the chambers to elevate the CO_2 level. Two types of controls were used: undisturbed control (control 1) and disturbance control (control 2) with unheated cables in the ground, OTC and circulating air. The experimental set up consisted of a total of 30 plots, which were randomly assigned to one of the five treatments (3 manipulations and 2 controls), which were repeated across 6 blocks, each of which contained each type of climate manipulation and controls.

Sampling and chemical analyses

Current year shoots of *V. myrtillus* were collected at tree occasions during 2001, i.e., in the end of June, in the end of July and in the middle of September (hereafter referred to as June, July and September, respectively). At each sampling occasion, two shoots from each plot were randomly collected. One of the shoots was frozen on dry ice for amino acid analysis and the other shoot was air-dried in room temperature for phenolic analysis. Amino acids were extracted and analysed as their 9-fluorenylmethylchloroformate (FMOC) derivatives using HPLC with fluorescence detection (Nordin & Näsholm 1997). The extraction and HPLC-analysis of phenolics was carried out according to the method described by Witzell *et al*. (2003). The most abundant individual amino acids and phenolics were quantified. Here, were report the results for four individual amino acids and phenolic compounds.

Quantification of fungal infections

In July 2001, the severity of fungal infections (i.e. presence of dark reddish or brownish spots or lesions) was visually estimated from shoots occurring along longitudinal transects on each plot. The number of shoots observed per plot varied from 18 to 21. In September 2001, leaves of 15 shoots were collected along longitudinal transects on each plot for a more detailed analysis of infection severity. The severity of fungal infestation on leaves was estimated by classifying the leaves to six groups according to the visual symptoms. The groups were as follows: no visible symptoms (group 0); infection symptoms covered less than 1 % of leaf area (group 0.5); estimated infected leaf area was about 1 % (group 1); 1–10 % (group 2); 10–30 % (group 3) or 30–80 % (group 4). The leaves on which the infections covered virtually the whole surface were classified to group 5.

To identify some of the potential causal agents of the symptoms, *V. myrtillus* leaves showing typical symptoms were collected from the immediate vicinity of the experiment, surface sterilized (4 % NaOCl for 1 min, 70 % EtOH 30 s, followed by rinsing with sterile water) and placed on potato dextrose agar (Sigma Chemicals Co, St Louis, MI, USA). On the basis of colony morphology, five of the most common fungi were selected for a more detailed identification at CBS (Centraalbureau voor Schimmelcultures, Utrecht, the Netherlands).

Statistical analyses

The MIXED -procedure of SAS (SAS Institute Inc., Cary, NC, USA, release 8.1) was used to study the treatment effects and within-seasonal (June, July and September 2001) fluctuations of the compound concentrations. The data was transformed to meet the criteria of normal distribution and homoscedasticity of variances. The main factors tested were block, time, eTEMP and eCO_2 using the repeated measurements option. The interaction between block, eTEMP and eCO_2 was used as a random factor. The control 2 (disturbance control) was chosen as the control-treatment to exclude disturbance effects from the results. The data on infection classes were analyzed with the same MIXED -model, which was used for the compound concentrations. The least squares means (LSM) of different factor combinations were compared with Tukey's post hoc test, and the slice-option of the MIXED -procedure was used to study the interactions between the factors. Disturbance by the experimental set-up, i.e. differences between controls 1 and 2, was tested with general linear model (GLM) -procedure at each sampling occasion with and without sample infection as covariate. The direct impact of infection frequency on the compound concentrations was tested with a parametric regression fit (SAS INSIGHT) between infection and concentrations of studied compounds in the controls.

Results and discussion

Fungal infections of V. myrtillus *leaves*

In July, only few symptoms were visible suggesting that the fungal infections were at the initiation phase. The proportion of the most severely infected leaves (group 3) was significantly increased in plants subjected to the combined eTEMP+e CO_2 treatment ($P_{eTEMP+e\,CO2} = 0.007$; Fig. 1a). In September, eTEMP significantly increased the proportion of healthy leaves ($P_{eTEMP} = 0.01$; Figure 1b) and reduced the proportion of leaves belonging to infection groups 2 and 3 ($P_{eTEMP} = 0.01$ and 0.009, respectively; Figure 1b). In addition, the proportion of leaves classified to the most severe infection group 5 tended to increase in eTEMP treatment ($P_{eTEMP} = 0.06$; Figure 1b). Significant main effects on fungal infections were not detected for eCO_2 (Figs. 1a, b) or for the combined eTEMP+eCO_2 treatment. The differences between controls were not consistent and significant, indicating that the OTC alone did

not systematically alter the infection patterns. Our results suggest that temperature elevation has a high potential to alter the fungal infection patterns on *V. myrtillus* leaves, whereas the effect of CO_2 enrichment on fungal infections appears to be negligible.

On basis of morphological features, at least ten different types of colonies could be separated among the fungi isolated on PDA medium. Of the isolates, *Hormonema prunorum* (C. Dennis and Buhagiar) and *Godronia cassandraea* Peck forma *vaccinii* (anamorph) could be identified to the species level, and *Melanconium* and *Isthmolongispora* to the genus level.

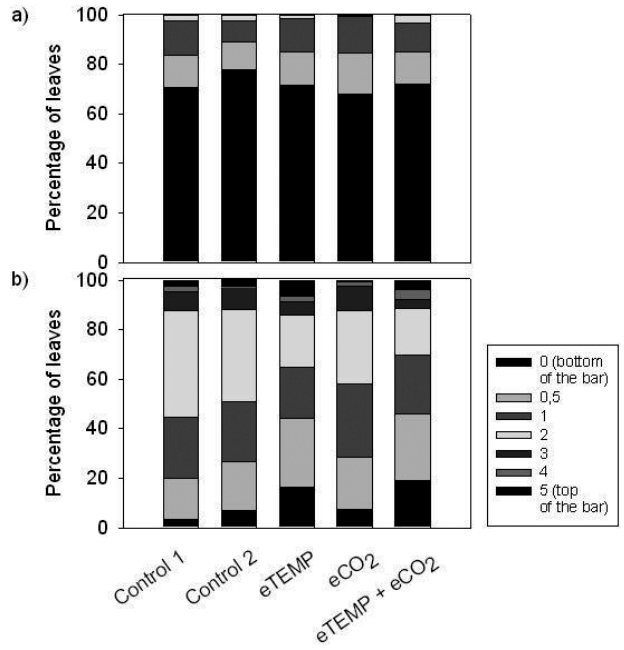

Fig. 1. Severity of fungal infections on V. myrtillus *leaves in June (a) and September (b) quantified as percentages of leaves (per shoot) classified to each infection group (0, 0.5, 1, 2, 3, 4 or 5). Shown are the mean values of 18–27 (June) and 15 (September) shoots. (n of treatments = 6).*

Within seasonal variation in plant chemistry

The concentrations of the main amino acids in *V. myrtillus* leaves (aspartate, serine, glutamate and alanine, Fig. 2) showed significant temporal variation (P_{TIME} =0.0001 for all amino acids). In addition, several of the analysed phenolic compounds showed individual seasonal kinetics (P_{TIME} = 0.0001 for arbutin and *p*-coumaric acid, as well as for two minor quercetin glucosides for which data is not shown). These results emphasize the marked within-seasonal variation in the primary and secondary chemistry of *V. myrtillus* (see also Witzell & Shevtsova 2004), and show that parasitic fungi must cope with a highly variable chemical environment during their developmental phases on *V. myrtillus* leaves. Temporal variations in plant chemicals may reflect the various functions of individual compounds in plants. For instance, aspartate and glutamate are both assimilatory and transport amino acids (Buchanan *et al*. 2000). Within-seasonal fluctuations of phenolic compounds may reflect the temporally varying allocation of carbon to either growth or defence (cf. Bryant & Julkunen-Tiitto 1995).

Treatment effects on plant chemistry

Elevated temperature, administered alone or in combination with eCO_2, decreased the concentration of glutamate especially in September (P_{eTEMP} = 0.04; $P_{eTEMP \times CO2}$ = 0.003; Fig. 2). The concentrations of some phenolics (e.g., *p*-coumaric acid and flavonoids) increased in eTEMP-treated plants in June, but in July we found reduced levels of some phenolics in eTEMP-treated plants (Fig. 3, P_{eTEMP} = 0.03 for *p*-coumaric acid; $P_{eTEMP \times TIME}$ = 0.01 and 0.002 for *p*-coumaric acid and the quercetin glycoside, respectively). We did not find significant main effects of eCO_2 on any of the analyzed amino acids or phenolics. Our results thus suggest that elevated temperature has the strongest capacity to affect the chemical quality of *V. myrtillus* leaves, whereas eCO_2 has no or only an additive effect. The lack of eCO_2 effect on amino acids suggest that there was no dilution of N concentration in *V. myrtillus* plants, although it is commonly reported in plants under elevated CO_2 (e.g. McGuire 1995). The carbon metabolism of *V. myrtillus* seemed to be generally unaffected by eCO_2, or rapidly acclimated to it, as indicated by the rather stable levels of phenolic metabolites under eCO_2.

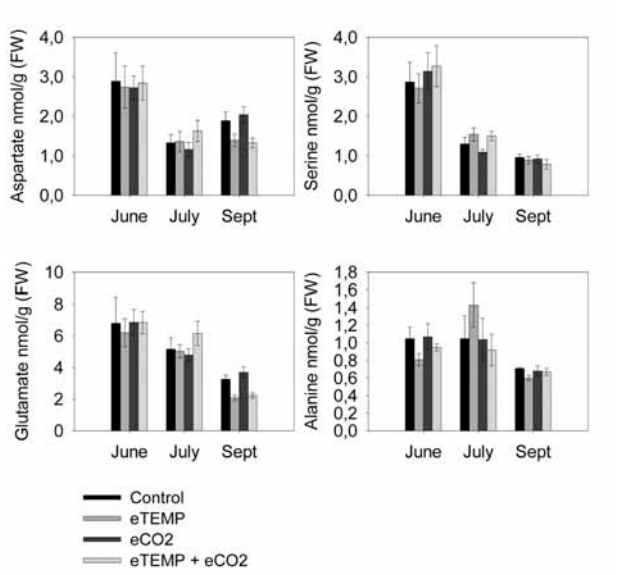

Fig. 2. Concentrations of four amino acids (nmol g^{-1} FW) in V. myrtillus *plants at different climate manipulation treatments during one growth season. Shown are the means of 6 replicates. Vertical bars represent standard error of the mean.*

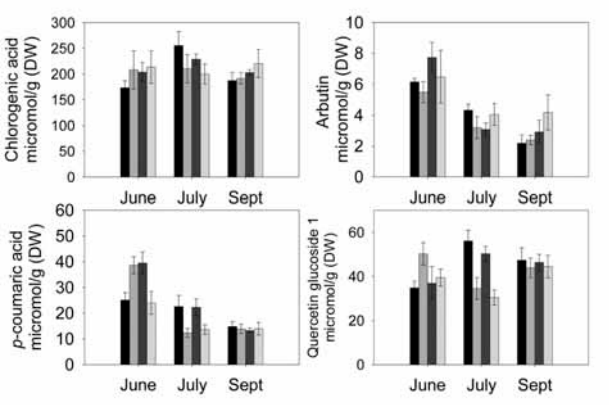

Fig. 3. Concentrations of four phenolic compounds (µmol g^{-1} DW) in V. myrtillus *plants at different climate manipulation treatments during the growth season. Shown are the means of 6 replicates. Vertical bars represent standard error of the mean. See figure 2 for the treatment legend.*

Associations between plant chemistry and fungal infections

At the study area, the outbreak of fungal infections occurred around mid of July and it is possible that the concurring eTEMP-associated decrease in phenolics (Fig. 3) rendered the plants to a better (less toxic) substrate for the parasites, allowing them to initiate leaf colonization. However, changes in amino acids and phenolics did not seem to explain the treatment-induced patterns in infections, such as the increased proportion of healthy leaves in plants treated with eTEMP (alone or in combination with eCO_2) in September. Rather, this response may have been associated with temperature-induced alteration in plant growth patterns (e.g., increased leaf biomass and area; data not shown) or to direct, microclimatic factors on the fungi. The lack of clear-cut and temporally consistent associations between the measured plant metabolites and severity of fungal infections suggests that the studied chemicals may not be major determinants of fungal success on *V. myrtillus* leaves. Thus, we conclude that the infection patterns on *V. myrtillus* plant under climate change conditions are likely to be more strongly dictated by other plant chemical characters, or by the direct effects of elevated temperature on the fungi.

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Hosts and distribution of *Armillaria* species in Serbia

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Abstract

Twenty-five tree species were recorded as hosts for five European *Armillaria* species in studies on forest ecosystems in Serbia. *Armillaria* was most frequently isolated from the conifers *Picea abies* and *Abies alba* and from the deciduous trees *Fagus moesiaca* and *Quercus petraea*. *A. mellea* and *A. gallica* coexisted in hardwood forests in northern and central parts of Serbia, while *A. ostoyae* and *A. cepistipes* were mostly present in coniferous forests in the southern mountain region of Serbia. The distribution depended on the *Armillaria* species, altitude, and the forest type.

Introduction

The genus *Armillaria* has a worldwide distribution from tundra in the north to the tropical forests around equator and the forests of Australia and Patagonia in the south. The genus includes at least 36 species (Watling *et al*. 1991; Volk & Burdsall 1995), with seven morphological species present in Europe (Guillaumin *et al*. 1985; Termorshuizen & Arnolds 1987). Six of the European *Armillaria* species have a wide distribution in forest ecosystems, while *A. ectypa* is growing only on peat bogs (Korhonen 2004). The European species differ in geographical distribution, ecological behaviour, host range, and pathogenicity (Guillaumin *et al*. 1993).

The economic significance of *Armillaria* derives from its role as a parasite of woody plants. *Armillaria* species can behave as primary and secondary pathogens causing root and butt rot on numerous coniferous and broadleaved trees species both in natural regenerated forests and in plantations (Guillaumin *et al*. 1993; Morrison *et al*. 2000). As parasites, *Armillaria* spp. can cause significant economic loss and influence the tree species composition of forests (Kile *et al*. 1991).

This study was performed to increase the knowledge about hosts and distribution of *Armillaria* species in forest ecosystems in Serbia.

Materials and methods

The study was conducted on 34 sites in Serbia and on one site in Montenegro (Fig. 1). The sites were chosen so, that they were distributed evenly throughout the country. The Site Durmitor in Montenegro was chosen because of its importance as a National Park under protection of UNESCO and because of its conserved forests.

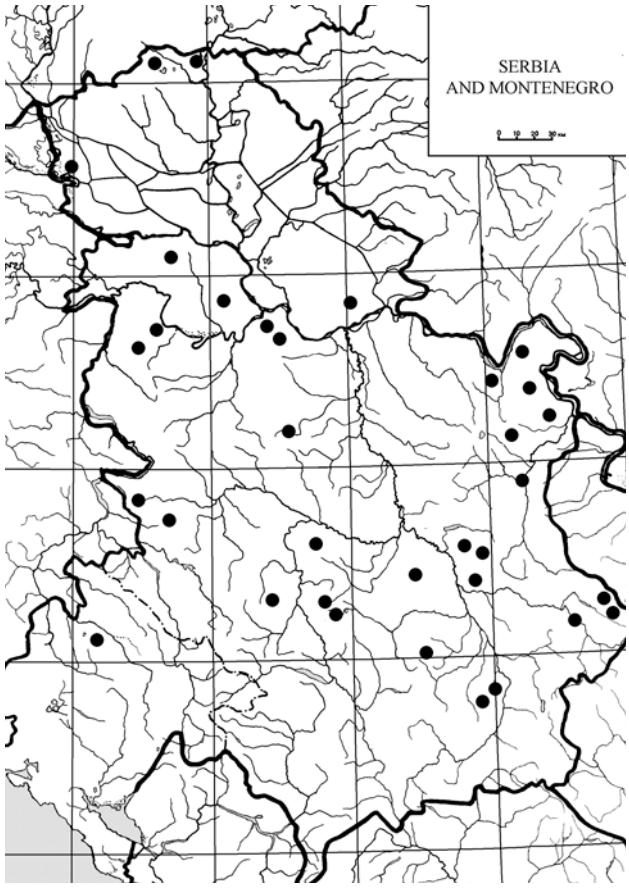

Fig. 1. Distribution of sites in Serbia from which Armillaria *species were found*

The sites studied included all dominant forest ecosystems. Different oak associations in the plain and beech associations in mountain regions were studied. Mixed forests of broadleaved and coniferous species (beech–fir, beech–spruce, beech–fir–spruce associations) were of special interest for this study, because of complex host – *Armillaria* spp. interactions.

Sampling

The sampling was done in 2002, 2003 and 2004. Sampling within the plots was systematic and focused on dominating tree species but if symptoms of *Armillaria* attack were present on other tree species samples were collected for those species as well. Sampling followed descending order of priority. Trees were examined for symptoms of decline such as crown dieback, early discolouration of needles or leaves, or presence of small leaves. If *Armillaria* species were suspected to be present, the root collar of major roots

was excavated. When potential signs or symptoms of cambial infection were observed on the living trees (resin flow, discoloration or sunken areas of bark), small areas of bark were removed to check for the presence of mycelial mats in cambial zone. Following examination of living trees, recently died trees, snags, stumps, wind-thrown and broken trees were also examined and sampled. Rhizomorphs, wood samples, mycelial mats and basidiomata were collected from 59 living trees, from 39 recently died trees and from 56 decaying trees.

Identification of isolates

Identification of isolates was performed by: a) the polymerase chain reaction (PCR) and sequencing (Chillali *et al*. 1998), b) haploid – diploid pairings according to the method of Korhonen (1978), and c) identification of basidiomata (Termorshuizen & Arnolds 1987).

Results

Species identification

Armillaria species were found on 34 sites studied (Fig.1), 152 plots or on 81 % of the controlled stands. There were no obvious differences between stands where *Armillaria* species were detected or not. A total of five *Armillaria* species were identified. *Armillaria gallica* was the species most commonly isolated (73 isolations from 27 sites), followed by *A. mellea* (51 isolations from 20 sites), *A. cepistipes* (36 isolations from 12 sites), *A. ostoyae* (25 isolations from 15 sites), and *A. tabescens* (4 isolations from 4 sites). Four isolates could not be identified as any of tested species.

Hosts

Armillaria species were found on 25 tree species that are dominant in the forest ecosystems on the studied sites. Different *Armillaria* species were isolated from 15 hardwood and 10 coniferous hosts (Table 1). Most of isolates were from spruce (45), fir (21), beech (19), and sessile oak (15).

Fifty-three percent of isolates were from conifers and 47 % from broadleaved hosts. Frequencies of isolates from conifers were: *A. cepistipes* (30 %), *A. ostoyae* (26 %), A. *mellea* (23 %) and *A. gallica* (21 %). On hardwoods *A. gallica* was the most common (58 %), followed by *A. mellea* (31 %). The other species were only occasionally found; *A. cepistipes* (7 %), *A. ostoyae* (2 %) and *A. tabescens* (2 %). *Armillaria tabescens* was observed only on hardwoods and only on oaks.

Armillaria gallica was found more frequently than expected by chance on beech and hornbeam, in 40 % of isolates, while *A. ostoyae* and *A. cepistipes* were more frequently observed on conifers. For *A. mellea* there was no statistically significant difference between association with conifers or hardwoods. Sessile oak and Austrian pine were the most frequent hardwood and conifer hosts for *A. mellea. Pinus nigra* was hosting only *A. mellea* and *A. ostoyae,* while *A. tabescens* was isolated only from *Quercus petraea* and *Q. robur*.

Table 1. Number of isolates of *Armillaria* spp. obtained from different tree species in Serbia

Hosts	**No.**
Conifers (10 species)	
Abies alba	21
Abies concolor	2
Cedrus atlantica	2
Larix europea	2
Picea abies	45
Picea omorika	4
Pinus nigra	10
Pinus sylvestris	3
Pinus strobus	7
Pseudotsuga taxifolia	6
Hardwoods (15 species)	
Acer heldreichii	1
Acer pseudoplatanus	3
Carpinus betulus	13
Fagus moesiaca	19
Fraxinus excelsior	3
Prunus domestica	2
Quercus cerris	3
Quercus farnetto	12
Quercus petraea	15
Qurcus robur	12
Quercus rubra	1
Robinia pseudoaccacia	2
Tillia argentea	1
Ulmus carpinifolia	2
Ulmus montana	1

Geographic and altitudinal distribution

Armillaria species were found in the range between 70 and 1820 m above see level (Table 2), where they accompanied trees in major forest ecosystems.

Armilliaria mellea was found in northern lowland forest types, and in eastern hilly region of Serbia with dominant forests of sessile oak, beech and hornbeam. It seems that in these ecosystems the fungus found optimal ecological conditions, characterized by forests with dominating hardwoods, especially oak species.

Armillaria gallica was found in all major regions except in the high mountains of Kopaonik, Stara Planina and Golija. It was present in beech and xerophilous forests of different oak species, but also on conifers at the higher altitudes. *A. gallica* was less frequent above 1.000 m altitude. *A. tabescens* was observed only in dryer forest ecosystems of Hungarian oak and Turkey oak at low altitudes. *A. cepistipes* was found only at altitudes above 590 m, and based on its frequency in different areas, the ecological

conditions favouring *A. cepistipes* locate in the mountain areas in the south central and eastern part of country.

Table 2. Altitudinal distribution of *Armillaria* species in Serbia

***Armillaria* sp.**	**Altitude (m)**		
	Minimum	**Optimum**	**Maximum**
cepistipes	590	1.000–1.500	1.820
gallica	60	– 1.000	1.450
mellea	70	– 800	1.040
ostoyae	850	900–1.600	1.820
tabescens	70	– 250	250

Armillaria ostoyae was predominantly found in southern part of Serbia between 44 and 43 ° N, which corresponds to the extension of Dinaric Alps and Balkan mountains. Distribution of this species overlaps with the occurrence of conifer species at higher altitudes.

Discussion

Five *Armillaria* species were now found during a survey of forest ecosystems in Serbia. Up to three *Armillaria* species were found in single sites, but on most sites two *Armillaria* species were coexisting. Combinations of *Armillaria gallica/A. mellea* and *A. ostoyae/A. cepistipes* were most frequently observed, and on some mountain sites the combination of *A. ostoyae/A. cepistipes/A. gallica* was common.

Armillaria species occurring in European forests have a wide distribution throughout the continent. *Armillaria borealis* has the northernmost distribution, its northern limit coinciding with the limit of woody vegetation in Scandinavia (Roll-Hansen 1985). The species has been found only in Europe, and the most eastern record is from Ural region in Russia (Korhonen 2004), while the southern limit is somewhere in Slovenian part of Alps (Munda 1997) and plains of Hungary (Szanto 1998).

Armillaria cepistipes has a very wide distribution from the Arctic Circle (66 °N) (Korhonen 1978) to the mountain Vernon (40°40' N) in Greece. In Serbia and Montenegro *A. cepistipes* follows the high mountain massif between 44° and 43° N. According to the data from Balkan (Tsopelas 1999; Lushaj *et al*. 2001) and Serbia, this species follows the woody vegetation to its disappearance, which has been also observed in the Alps in central Europe (Rigling 2001).

Armillaria ostoyae occurs independently of latitude or altitude in European coniferous forests with continental or oceanic climate type (Guillaumin *et al*. 1993). As observed in Mediterranean countries, *A. ostoyae* was now found only at high altitudes in Serbia. High mountains of Dinaric Alps (south-western part of Serbia) and Balkan Mountains (south-eastern part of Serbia) massifs were the only sites where this species was recorded. *A. ostoyae* appeared above 800 m, but its optimal growth conditions seem to locate between 1000–1600 m. On higher altitudes its occurrence decreased, but still it accompanied coniferous forest types to the end of vegetation. It seems that the altitudinal distribution of *A. ostoyae* is similar between southern and central part of Europe and influenced by the distribution of conifers.

Armillaria gallica is widely distributed throughout the European continent, but its distribution is highly dependent on altitude (Guillaumin *et al*. 1993). In the French Massif Central *A. gallica* is predominant in forests up to 850 m, but becomes rare at higher altitudes, though it still is present up to an altitude of 1100m. Because of the continental climate type prevailing in northern and central part of Serbia this species is rare at altitudes above 1000 m and absent from altitudes above 1400 m.

Armillaria mellea occurs in central and south Europe, but is common only in the southern and western parts of this area (Korhonen 2004). In central part of France the species is present in all predominant forest types at altitudes below 900 m (Legrand & Guillaumin 1993) but further south the species can occur at altitudes up to 1400 m in Albania (Lushaj *et al*. 2001) and up to 1750 m in Greece (Tsopelas 1999). Records from Serbia show that this species is distributed throughout the country, except in high mountain region.

Armillaria tabescens is the most thermophilic species and it was found in Serbia only in the altitude range between 70–250 m. This does not correspond with the data from Greece (Tsopelas 1999) and Albania (Lushaj *et al*. 2001), where the species has been found at altitudes up to 1150 m and 1300 m, respectively. Climatic conditions may explain this difference since Serbia has a more continental climate than the others.

Due to their wide host range *Armillaria* species can survive for a long time on an occupied forest area (Kile *et al.* 1991). These fungi can successfully survive on plant remains and wait for an opportunity to colonize new substrate, either as opportunists or primary pathogens. A simplistic view of interactions between hosts and *Armillaria* species is that *A. mellea*, *A. gallica* and *A. tabescens* occur primarily on hardwood species, while *A. ostoyae*, *A. cepistipes* and *A. borealis* prefer conifers (Kile *et al*. 1991, Fox 2000). However, it should be kept in mind that all these species can successfully colonize both conifers and broadleaved trees.

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Discolouration of birch after sapping

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Abstract

Discolouration in the wood of silver birch (*Betula pendula* Roth) was studied in a 60-year-old birch stand in eastern Finland. Altogether 45 trees were analysed two and five years after sapping.

The boring hole made for sapping caused a strongly flattened, conical- shaped discolouration column down- und upwards from the hole. The discolouration spread only very slightly in the radial or the crosswise directions, but increased rapidly in the longitudinal direction. In many trees the discolouration caused by the sapping hole joined with discolouration originating from branches and butt. After five years, the estimated volume of the discolored area was almost four times bigger in these trees.

486 microbial pure cultures were isolated (191 bacteria, 224 fungi, 77 yeasts or yeast-like fungi). The samples from the base of the tree contained a larger proportion of fungal isolates than samples from the highest point of discolouration. The number of pure cultures containing bacteria and yeasts was less after five years than after two years since sapping. Even the samples from sound-looking wood contained microbes, mostly bacteria. Most of the identified fungi belonged to *Phialophora* sp. (especially *Phialophora fastigiata*). *Penicillium* sp.and *Cladospora* sp. were also common. Only three of the isolates contained suspected basidiomycetous decay fungi. Most of the identified bacteria belonged to genera *Serratia*.

Introduction

Sapping of broadleaved trees, like birch species (*Betula* sp.) has been a long tradition. Birch sap can be used for a variety of purposes. The production, composition and properties of the sap, birch syrup, have been rather intensively studied (e.g. Kallio *et al*. 1989). Sap can be collected from a bundle of narrow, cut branches, from one larger branch, or from a hole bored near the base of the trunk. The latter is the most efficient way in terms of sap production. From the forest pathological point of view, however, wounding the tree in this way unavoidably causes wood discolouration and decay later on (Vuokila 1976). Therefore, this method is commonly exploited 5–10 years prior to the felling of the trees. However, the extent or the rate of spread of the discolouration is not well known. The first colour changes in the wood are due to oxidative processes. Micro-organisms appear later, if the environmental conditions are favourable for them (Scheffer 1969, Wilhelmsen 1975). The literature on the microbial flora and its succession at the early stages of injury on birch is relatively scarce. The later stages, decay of birch trees and the microbes from decayed birches are known much better also in Fennoscandia (Björkman 1953, Henningsson 1967).

Material and methods

The study was carried out in a 60- year-old silver birch (*Betula pendula* Roth.) stand in the Koli research forest, eastern Finland (63º 7.3’ N, 29º46,7’ E). The stand was growing in a grove-like, grass-herb mineral site type (Oxalis-Myrtillus site type). The stand was born naturally after prescribed burning, and thus resembles the typical birch stands in the area. A permanent study plot was established in the stand, and three groups of log-sized trees, 20 trees in each, were selected for sapping. The trees in the groups were subjectively selected to resemble each other by their diameter, crown condition and general vigour. Conventional stand and sample tree measurements were carried out. Possible defects such as frost cracks and conks of rot fungi were also recorded.

Sapping was conducted during early summers in two consecutive years. The exact dates were from 6th May until 3rd of June in 1996 and from 12th of May until 3rd of June in1997. 30 trees were tapped in each year. A slightly upwards-slanting hole with a length of 6–7 cm was made near the base of the trunk in each tree with an incremental borer, and sap was tapped through sterilized plastic tubes. The mean height of the hole was 42 cm from the ground. The results such as sap production etc. are reported elsewhere (Salo 2000). After sapping, the holes were either i) left open ii) closed with a plug of birch wood or iii) sealed with beeswax.

Altogether 45 trees were felled two and five years after the sapping year, in 1998, 1999, 2001 and 2002, in the beginning of November. Trees with signs of external injuries or conks were rejected. The average data of the felled trees is presented in Table 1. A disc of about 10 cm containing the sapping hole was first taken. The extend of the discolouration column was then followed down- and upwards. The dimensions of the discoloured area were measured also in radial direction (i.e. the direction of the boring hole) and at right angles to it (in «tangential» direction). A disc containing the highest point of the column was also sawn.

Table 1. Average data of the felled sample trees.

Year of felling	Years from sapping	Dbh, cm	Volume,dm^3	Height,m	Crown base height, m	Crown width, dm	Number of trees
1998	2	25.54	542.98	23.08	10.12	56.00	14
1999	2	24.18	495.43	23.30	10.41	56.90	10
2001	5	20.67	360.60	22.62	10.41	47.50	10
2002	5	22.70	436.66	23.16	11.44	51.91	11

In the laboratory the two discs were aseptically dissected, and small chips of wood were cultured on malt extract agar for the isolation of microbes. The samples were taken from discolored wood just above the hole (sample a), from sound-looking wood at the same height (sample b) and near the highest point of the discolouration (sample c). The microbes were grouped, and some of the groups were identified morphologically using the identifications and descriptions e.g. in Cole & Kendrick 1973, Domsch *et al*. 1983 and Wang & Zabel 1990. Some bacterial cultures were identified by the VTT Technical Research Centre of Finland using the Riboprinter method (DuPont Qualicon, USA).

Fig. 1. A typical discolouration at the height of the boring hole, five years from sapping. The discolored area has spread a little in the radial and tangential directions.

Results

Discolouration

The boring hole made for sapping caused a very narrow, strongly flattened, conical- shaped discolouration column down- and upward from the hole. In most cases, the discolouration widened only a few millimetres in the tangential – or radial dimensions after two and five years (Fig. 1). The dimensions increased greatly, and statistically significantly, in the vertical direction between the dates (Tables 2 and 3). The column was at its widest at the height of the boring hole, narrowing quickly downwards- and also upwards within a distance of 60–70 cm. The typical shapes of the discolouration column caused by the sapping hole after five years are described schematically in Fig. 2.

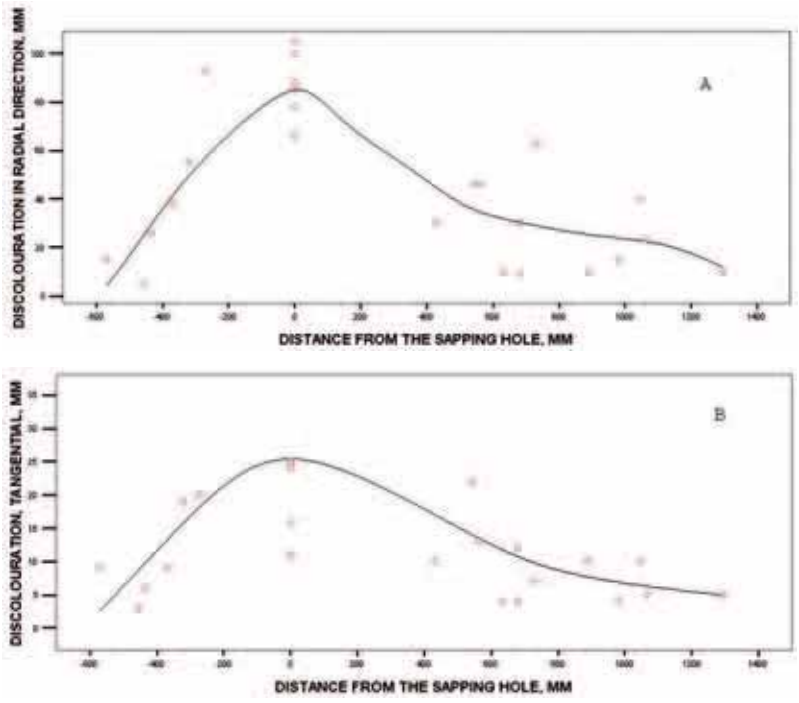

Fig. 2. Schematic presentation of the width of the discolouration column at different heights from the sapping hole. A. Radial direction B. Tangential direction

Table 2. Dimensions of discolouration two and five years after sapping. Data: all felled sample trees.

Time	Dimensions of discolouration, mean ± s.d.			
	Height, cm	Width, radial, cm	Width, tangential, cm	Volume of discolored area, cm^3
2 years after sapping	109.3 ± 93.2	4.7 ± 1.8	1.9 ± 13	
5 years after sapping	245.0 ± 243.5	6.6 ± 2.3	2.3 ± 1.3	7152.1 ± 11672.8
M-W U significance	0.004	0.013	0.059	

In 15 trees (33.3 %) the discolorated area was quite wide, sometimes also near the base of the trunk. Without exception, these were the cases where discolouration originating from branches/ branch stubs or butt of the tree joined the discolouration caused by the sapping hole. This phenomenon complicated the analyses and caused much variation in the dimensions of the discolouration. All the dimensions of the discoloured area were much smaller in the trees in which the discolouration originated from the tapping hole alone. For instance, the estimated volume of the area was 16 x smaller in these trees (Table 3).

Table 3. Dimensions of the discolouration five years after sapping.

Origin of discolouration	Discolouration five years after sapping, mean ± s.d			
	Height, cm	Width, radial,cm	Width, tangential, cm	Volume of discoloured area, cm^3
Sapping + branches and butt	402.9 ± 309.8	8.1 ± 1.8	3.1 ± 1.5	15433 ± 444.4
Only from the sapping wound	126.5 ± 47.9	3.7 ± 0.9	1.3 ± 5.4	941.4 ± 238.4

There were some differences in the dimensions of the discolouration according to the closing method. Due to the difficulties described in the previous chapter, these could not be analysed reliably in all trees. Therefore, the differences between the closing methods were not statistically significant after five years (Table 4).

Table 4. The dimensions of the discolouration by different closing method, five years after sapping

Closing method	Dimensions of the discolouration (in mm) 5 years after sapping		
	Mean height	Width, radial direction	Width, tangential direction
Control	1178	36	14
Wood	1637	63	22
Wood + wax	1095	74	33
Kruskal- Wallis Chi-Square	.831	3.568	.695
K-W significance	.660	.168	.707

Microbes

486 microbial pure cultures were obtained (191 bacteria, 224 fungi, 77 yeasts or yeast-like fungi). The greatest change between the two dates of sampling (two and five years after sapping) was the reduction in the number of cultures containing bacteria (from 183 to 65 cultures). The number of cultures containing fungi also reduced slightly, from 122 to 102. The numbers containing yeasts or yeast-like fungi were 44 and 33, respectively. After five years, 90 % the a- samples (samples from the discoloured wood just above the boring hole) contained fungi. Even the b-samples (from sound-looking wood) contained microbes, mostly bacteria, although over 40 % of them were sterile (Table 5). After five years, only 3 % of the cultures contained fungi, which were suspected to be decay fungi. These were found in trees with discolouration originating from branches.

Table 5. Proportion of microbial groups in different sampling points (a, b,c)*.

	2 years after sapping			5 years after sapping		
	a	b	c	a	b	c
	Proportion of samples containing…					
Bacteria	.88	.46	.63	.38	.29	.57
Fungi	.63	.00	.21	.90	.00	.33
Yeasts	.54	.04	.29	.38	.19	.48
Sterile	.08	.42	.33	.05	.43	.24

*The samples were taken a) from discolored wood just above the hole, b)from sound-looking wood at the same height, c) and near the highest point of the discoloration

Phialophora sp. was the most common of the fungal genera (65 isolations). Some of these resembled morphologically *Phialophora fastigiata* (Lagerberg & Melin) Conant (Fig. 3). 51 (80 %) of the *Phialophora* sp. samples were obtained in sampling point a. *Penicillium* sp. (in 21 cultures) and *Cladosporium* sp. (in 8 cultures) were also common fungal genera. Yeasts and yeast-like fungi were also common, but it was not possible to identify them at this stage. Moreover, it was very difficult to separate bacteria/fungi/ yeasts in some samples with conventional culturing- subculturing methods (e.g. dilution plates etc.).

Fig. 3. The most common fungal isolate, morphologically identified as Phialophora fastigiata*, with funnel-shaped collaret's (1000 x).*

Of the samples taken 2 years after sapping, 18 bacterial pure cultures were selected for identification with the Riboprinter method. 10 of these were identified as *Serratia proteamaculans* subsp. *quinovora*. The proper name should now be *Serratia quinivorans* (Ashelford *et al.* 2002). Five of the isolates remained unidentified, and the remaining three were *Serratia proteamaculans* subsp. *proteamacula, Rahnella aquatilis* and *Hafnia alvei*.

Discussion

The present study gives support to the hypothesis that bacteria, yeasts, and other nonhymenomycetes are the primary colonists of discolored tissues. Most likely the early colonizers such as non-decay fungi (*Phialophora*) alter cell wall components, and degrade wound-initiated vessel plugs The may also modify phenolic substances in the reaction zone. All these primary degradations may modify wood xylem sufficiently for the decay fungi to break down the main part of the cell walls (lignin and cellulose). Mutualistic associations of bacteria and yeasts with wood-destroying hymenomycetes are also possible, since Basidiomycetous hyphae have been observed only in tissues where amorphous vessel deposits had been degraded by pioneer microorganisms (Shortle & Cowling 1978, Blanchette & Shaw 1978, Blanchette 1979). *Phialophora*- species have been found to be the predominant non-decay fungal species in wood a long time ago (Shigo 1967, Stewart *et al.* 1979).

Serratia appears to be a ubiquitous bacterial genus in nature, and ten species are currently recognized. Serratia species have been isolated from water, soil, animals (including man), and from plant surfaces (Grimont & Grimont, 1992). Their role in the discolouration process of wood is however unknown to the author.

There was no indication that the wounds made for sapping are infected by typical decay fungi of birch in this study. Hallaksela and Niemelä (1998) did not find typical birch decayers in their study on planted silver birch either, although some decay fungi were isolated from discolored wood. Lilja and Heikkilä (1995) found decay fungi, esp. *Chondostereum purpureum* in older defects in young birch trees broken by moose. *Phialophora fastigiata* was a common isolate in their material, and it also grew together with bacteria.

The results of this small-scaled study showed that the boring hole made for sapping caused only a minimal risk to the technical quality of the birch trees after five years, assuming that there are no other pathways for the infection of decay fungi.

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Top shoot dieback on Norway spruce seedlings associated with *Gremmeniella* and *Phomopsis*

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Abstract

In spring 2002, extensive damage was recorded in southeast Norway on nursery-grown Norway spruce seedlings that had either wintered in nursery cold storage or had been planted out in autumn 2001. The damage was characterised by a top shoot dieback. Two visually distinct types of necroses were located either on the upper or lower part of the 2001-year-shoot. Isolations from the upper stem necroses rendered *Gremmeniella abietina*, while *Phomopsis* sp. was isolated mostly from the lower stem necroses. RAMS (random amplified microsatellites) profiling indicated that the *G. abietina* strains associated with diseased nursery seedlings belonged to LTT (large-tree type) ecotype, and inoculation tests confirmed their pathogenicity on Norway spruce seedlings. *Phomopsis* sp. was not pathogenic in inoculation tests, this implying it may be a secondary colonizer. We describe here the *Gremmeniella* – associated shoot dieback symptoms on Norway spruce seedlings and conclude that the unusual disease outburst was related to the *Gremmeniella* epidemic caused by the LTT ecotype on large Scots pines in 2001. The role of *Phomopsis* sp. in the tissue of diseased Norway spruce seedlings is yet unclear.

Introduction

In the spring of 2001 a devastating epidemic of *Gremmeniella abietina* (Lagerb.) M. Morelet on large Scots pines (*Pinus sylvestris* L.) occurred in the south-eastern part of Norway (Solheim 2001) and in adjacent parts of Sweden (Elna Stenström, personal comm.) which probably was the strongest outbreak recorded in these areas.

The following spring, in 2002, a frequent occurrence of diseased Norway spruce (*Picea abies* (L.) Karsten) seedlings was registered in forest nurseries in the south-eastern part of Norway. The damage was detected mostly on 2-year-old seedlings that were either planted out in the autumn 2001 or taken out from cold storage, ready to be planted out in the spring 2002. The seedlings showed various degrees of top shoot dieback. When surveying plant nurseries with heavy damage, also 1-year old seedlings were seen with similar symptoms, but to a lesser extent. At a closer examination principally two different types of stem necroses were observed. The two types of necroses yielded *Gremmeniella abietina* and *Phomopsis* sp. respectively.

Damages caused by *Gremmeniella abietina* are well documented and described on large pines as well as on Norway spruce. In Northern Europe *G. abietina* consists of two ecotypes, A and B (Uotila 1983), also described as «the small tree type» (STT) and «the large tree type» (LTT), respectively (Hellgren & Högberg 1995). The LTT is most common in 15–40 year-old Scots pine trees in southern Scandinavia and Finland (Hellgren & Barklund 1992, Uotila 1992), where it causes dieback of current year shoots in the entire crown. The STT occurs on young Scots pine trees in northern Scandinavia and at higher elevations in the south, where it causes perennial cankers on the parts of the tree covered by a lasting snow layer during the winter (Karlman *et al.* 1994).

On pine seedlings it causes the typical umbrella-like folding of needles on the leader (Nef & Perrin 1999). However, to our knowledge, neither *Gremmeniella abietina* nor *Phomopsis* sp. infections have been described on Norway spruce seedlings in nursery production.

Here we report on the *Gremmeniella* and *Phomopsis* associated symptoms on Norway spruce seedlings that occurred after the epidemic *Gremmeniella*-outbreak in spring 2001. The objectives of this work were (i) to describe the disease symptoms on Norway spruce seedlings; (ii) to isolate and identify the fungi associated with this damage and further determine their pathogenicity *in vivo* and *in vitro*; (iii) to assess survival and development of the outplanted symptomatic seedlings.

Materials and methods

Plant material and fungal isolation

Norway spruce seedlings (2-year-old) were collected from affected nurseries in south-east Norway. The length and location of the necroses were measured. Tissue chips were cut out from the necrose margins, sterilized and plated on the malt (1.25 %) agar (2 %) medium, incubated at 210C in the dark for 3–5 weeks, then fungi were identified.

Pathogenicity test in vitro *and* in vivo

The fungi isolated from the diseased seedlings were tested for their ability to induce dieback on fresh living tissue *in vitro* and *in vivo*. For both tests, three isolates of *Gremmeniella abietina* (2002–48/2, 2002–26/2, 2002–47/1), and *Phomopsis* sp. (2002–53/3, 2002–117/3, 2002–62/1) were chosen. The *in vitro* test compared the ability of the fungi to kill the tissue of freshly detached, aseptic spruce needles. Needles from aseptically grown spruce seedlings (about 5 weeks old) were detached, placed in a petri plate containing malt agar medium, together with the actively growing culture of the fungus. The needles were positioned in front of the advancing mycelium. Needles on malt agar without any fungal culture were used as controls. The petri plates were incubated in the darkness at room temperature. The visual inspection of all needles was done

once per day. The relative amount of discoloration on each needle was recorded and the percentage of damage for each needle was registered. There were three replicates for each fungal culture with 10 needles in each petri dish. The pathogenicity for each fungal culture was estimated as a time necessary for the fungus to kill 50 % of the needles.

To determine the pathogenicity of the isolated fungi *in vivo*, healthy looking seedlings were inoculated with the same fungi as in the pathogenicity test *in vitro*. Both 1-and 2-year-old seedlings of Norway spruce, were delivered from the nursery production in November 2003. Ten seedlings of each kind were inoculated with 3 isolates of *Gremmeniella*, 3 isolates of *Phomopsis* sp., respectively. A scalpel incision (2 mm) was made in the middle of the stem and a piece of fungal mycelium (about 1 mm^3) on agar medium was placed inside. The wound was sealed with parafilm. Control seedlings were mock inoculated with agar only. Seedlings were then placed in containers and moved over to a climatic chamber where cold storage conditions (2–50 C, 80 % humidity and darkness) were simulated. Eighteen weeks later extend of the necroses and the shoot lengths were measured.

Outplanted symptomatic seedlings

In order to investigate and follow the further development of diseased seedlings, an outdoor outplanting experiment was set up. One year old Norway spruce seedlings, originating from the nursery with large amount of typical *Gremmeniella*-diseased seedlings, were selected for outplanting. Thirty-six seedlings with the same symptoms were taken to Hoxmark, the experimental garden of Norwegian Forest Research Institute, and outplanted during the summer 2002. All seedlings had dead top shoots. Total shoot length, the length of the diseased shoot and the extent of the necrotic part of the shoot were measured, in spring 2003. All outplanted seedlings showed a tendency of the side shoot taking over the dead leader. In 8 cases out of 36, there was a tendency to develop a double leader (double stem). The seedlings were regularly observed during the following growing seasons, and development of fungal fruitbodies was monitored. In January 2005 all seedlings were cut off , their health condition, shoot length and fungal fruitbody development was evaluated.

RAMS-PCR-assay of Gremmeniella *isolates*

Random amplified microsatellite (RAMS) technique was used to further characterize the *Gremmeniella* – isolates and determine which biotype they represented. The *Gremmeniella* – isolates were grown on cellophane-coated malt and V8 juice agar, and the mycelia harvested were ground with a pestle in liquid N_2 chilled mortars. DNA isolation was performed by using Plant DNA Mini Isolation Kit (Qiagen) according to the manufacturer's instructions. The PCR reactions were carried out in the reaction conditions recommended by the manufacturer of the HotStarTaq™ DNA Polymerase by using 2 µM concentration of the degenerate CCA primers described by Hantula and Müller (1997). The PCR cycling parameters were also as described in that study. Amplification products were separated by gel electrophoresis in 1.5 % agarose gels using TAE running buffer and visualized under UV-light after ethidium bromide staining.

Statistical analysis

The data for necrosis length on 1- and 2-year-old Norway spruce seedlings in the *in vivo* pathogenicity test were subjected to analysis of variance by using Oneway ANOVA (JMP, SAS institute)

Results and discussion

The symptoms on Norway spruce seedlings became visible during the spring of 2002, one year after the *Gremmeniella* epidemic on large Scots pines. Both 1- and 2-year-old plants showed symptoms of desiccated leader shoot (Fig. 1) and had necrotic stem lesions on the 2001-year shoot. The first visible signs of a stem lesion were a local indentation in the bark, and greyish green foliage on the lesion area. Later the foliage and branches distal to the lesion area became yellow and brown. Some lesions were located only on one side of the stem, while others ringed the whole stem, causing top dying of the shoot. Occasionally there were 2–3 separate necroses on one stem. Generally, two types of necroses, «upper stem necroses» and «lower stem necroses», could be distinguished (Fig. 2).

Fig. 1. Top shoot dieback caused by G. abietina *on 2-year-old Norway spruce seedling. Photo: H. Solheim.*

Fig. 2. Characteristic location and appearance of the necroses on stems of the 2-year-old Norway spruce seedlings. Typical upper stem-necrosis (photo on the left), with brown, resinous tissue, where G. abietina *was isolated. Lower stem necrosis (photo on the right), with light brown and waterlogged tissue, were often located close to the stem node.* Phomopsis *sp. was frequently isolated here. Photos: H. Solheim.*

Upper stem necroses: associated with Gremmeniella

Mean 2001-shoot length on 2-year-old seedlings with this type of necroses was 25 cm. Necroses on the upper stem were located 14.9 cm (mean distance) above the 2000–2001 stem node and their average length was 4.3 cm. The necrotic, dark brown coloured bark was profusely impregnated with resin (Fig. 2). In this area, the stem was usually girdled, the nearby needles were brown at the base, and the shoots above the necrosis were dead or dying. The edges of the necroses were sharp and distinct. In most cases, *G. abietina* was isolated from the advancing edge of the necrotic tissue. *G. abietina* alone was isolated predominantly from seedlings sampled in April-May period. In isolations performed later (June and later), also *Phomopsis* was occasionally recovered from this type of necroses. No other potentially pathogenic fungi were isolated from the upper stem necroses. Most of the seedlings with upper stem necroses yielding *Gremmeniella* originated from a nursery, where large pine trees were in close vicinity to the nursery area.

Lower stem necroses: associated with Phomopsis

Mean 2001-shoot length on 2-year-old plants with this type of necroses was 21 cm. The mean distance from the lower edge of the necroses to the 2000–2001 stem node was 3.9 cm. These necroses were often located at the base of the 2001-shoot or partially at the end of the 2000-shoot. Necroses on lower stem were lighter in colour compared to the upper stem necroses, and had a characteristic water-soaked appearance without any resin flow (Fig. 2). The edges of necroses were diffuse, non-distinct. Occasionally, such necroses were found also on the upper part of the 2001-shoot. The most frequently isolated fungus from these lesions was *Phomopsis* sp., which was recovered in the period from April to December. Apart from two cases where *Botrytis* sp. was recovered, no other potentially pathogenic fungi were isolated from these necroses. Fruitbodies of *Phomopsis* sp. developed readily on plants after storage at + 4×C. Seedlings with lower stem necroses originated mostly from nurseries, where there were no pine trees in the immediate vicinity.

The stem necroses may have originated from the bark fissures, cracks in the bark associated with rapid growth, usual for plants in nurseries. The damage above the necroses first became visible in 2002. The seedlings were probably infected during spring or summer 2001 and the disease was already latent during their moving to cold storage or outplanting, in autumn 2001. Presumably, the seedlings at this point had no visible symptoms, which would explain why infected plants were not discarded.

Pathogenicity tests

In the pathogenicity test *in vitro* with needles (Fig. 3), *G. abietina* strains killed 50 % of the needle tissue within 4–6 days, strain 2002–48/2 (G3) being the most aggressive. The *Phomopsis* strains (P1 and P3) caused 50 % damage on needle tissue after 9 days, while P2 showed no signs of pathogenicity at 10 days after the inoculation, when the experiment was ended.

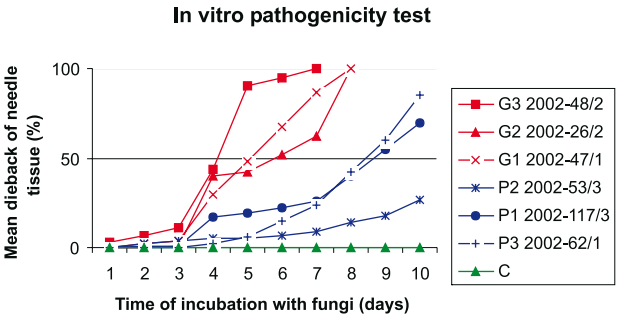

Fig. 3. Pathogenicity test in vitro. Dieback of aseptic spruce needles inoculated with three isolates of G. abietina *(G1-G3) and* Phomopsis *sp. (P1-P3) compared to non-inoculated control needles (C). All fungi were isolated from Norway spruce seedlings with top dieback symptoms.*

In the pathogenicity test *in vivo*, seedlings were stored in climatic chambers for 18 weeks in the period from mid November to the end of March. In one-year-old seedlings, *G. abietina* strains 2002–48/2 (G3) and 2002–26/2 (G2) caused significantly longer necroses than the other strains (Fig. 4). The necroses produced by the other strains were not significantly different from the control. In two-year-old seedlings, the longest necroses were caused by *G. abietina* strains 2002–26/2 (G2) and 2002–48/2 (G3), but only *G. abietina* strain 2002–26/2 (G2) differed significantly from the control. (Fig. 4).

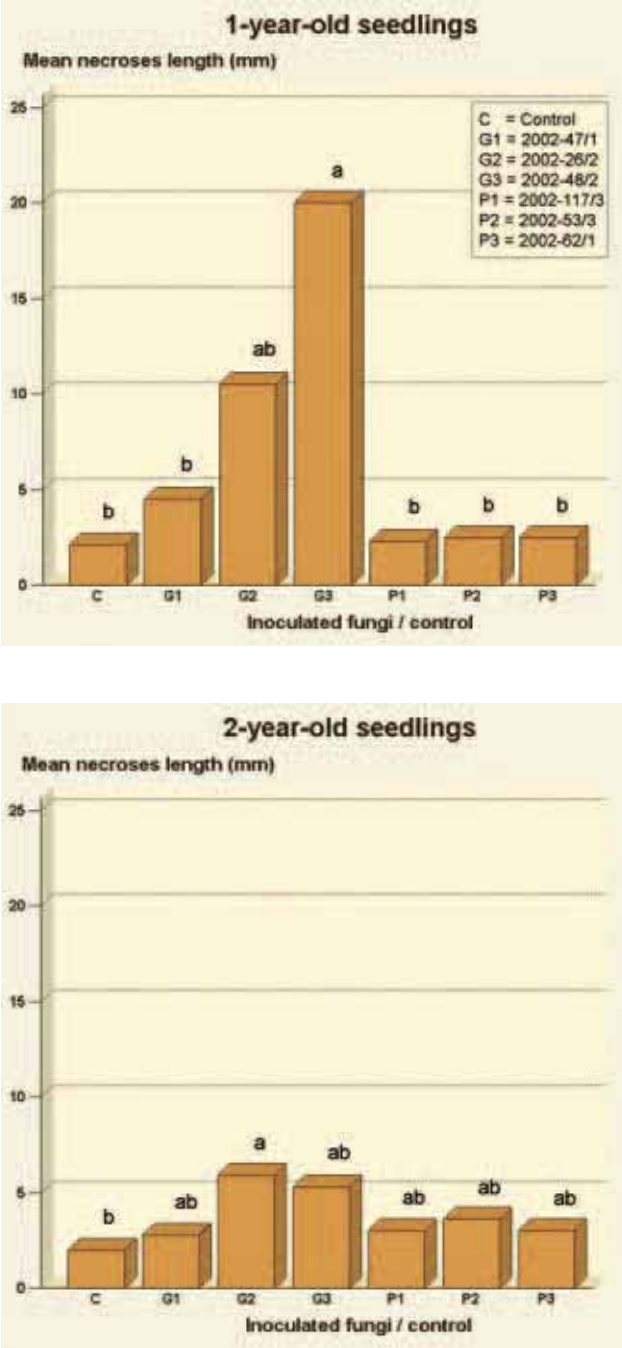

Fig. 4. In vivo *pathogenicity test. Length of necroses in one- and two-year-old Norway spruce seedlings 18 weeks after inoculation with different isolates of* G. abietina *(G1-G3),* Phomopsis *sp. (P1-P3) and mock inoculated control (C).*

Both pathogenicity tests confirmed the virulence of the *Gremmeniella abietina* isolates on Norway spruce seedlings. Most of the literature on nurseries reports *Gremmeniella* exclusively as a pathogen on pine seedlings, and if associated to Norway spruce, *G. abietina* is mentioned as a pathogen on saplings (Kaitera *et al.* 2000) and on larger seedlings in plantations (Roll-Hansen 1967). In pine seedlings, the disease is easily recognized by the characteristic umbrella-like folding of needles on the leader shoot (Björkman, 1959, Nef & Perrin 1999), whereas the symptoms of *Gremmeniella* infection on Norway spruce seedlings, necroses and shoot dieback, are rather non-specific and can be caused by several pathogens as well as by abiotic stresses, such as frost, drought or cold storage. Since multiple factors can cause these symptoms in Norway spruce seedlings, incidents of *Gremmeniella*-infection may be misidentified.

Symptomatic seedlings in outplanted plots

In spring 2003, at the time of the first assessment, 23 % of the seedlings (8 seedlings out of 36) had a tendency to develop a double shoot, i.e. two sideshoots were competing for the dominance. At this time, four dead shoots had pycnidia of *Brunchorstia pinea* (P. Karst.) Höhn., the anamorph stage of *G. abietina*, with conidia still present. In January 2005, at the time of final harvesting, 64 % (9 seedlings out of 14) of the seedlings had developed a double stem (unfortunately, 22 seedlings were destroyed by accident before the last evaluation, and thus only 14 remaining seedlings were inspected at the end of the experiment). The seedlings were alive, and showed good growth (Fig. 5). The originally diseased leader shoots had been taken over by a new leader. Out of the 14 dead shoots collected at the last inspection, four had old, empty pycnidia still present, while ten had only visible scars after pycnidia. No apothecia were observed in any seedling.

The outplanting experiment confirmed that the infected Norway spruce seedlings survive the damage. Even if the part above the stem necrosis dies, in young plants usually the side shoot takes over the dead leader. Some of the seedlings develop double leaders after the *Gremmeniella* infection.

Fig. 5. Long term field performance of damaged 1-year-old Norway spruce seedlings. Left: Seedlings were 1-year-old (in 2002) when top shoot damage occurred (arrow). One year later (in 2003) the dead shoot was taken over by side-shoots. Right: The same seedling in 2005. Photos: H. Solheim

The RAMS-PCR assay

The RAMS-CCA banding patterns were identical among the *Gremmeniella* isolates from Norway spruce seedlings, while the included reference strains of LTT and STT ecotypes showed type specific banding patterns (Fig. 6). The assay confirmed that the *Gremmeniella* isolates from Norway spruce seedlings belonged to the LTT ecotype, as their banding patterns were identical to those from the reference strains of the type and differed from the STT reference strains. With the CCA primer, only the LTT reference strains and the strains from the seedlings had a 1500-bp band.

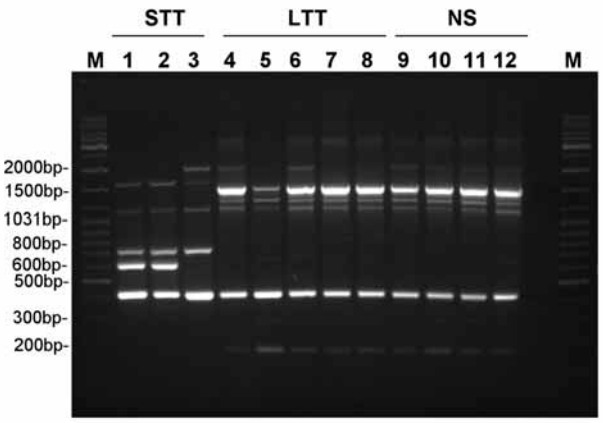

Fig. 6. RAMS patterns (CCA primer) of three small-tree type (STT) (1988–306/1, 1988–307/3 and 1974–46/1, respectively) and five large-tree type (LTT) (2002–20/4, 2002–47/1, 1985–111/6, 1985–393/16/1 and 1966–163/2, respectively) G. abietina *reference strains, and four isolates (2002–4/4, 2002–79/2, 2002–107/2 and 2002–124/1, respectively) obtained from diseased Norway spruce seedlings from nurseries (NS) with* Gremmeniella *problems . Lane M: DNA size marker (GeneRulerTM DNA ladder mix).*

These data confirmed that the strains associated to nursery-grown Norway spruce seedlings belonged to the LTT ecotype of *G. abietina*. Our nursery samples were collected from the geographical area in south-eastern Norway, where a devastating epidemic of *G. abietina* had occurred on large pines the previous year. This epidemic was a typical LTT outbreak characterised by dieback of shoots in the entire crown (Solheim 2001). As the *Gremmeniella* strains from diseased nursery seedlings of Norway spruce grouped to the LTT, we conclude that the unusual disease outburst on Norway spruce seedlings in 2002 was related to the previous year's epidemic on Scots pines. Apparently similar damages in Norway spruce seedlings after the pine epidemic were observed in Sweden (Stenström, pers. comm.) and in Finland (Petäistö 2003) as well.

During the periods of high inoculum density, the pathogen can also infect the Norway spruce seedlings in the neighbouring nurseries. In order to avoid infection from the pines, it is important to keep the pines away from the forest nurseries and Christmas tree plantations.

Besides *Gremmeniella*, a *Phomopsis* species was frequently associated with the shoot dieback-stem necrosis symptoms in the Norway spruce seedlings now examined. Compatible with our observations on *Phomopsis*, Hansen & Hamm (1988) report on *Phomopsis* associated with top-kill symptoms of Douglas fir seedlings, where necroses were formed at the base of new shoots. They suggested that the infection takes place during the summer, possibly through the bud scales. In addition to location, also the appearance of necroses associated with *Gremmeniella* and *Phomopsis* differed. Resin flow, a characteristic conifer response upon pathogen attack, was commonly observed in necroses hosting *Gremmeniella*, whereas *Phomopsis*-associated necroses were water soaked and without any resin flow.

Based on the ITS rDNA sequence analysis performed, the *Phomopsis* isolates do not represent any previously characterized *Phomopsis* species associated to conifers (Børja *et al.*, submitted). Since the ITS sequence similarity of the *Phomopsis* strains from Norway spruce seedlings to deposits at the NCBI GenBank Sequence Database was also relatively low (£ 95 %), it is likely that these *Phomopsis* strains now studied represent an yet uncharacterized species on Norway spruce. This complicates comparison to other studies. Bearing this caution in mind, *P. occulta* (Sacc.) Traverso has been associated with stem cankers (Donaubauer 1995, Hahn 1943), while *P. conorum* (Sacc.) Died has been observed in correlation with shoot dieback of young spruce trees in Austria (Donaubauer 1995, Cech & Perny 1995). In British Columbia, P. *occulta* is considered as a pathogen on spruce seedlings in nurseries (Thompson *et al.* 2002). Cech (pers. comm.) confirms the occurrence of *Phomopsis* spp. on spruce, but has the opinion that *Phomopsis* is a secondary fungus, infecting after e.g. *Sirococcus* or *Gremmeniella*. Consistently, Perny *et al.* (2002) described also *Phomopsis* species as merely a weak parasite of spruce that is favoured only in cases of adverse climatic conditions, wrong provenance or localization. Our own data are consistent with the latter two cases as in the included pathogenicity tests the *Phomopsis* strains were non-pathogenic. Our current hypothesis is that in order to become pathogenic, the now examined *Phomopsis* strains need specific host-predisposing conditions, such as infection by other pathogens and/or abiotic stress.

The occurrence of the disease is not new, but overlooked. The unique event of *Gremmeniella* epidemics on large pines, which occurred in 2001, allowed us to follow and describe the *Gremmeniella*-disease development on Norway spruce seedlings in nurseries.

Conclusions

In conclusion, the massive *Gremmeniella* infection in nursery-grown Norway spruce seedlings is reported here for the first time. The incidence of the disease is correlated with the serious *Gremmeniella* epidemic on large Scots pine and Norway spruce trees the previous season. The

resulting extreme infection pressure combined with predisposing weather conditions, cold and high rainfall periods in the summer followed by mild winter account for the atypical outbreak of *Gremmeniella* on nursery-grown Norway spruce seedlings. Removal of the large Scots pines, a source of *G. abietina*-inoculum, from the immediate vicinity of the nursery, may diminish the damage on seedlings. In years with high infection pressure of *G. abietina*, selective chemical treatment of Scots pine but also Norway spruce seedlings seems warranted. We report here on *Phomopsis* sp., associated with lower stem necroses in Norway spruce seedlings, yet the pathogenicity potential and function of this fungus is unclear.

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Colonisation profiles of *Thekopsora areolata* and a co-existing *Phomopsis* species in Norway spruce shoots

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Abstract

The difficulty in sub-culturing biotrophic fungi complicates etiological studies related to the associated plant diseases. By employing species-specific ITS sequence stretches, we used real-time PCR to investigate the spatial colonization profiles of *T. areolata* and a co-existing *Phomopsis* species in seedlings and saplings of Norway spruce showing bark necrosis. There was a strong gradient in the colonization level of *T. areolata* DNA along the lesion length, with the highest DNA amount levels being recorded in the area with dark brown phloem. The separate analysis of bark and wood tissues indicated that the initial spread of the rust to healthy tissues neighbouring the infection site presumably takes place in the bark. *A Phomopsis* species co-existing together with *T. areolata* in several cases showed very high DNA levels in the upper part of the lesion outside the brown phloem area, and even in the visually healthy proximal tissues above the lesions. This indicates that this ascomycete has a latent stage during early colonization of Norway spruce shoots. This mode of infection most probably explains the successful co-existence of *Phomopsis* with a biotrophic rust, as their mutual interest would be to avoid triggering host cell death.

Introduction

Thekopsora areolata (Fr.) P. Magn. [*Pucciniastrum areolatum* (Fr.) Otth, *Pucciniastrum padi* (Schm. & Kunze) Diet.] is a Eurasian rust fungus recorded from England through the whole of Europe and from Russia to Kamtschatka and Japan (Gäumann 1959). The fungus alternates between conifers and broadleaved trees in order to complete its life cycle with five distinct spore stages. Its main hosts are Norway spruce [*Picea abies* (L.) Karst.] and wild bird cherry (*Prunus padus* L.) (Roll-Hansen 1965).

Thekopsora areolata overwinters as telia in the leaves of wild bird cherry shed on the ground. In spring during rainy weather the teliospores germinate and form basidiospores in synchrony with the flowering of Norway spruce. The basidiospores are carried by air currents to infect female flowers of spruce that eventually give rise to cones. Following the formation of pycnia on the outer sides of the cone scales and spermatization, dikaryotic hyphae form aecidia on both sides of the cone scales during the infection summer (Gäumann 1959). The aecidia mature and open next spring and release aecidiospores, which infect cherry leaves. Basidiospores of *T. areolata* may also infect actively growing shoots of spruce, but this takes place more seldom than the infection of cones. The fast-growing terminal shoots of spruce saplings are especially susceptible. Infected shoots usually become crooked, S-formed, with some dead tissue in the crooked part and often the shoots are dead also above the crook (Roll-Hansen 1947).

In a project focused on diseases of Norway spruce, we have been investigating the etiology of bark necrosis in nursery seedlings. Seedlings showing typical symptoms of *T. areolata* infection were often observed in forest nurseries but no fruit bodies of the rust were observed in these seedlings. An ascomycete, a *Phomopsis* species, was commonly co-detected with *T. areolata* in these diseased shoots of Norway spruce. To study the interaction of *T. areolata*, *Phomopsis* sp. and the hosting Norway spruce, the diseased shoots were spatially sampled at the advancing margins of the lesions, and the DNA pools of the three organisms were quantified by real-time PCR.

Materials and methods

Sampling, DNA isolation and real-time PCR

Nursery seedlings of Norway spruce that showed necrotic stem lesions were sampled spatially by taking 5-mm-long samples from the edges of the lesion area.

For DNA isolation, infected bark and wood samples from Norway spruce were excised, frozen immediately in liquid N_2 and ground in liquid N_2-chilled containers for 2 min in an MM 300 mill (Retsch Gmbh, Haan, Germany). DNA isolation was performed by using Plant DNA Mini Isolation Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

The real-time PCR primers used for monitoring *T. areolata* colonization in infected seedlings were designed with the Primer Express software 1.5a provided with Applied Biosystems real-time quantitative PCR systems (Applied Biosystems) by employing a conserved and species-specific sequence area in the ITS rDNA gene cluster. The amount of Norway spruce DNA in analysed samples from infected nursery seedlings was estimated by using the polyubiquitin primer/probe set previously described (Hietala *et al.* 2003). In addition, we monitored the presence of *G. abietina* and *Phomopsis* sp., pathogenic fungi commonly associated with necrotic lesions in Norway spruce seedlings, with primer/probe sets described by Børja *et al.* (submitted).

The real-time PCR detection of *T. areolata* DNA was performed in SYBR Green PCR Mastermix (P/N 4309155; Applied Biosystems), while amplification of Norway spruce, *G. abietina* and *Phomopsis* sp. DNA was performed with TaqMan Universal PCR Master Mix (P/N 4304437; Applied Biosystems). A primer concentration of 50 nM was chosen for the *T. areolata* primer pair, while the primer and probe concentrations of 150 nM and 333 nM (Hietala

et al. 2003), respectively, were used for detecting the DNA of Norway spruce. For *G. abietina* and *Phomopsis* sp. a primer concentration of 300 nM and a probe concentration of 400 nM were used (Børja *et al.* submitted). All PCR reactions were performed in singleplex conditions.

Dilution series were prepared for the monitored DNA pools to obtain standard curves. A 4-log-dilution series were prepared for each experimental sample to examine the presence of substances inhibitory to PCR amplification and ensure that the cycle threshold values (Ct; Ct determines the PCR cycle at which the reporter fluorescence exceeds that of the background) from the experimental samples fell within the standard curves. Each experimental sample had undiluted DNA as the most concentrated, and all four concentrations were used as templates in real-time PCR. For both of the series, the experimental and standard curve samples, 3 µl of the DNA solution was used as the template for each 25-µl PCR reaction. Each reaction was repeated twice. PCR cycling parameters were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Fluorescence emissions were detected with an ABI Prism 7700 (Applied Biosystems). The data acquisition and analysis were performed with the Sequence Detection System software package (1.7a; Applied Biosystems).

Results

The standard curves constructed

The primer set developed for monitoring *T. areolata* did not detect the DNA of Norway spruce, and the primer/probe set used for detecting DNA of Norway spruce did not detect the DNA of *T. areolata*. The DNA amount standard curves for Norway spruce and *T. areolata*, based on the relationship of Ct values (x) and the amount of template (y) generated from known host and pathogen DNA concentrations, were $\log y = 8.47 - 0.281x$ and $\log y = 3.192 - 0.278x$, respectively. For quantifying DNA of *G. abietina* and *Phomopsis* sp., we applied the standard curves, $\log y = 5.02 - 0.288x$ and $\log y = 4.64 - 0.282x$, respectively, constructed by Børja *et al.* (submitted).

Symptoms of the disease and colonization profiles of T. areolata and other fungi monitored

The diseased seedlings and saplings of Norway spruce showed a few centimetre long dark brown, slightly swollen bark area with resin flow, and many plants were crooked in the infected area (Fig. 1). In the areas with dark brown bark, the phloem was also dark brown, while at proximal areas above and below this region the phloem was light brown, eventually showing a green colour when examining more distal areas. The change in the phloem colour from dark brown to light brown was abrupt, while the transition from light brown to green phloem was often gradual. Fruit bodies (aecidia, pycnia) were not observed in the examined seedlings. Similar symptoms as observed in the nursery seedlings were also noted in the 5–10 m long saplings included as reference material. Aecidia were observed in some of the leader shoots of these saplings (Fig. 2).

Fig. 1. Typical symptoms of T. areolata *infection in a nursery-grown Norway spruce seedling: crooked stem with dark brown, slightly swollen bark area with resin flow. The crooked section is ca 5 cm long. (Photo: H. Solheim).*

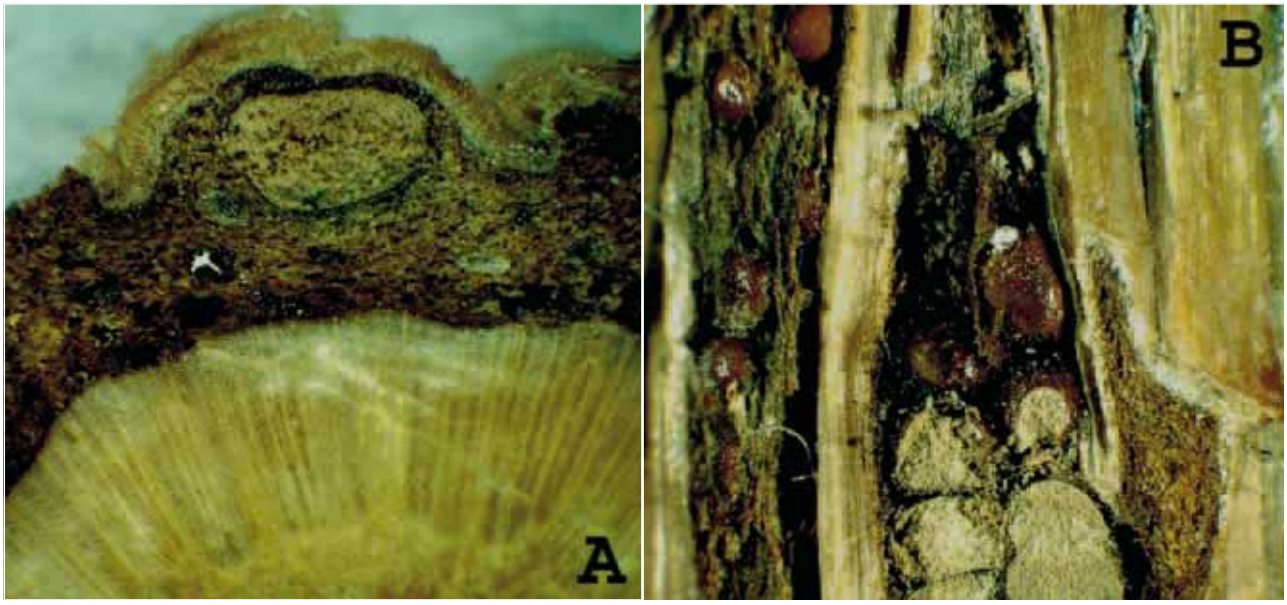

Fig. 2. Aecidia of T. areolata *in phloem of Norway spruce saplings. A) Cross section through an aecidium embedded in the phloem. B) Longitudinal cut into the phloem revealed many red brown aecidia, some of them sliced. (Photos: H. Solheim).*

In all the seedlings studied, the highest DNA amount estimates for the rust were observed in the area with dark brown phloem (Fig. 3). The levels of *T. areolata* DNA declined steeply in the area where the phloem changed from dark brown to light brown. Some seedlings were sampled in such a way that the bark was separated from the wood and these tissues were processed separately. Both above and below the dark brown lesion the rust progressed further away from this zone in the bark than in the wood (Fig. 4). Regarding the leader shoot of the diseased sapling analysed, the maximum amount of *T. areolata* DNA in respect to host DNA was at a similar level compared to those recorded for the seedlings, but unlike in the seedlings, the amount of *T. areolata* DNA was relatively equal across the area with visible symptoms (data not shown).

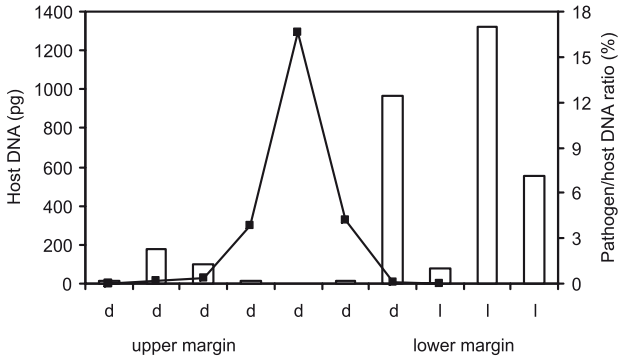

Fig. 3. The host DNA yields (columns) and Thekopsora*/ host DNA ratio (%) (line with filled squares) in a stem lesion of Norway spruce seedling. The lesion area was sampled spatially by taking 5-mm-long stem sections. The colour of phloem in each sampled section is indicated by letters (d, dark brown; l, light brown).*

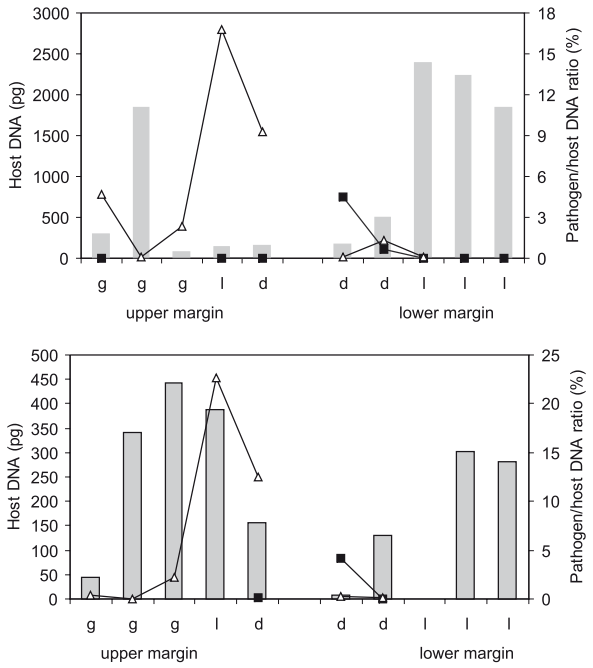

Fig. 4. The host DNA yields (column), Thekopsora*/host DNA ratio (%) (line with filled squares) and* Phomopsis*/host DNA ratio (%) (line with open triangles) within bark (upper) and wood (lower) in the upper and lower margin of a stem lesion of a Norway spruce seedling. The lesion margins were sampled spatially by taking 5-mm-long stem sections, and by processing then the bark and wood separately for each section. The colour of phloem in each sampled section is indicated by letters (g, green; d, dark brown; l, light brown). Note that the middle of the lesion (5.5 cm long area with dark brown phloem) with missing data was not analysed.*

Phomopsis sp. was co-detected with *T. areolata* in seedlings from Skjerdingstad (Fig.4) and in the sapling (data not shown), but in the latter its presence was restricted to a single sampling point. Like *T. areolata*, *Phomopsis* sp. also progressed further away from the dark brown lesion within the bark than within the wood (Fig. 4). In contrast to *T. areolata*, high levels of *Phomopsis* sp. DNA were observed in the upper part of the dark brown lesion and even in healthy-appearing bark with green phloem. Consistently, in general low levels of *Phomopsis* sp. DNA were observed in the lower parts of the dark brown lesion areas, where *T. areolata* was thriving. The other monitored species, *G. abietina*, was not detected in any of the examined Norway spruce material.

Discussion

We now showed that *T. areolata* is commonly associated with stem lesions in nursery-grown spruce seedlings. The symptoms observed in these seedlings are similar to those observed in saplings infected frequently by the rust in forest conditions. Based on fruit body observations, Roll-Hansen (1947) showed the presence of *T. areolata* on 3–4 year-old nursery seedlings of Norway spruce. In laboratory conditions, Klebahn (1900) was able to artificially inoculate shoots of Norway spruce with basidiospores of the pathogen; no fruit bodies were formed in these experiments, but the author noted the strong smell characteristic of sugary liquid exuded by pycnia. Otherwise there are no reports of young spruce seedlings hosting this rust. This is most likely due to the fact that the rust is difficult to culture in artificial media, and that fruit bodies allowing conventional identification of the fungus are not formed in infected seedlings.

There was a strong gradient in the amount of *T. areolata* DNA along the lesion length, with the highest levels being recorded in the area with dark brown phloem. The steep decline in DNA levels of *T. areolata* in the margin areas of the lesion coincided with the change of the phloem colour from dark brown to light brown, this indicating a host response to infection. It is obvious that the dark brown phloem represents initial infection sites from which *T. areolata* is spreading both upwards and downwards to the neighbouring healthy tissues. The analysis of bark and wood tissues separately indicated that the rust is able to colonize also wood in the area with dark brown phloem, but its initial spread to healthy tissues neighbouring the infection site presumably takes place within the bark.

The host DNA yields from diseased seedlings were in general lower in the upper part than in the lower part of the lesions. This pattern was observed also in seedlings, where no other fungi were co-detected with the rust. This is compatible with the observation that the shoots of Norway spruce attacked by *T. areolata* often die above the infection site, possibly because of interruption of nutrient and water flow to shoots above the infection site. Based on fruit body observations and fungal isolations, Cech and Perny (1995) showed that *Phomopsis* spp. are commonly present in *T. areolata* infected shoots of Norway spruce saplings in forest conditions. Compatible with their study, a *Phomopsis* sp. was now co-detected with *T. areolata* in diseased nursery seedlings. Based on ITS rDNA sequence data, the *Phomopsis* sp. associated with diseased Norway spruce seedlings in Norwegian forest nurseries is a previously uncharacterised species (Børja *et al.* submitted). Hahn (1943) describes *Phomopsis occulta* as a weak pathogen in conifers following injuries caused by frost, transplanting, drought and parasitic fungi such as the white pine blister rust (*Cronartium ribicola*). We consider it highly likely that the *Phomopsis* sp. now co-detected with *T. areolata* is a secondary invader benefiting from the weakened condition of the host due to rust infection. In the seedlings where *Phomopsis* coexisted with *T. areolata*, the rust showed higher DNA levels than *Phomopsis* in the lower margin of the lesions, while the opposite was true in the upper margin of the lesions. Taking into account the typical dieback of the shoot above the infection site of *T. areolata*, this pattern of colonization is fully compatible with the presumed pathogenic modes of these two fungi. However, the mode of infection of the now studied *Phomopsis* sp. resembles that of a biotroph as the fungus is apparently able to colonize spruce bark without triggering host cell death. This colonization mode undoubtedly contributes to the successful coexistence of *Phomopsis* with a biotrophic rust.

Real-time PCR is currently the most sensitive quantification method for nucleic acids. Regarding quantification of infection in plants, the tool has so far been utilized for monitoring infection by singular pathogens. The multiplexing option provided by different fluorescent labels of the probe would allow simultaneous monitoring of several DNA pools in a single tube (Hietala *et al.* 2003). Due to the high throughput nature of real-time PCR, we anticipate that the tool will become widely used also in ecological studies when monitoring events such as colonization of a common niche by several microorganisms.

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Phytophthora spp. a new threat to tree seedlings and trees

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Abstract

At least 60–80 *Phytophthora* species has been described and most of them are soil-borne pathogens causing damping off, root rot, collar and stem rot and foliar blight on different woody plant species. These microbes are sometimes difficult to isolate and even more difficult to identify. A general review of isolation, detection and some newly identified species, including *Phytophthora alni* complex and *P. ramorum*, is presented in this article. The disease symptoms, host species and geographical range are also shortly described.

Phytophthora

Phytophthora and other oomycetous micro-organisms were long included within the fungi, but today, because of evolutionary phylogeny and structure of biflagellate zoospores, they are grouped in the kingdom Chromista, which includes e.g. brown algae (Erwin & Ribeiro 1996, Baldauff *et al.* 2000). *Phytophthora* is a genus that is mainly parasitic on plants including trees and tree seedlings. Tsao (1990) has presented that most crown diseases of woody plants can be attributed to *Phytophthora* although in most cases proper techniques have not been used to reveal these pathogens behind the symptoms.

Phytophythora spp. produce mainly diploid hyphae, oospores and chlamydospores within plant tissue. Although oospores can survive in organic part of soil for a long time the asexual chlamydospores are the main resting stage of oomycetes. The asexual, biflagellate, swimming zoospores, produced in vessels called sporangia, are responsible for plant infection under wet conditions. Some homothallic species are self-fertile and they produce oospores after fusion of oogonium and antheridium. In heterothallic species, oospore production needs a presence of two mating types called A1 and A2. Sexual recombination or somatic fusion might create new races having higher pathogenic ability than the parents. Typical for *Phytophthora* are also hybrids, a new combination produced by parents representing two different *Phytophthora* species as in the case of *P. alni*-complex (Brasier *et al.* 1999, 2004a).

Identification

At least 60–80 *Phytophthora* species have been described and most of them are soil-borne causing damping off, root rot, collar and stem rot and foliar blight on different woody plant species (Erwin & Ribeiro 1996). The traditional identification of *Phytophthora* spp. is based on the morphology of sporangia, oogonia and antheridia, presence or absence of chlamydospores, and the growth and colony characters of cultures on special agars (Waterhouse 1963, Stamps *et al.* 1990). Morphological grouping segregated the species into six main groups based on 1) the structure of the sporangium apex and the width of the exit pore, 2) the caducity of sporangia and the length of pedicel and 3) the antheridial attachment. [A sporangium may be papillate, semi-papillate or non-papillate, caduous sporangia shed at maturity and an antheridial attachment may be paragynous, amphigynous (Fig. 1) or both]. However, these morphological keys are not distinct and stable and might differ within a species or be similar between species. In addition the traditional taxonomic grouping does not reflect true phylogenetic relations (Kroon *et al.* 2004).

Fig. 1. Amphigynous antheridium on oospore.

Many molecular techniques such as protein electrophoresis, isozymes and PCR-based methods such as DNA fingerprinting and direct sequencing have been investigated in the search for more effective and rapid identification of the species within the genus *Phytophthora*. (eg. Bielenin *et al.* 1988, Oudemans & Coffey 1991, Cooke *et al.* 2000). Today, the internal transcribed spacer (ITS) sequence of most *Phytopthora* species is available in the GenBank, and thus this information can be used to determine the identity of unknown isolates.

Detection

Most *Phytophthora* spp. cannot be isolated directly from diseased plants, soil or water as easily as many other pathogens. The affected material should be in a stage of active infection since the ability of *Phytophthora* to compete with other microbes is restricted (Erwin & Ribeiro 1996, Martin

et al. 2004). A common reason for the failure of isolation procedure is also a dry season or too dry samples (Kox *et al.* 2002, Garbelotto 2003).

The main idea of baiting is the activation of the pathogen. The generally used baits are highly susceptible hosts such as unripe fruits (apples, pears etc.) or seedlings (lupine, alder etc.). Small cores are made in fruits and they are stuffed with soil or small fragments of wood tissue taken from a necrotic lesion on roots or bark. After incubation a *Phytophthora* 'rot' will develop on the host's exterior (Fig. 2) and isolation by e.g. plating on agar medium (with or without selective chemicals) can be done from this 'fresh', active infection (Jeffers & Martin 1986). Another option is to add water to the samples and use suitable living plant tissue floated on the surface or fruits in the water as baits (Streito *et al.* 2002, Themann *et al.* 2002).

Thus the need for more reliable approaches has created new methods. For example PCR- techniques used in studies on many *Phytophthora* spp. take advantage of the sequence in the ITS region of the ribosomal DNA or are based on the sequences for nuclear genes such as beta-tubulin or mitochondrial genes such as cytochrome oxidase subunits coxI and coxII and NADH dehydrogenase subunit 5 nad5 (Schubert *et al.* 1999, Nechwatal *et al.* 2001, Grote *et al.* 2000, 2002, Ivors & Garbelotto 2002, Kox *et al.* 2002, Garbelotto 2003, Martin *et al.* 2004).

Fig. 2. Phytophthora *'rot' in apple baits after incubation. Before inoculation small cores were made in raw, green fruits and they were stuffed with tissue taken from a necrotic lesion on diseased plants.*

Alder *Phytophthora*

Symptoms and distribution

During 1993 and 1994 an unusual *Phytophthora* was consistently isolated from bark lesions at the stem bases of dying *Alnus glutinosa* along riverbanks, in orchard shelter belts and in woodland plantations in southern Britain (Brasier *et al.* 1995, Gibbs 1995). Typical for affected trees were abnormally small, yellow and sparse leaves and the presence of tarry or rusty colored exudations on stem lesions. In the following years, the disease was also found on *A. incana* and *A. cordata*, and it has been reported to be present in many countries in Europe: Austria, Belgium, France, Estonia, Germany, Hungary, Italy, Lithuania, Netherlands and Sweden (Gibbs *et al.* 2003). Field studies showed that it might be locally very damaging and an easily spreading disease.

Origin and variants

The microbe behind the disease is a group of heteroploid hybrids. Nucleotide sequence of the ITS-region and amplified fragment length polymorphism (AFLP)-analysis of total DNA have shown that the parents of these hybrids are probably *P. cambivora* and *P. fragariae* (Brasier *et al.* 1999). The hybrid variants (standard, Swedish, German, Dutch and UK) differ in their chromosome numbers (n=11–22), oogonial and antheridial morphology, oospore viability and colony characters. The origin of different variants may be the breakdown products of the first isolated standard hybrid or products of subsequent back-crosses or inter-crosses (Brasier *et al.* 1999, 2004a). However all variants seem to be relatively host specific pathogens of alders (Gibbs *et al.* 2003). The most aggressive are the standard- and Dutch-type variants. Recently the standard-type was described as *P. alni* subsp. *alni* and the Swedish variant as *P. alni* subsp. *uniformis*. Although the German, Dutch and UK variants have shown phenotypic diversity, they have identical ITS-profiles and thus they have been grouped together as *P. alni* subsp. *multiformis* (Gibbs *et al.* 2003, Brasier *et al.* 2004a).

Phytophthora ramorum

Morphology and distribution

In 2001 *Phytophthora ramorum* associated with twig blight disease in *Rhododendron* and *Viburnum* in Germany and Netherlands was described as a new species (Werres *et al.* 2001). This heterothallic *Phytophthora* was first characterized by abundant production of chlamydospores and elongate, ellipsoid, deciduous sporangia. Oogonia with amphigynous antheridia were produced by parings with *P. chryptogea* representing mating type A2 (Werres *et al.* 2001). Later the same pathogen was found to be responsible for the Sudden Oak Death disease (SOD) of *Quercus* and *Lithocarpus* spp. in California (Rizzo *et al.* 2002). The disease was first discovered on *Lithocarpus* spp. near Mill Valley in 1995. Since that time, it has spread throughout coast counties around the San Franscisco Bay area and numbers of *L. densiflorus*, *Q. agrifolia*, and *Q. kelloggii* have died (Rizzo *et al.* 2002, Davidson *et al.* 2002, 2005). Later the pathogen has been found in Oregon, Washington, and British Columbia (Anon 2003, Davidson *et al.* 2005, Hansen *et al.* 2003a). Recent findings of *P. ramorum* in North American nurseries and in trees in Europe have shown that the pathogen is a real threat to forests in both continents (Anon 2004a,b, 2005).

In the course of time *P. ramorum* has been found in many European countries: Germany, Netherlands, Belgium, Denmark, Ireland, Italy, France, Norway, Slovenia, Spain, Sweden, Switzerland, the UK and Poland (Werres

et al. 2001, Delatour *et al.* 2002, Moralejo & Werres 2002, Orlikowski & Szkuta 2002, De Merlier *et al.* 2003, Heiniger *et al.* 2004, Zerjav *et al.* 2004). In 2004 the Finnish Food Safety Authority, Evira found *P. ramorum* on *Rhododendron* in one Finnish nursery producing horticultural plants. It was detected by species-specific PCR and identified morphologically (Fig. 3).

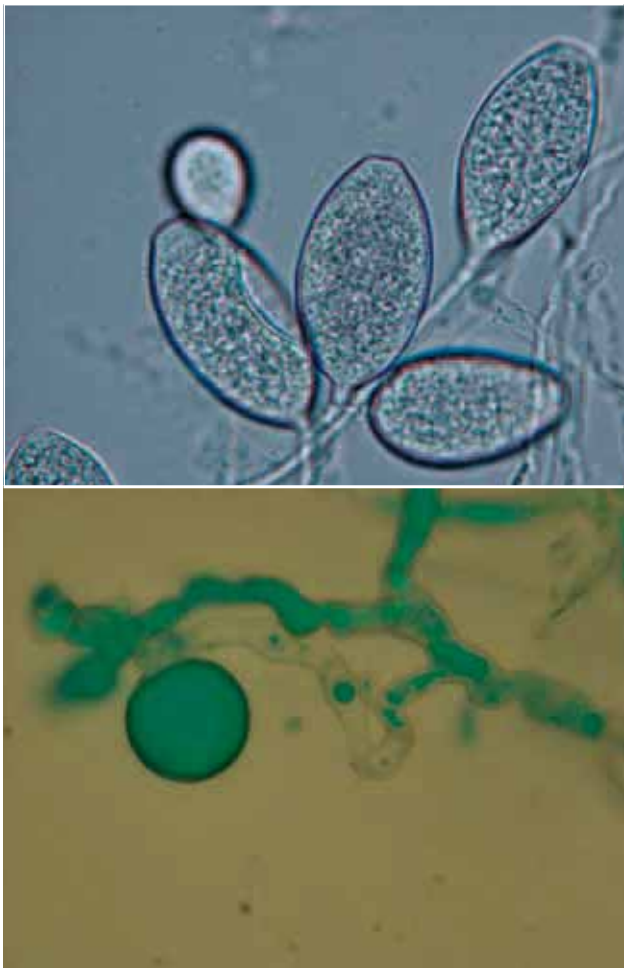

Fig. 3. Sporangia (a), chlamydospores and coralloid hyphae (b) typical for Phytophthora ramorum.

Symptoms and hosts

P. ramorum invades susceptible trees through the bark on which cankers with tarry or rusty colored exudations are developed. Later the leaves of infected trees may turn to brown over a short period (Garbelotto *et al.* 2001). Non-lethal foliar infections on woody shrubs or other hosts in understorey serve as a source of inoculum for trees (Davidson *et al.* 2005). Today over 40 plant genera have been found to be susceptible for *P. ramorum* (Rizzo *et al.* 2005). These include in North America besides *L. densiflorus*, *Q. agrifolia*, *Q. kellogii* and *Q. parvula* var. *shrevei* species such as *Q. chrysolepis*, *Umbellularia californica*, *Sequoia sempervirens, Pseudostuga menziesii, Acer macrophyllus* and *Aesculus californica* . The pathogen was also found on *Vaccinium ovatum, Arbutus menziesii, Arctostaphylos manzanita, Heteromeles arbutifolia, Lonicera hispidula, Maianthemum racemosum, Rhamnus californica, Rosa gymnocarpa, Toxicodendron diversilobatum, Rubus spectabilis, Rhamnus purshiana, Corylus cornuta, Pittosporum undulatum, Trientalis latifolia* (Davidson *et al.* 2002, Goheen *et al.* 2002, Rizzo *et al.* 2002, Knight 2002, Hong 2003, Hüberli *et al.* 2004, 2005, Murphy & Rizzo 2003, Maloney *et al.* 2005). In Europe, *P. ramorum* was first found on *Rhododendron* and *Viburnum*, but later it has also been isolated e.g. from *Arbutus*, *Camellia, Hamamelis, Kalmia, Leucothoe, Pieris* and *Syringa* (Werres & De Merlier 2003, Beales *et al.* 2004a,b). In 2003 the pathogen was found on *Quercus falcata* in the UK, and shortly after on *Fagus sylvatica*, *Quercus ilex*, *Q. cerris*, *Castanea sativa, Taxus baccata* and *Aesculus hippocastanum* (Anon 2004a, Brasier *et al.* 2004b, Lane *et al.* 2004). In the Netherlands infection has also been identified on *Q. rubra* near diseased *Rhododendron*s (Anon 2004b).

Mating type and origin

At first it was believed that the reason why we have not had a same kind of epidemic in Europe than in North America was that different mating types were found in Europe (A1) and in North America (A2). However, in 2003 the occurrence of isolates of *P. ramorum* belonging to A1 and A2 mating types was respectively reported in North America and Europe (Hansen *et al.* 2003a, Werres & De Merlier 2003). The AFLP-fingerprinting clustered European and American isolates separately within individual clades according the mating type (Ivors *et al.* 2004). Also the morphological characters separated the mating types in most cases so that the European isolates were much more homogenous than the North American isolates (Werres & Kamiski 2005). However, the genetic diversity among European isolates was greater than among *P. ramorum* isolates from North America (Brasier 2003, Werres & Zielke 2003, Brasier & Kirk 2004, Ivors *et al.* 2004). The A1 isolates grew faster, had larger chlamydospores and did not produce gametangia with *P. cambivora* (Werres & Kaminski 2005). This might prove that the pathogen was separately introduced into North America and Europe from a third area, which remains unknown, but probably locates in Asia.

Other *Phytophthora* spp.

A new *Phytophthora* species, described few years ago, is *P. inundata*, which infects *Salix* in riparian ecosystems (Brasier *et al.* 2003). It has also other woody hosts as *Aesculus, Olea* and *Prunus*, and might be highly pathogenic after flooding or waterlogging (Brasier *et al.* 2003). The extensive study on oak decline has revealed *P. quercina, P. psychrophila, P. europaea, P. uliginosa* and *P. pseudosyringae* (Jung *et al.* 1999, 2002, 2003). The latter *Phytophthora* was also found in necrotic fine roots and in stem lesions of *F. sylvatica* and *A. glutinosa* (Jung *et al.* 2003). *P. quercina* was the most frequently recovered species from rhizosphere soil near declining oaks in Sweden (Jönsson *et al.* 2003). There was also a correlation between

the presence of the pathogen and the vitality of oak stands (Jönsson *et al.* 2005). *P. nemorosa* is also a newly described species, which was found during an intensive survey on sudden oak death and *P. ramorum* in California and Oregon (Hansen *et al.* 2003b). A similar survey in the UK found *P. kernoviae*, which was isolated most frequently from *F. sylvatica*, but it has also been present on necrotic lesions of *Q. robur* and *Liriodendron tulipifera* (Brasier *et al.* 2005).

In Finland, a new homothallic *Phytophthora* sp. from *Rhododendron* was found to be highly pathogenic to many woody hosts including Norway spruce (Fig. 4).

Fig. 4. Norway spruce seedlings inoculated with a homothallic, unidentified Phytophthora *sp.*

Conclusion

The past decade has shown, that many new *Phytophthora* species are associated with diseased trees. Most of them are not native in the area where they are a serious problem: e.g. *P. ramorum*, the cause of sudden oak death, was introduced separately to North America and Europe. Even old, native species might create through sexual recombination or somatic fusion new combinations with higher pathogenic ability than their parents have. Typical for *Phytophthora* are also hybrids, a new combination produced by parents representing two different *Phytophthora* species, as was in the case of *P. alni*-complex, which has caused changes in riparian ecosystems all around the Europe. The fact that *P. ramorum* is present in large forest area in Oregon shows that the assumption that *Phytophthora* spp. cannot adapt to weather conditions in Nordic countries is not true. Thus we must be ready to prevent the spread of these introduced pathogens. The movement of infected plants should be avoided by strict quarantine regulations and control of all suspicious ornamentals and seedlings.

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Root systems of declining conifer seedlings are colonised by a highly diverse fungal community

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Abstract

Fungi of roots of declining pine and spruce seedlings were assessed by pure culture isolations and direct sequencing. The isolation from 1440 roots of 480 seedlings (240 per each tree species) yielded 1110 isolates which, based on mycelial morphology and ITS rDNA sequences, were found to represent 87 distinct taxa. Direct ITS rDNA sequencing from decayed sections of 140 roots (70 per each tree species) yielded 160 sequences representing 58 taxa. In respect to the amount of examined roots, direct sequencing revealed significantly larger fungal diversity (chi-squared test; $p<0.0001$). A total of 131 taxa were found, 92 of which (70.2 %) were identified at least to a genus level. Only 14 of the total number (10.7 %) were detected by both methods, while 73 (55.7 %) were detected exclusively by isolation, and 44 (33.6 %) exclusively by sequencing. Fungi most commonly isolated were the pathogens *Fusarium oxysporum* (25.6 %) and *Nectria radicicola* (14.9 %). On the contrary, direct sequencing most frequently revealed presence of the endophyte *Phialocephala fortinii* (33.1 %) and the unidentified sp.NS234A2 (10.0 %). Our results demonstrate that a diverse fungal community inhabits roots of declining conifer seedlings, and that pure culture isolations combined with direct sequencing provides complementary data in studies of fungal communities.

Introduction

Fungi colonising roots have a significant impact on health and productivity of tree seedlings, as they are able to form beneficial, neutral or pathogenic types of associations (Wilcox 1983). In recent years, root dieback of pine and spruce was reported to be a serious problem in a number of forest nurseries over the Europe. Diseased seedlings were usually occurring in patches, exhibiting stunted growth, discoloration of needles and partial or total death of the root systems (Venn *et al.* 1986, Lilja *et al.* 1988, Unestam *et al.* 1989, Ericson *et al.* 1991, Lilja *et al.* 1992, Kacprzak 1997, Camporota & Perrin 1998, Hietala *et al.* 2001). As a rule, this led to a significant decrease in quality of plants, and in some cases resulted in loss of stock production up to 40 % (Lilja 1994). Most often, fungi from the genera *Fusarium*, *Nectria, Rhizoctonia*, and *Pythium* were reported as causal agents of the disease (Galaaen & Venn 1979, Lilja *et al.* 1992, Lilja & Rikala 2000).

Seedlings, infected with root-decay fungi, might exhibit reduced survival rates following outplanting. Consequently, the success of plantation might be also dependent on the presence of root pathogens in afforested areas, as transferred seedlings are likely more susceptible to infection due to recent replanting stress. Such risks are indeed real, as couple of studies had already shown that potential pathogens are able to persist both in forest soils on clear-cut sites and on abandoned farmland (Perry *et al.* 1987, Wilberforce *et al.* 2003). Therefore, it is important to assess root disease hazard also in different types of planting terrain.

To date, such studies are scarce, and previously fungal communities in decayed roots of conifer seedlings were mainly assessed by fungal isolations into pure culture (Lilja *et al.* 1992, Kope *et al.* 1996). However, despite the large number of isolated fungi, it was noted that this method could be biased towards fast growing species and provide only portion of total fungal community inhabiting diseased roots. More recently, it has been demonstrated that PCR based molecular methods could be a powerful tool for identification of fungi (Donaldson *et al.* 1995, Hamelin *et al.* 1996, Hantula *et al.* 2002). For example, the direct sequencing of fungal DNA from roots has proved to be a sensitive method for the detection of potentially all root-inhabiting fungi, in particular species that are usually overlooked by isolation, e.g. latent pathogens, slow-growing endophytes and unculturable species (Kernaghan *et al.* 2003). The main aim of the present work was to determine species composition and relative abundance of fungi colonising roots of decayed *P. sylvestris* and *P. abies* seedlings in three types of terrain: bare root forest nurseries, afforested clear-cuts and abandoned farmland. In order to achieve this, pure culture isolations were combined with direct sequencing of fungal DNA from decayed root tissue.

Materials and methods

Diseased *Pinus sylvestris* and *Picea abies* seedlings were collected from three bare-root forest nurseries, three replanted clear-cuts and one afforested farmland. All four plantations were established during spring of the same year. The aboveground symptoms of all sampled seedlings were needle discoloration and stunted growth. Following excavation, all of them showed root dieback and decay. From each root system, three to five core roots with decay symptoms were randomly selected, and from each selected root, a single segment about 5 mm in length was cut at the zone of advancing decay. Three of those were immediately used for isolation of fungi into pure culture. In addition, from 10 randomly selected plants from each site, one segment per root system was designated for direct sequencing.

The isolation of fungal cultures was attempted from 1440 core roots derived from 240 pine and 240 spruce seedlings. For isolation from the nursery plants we used three

different types of agar medium (one type per each root from a single plant), 2 % water agar, vegetable juice agar (Barklund & Unestam 1988) and Hagem agar (Stenlid 1985). All isolations from replanted clear-cuts and afforested farmland, as well as all subsequent subculturings of all obtained strains were done exclusively on Hagem agar. The cultures obtained were grouped into mycelial morphotypes based on mycelial morphology. For identification, one to ten representative cultures from each morphotype were ITS rDNA sequenced. Moreover, a total of 140 segments of core roots representing 70 pine and 70 spruce seedlings was selected for direct ITS fungal rDNA sequencing from root tissue. In all procedures, extraction of DNA, amplification and sequencing followed the method described by Rosling *et al.* (2003).

Databases at both GenBank (Altschul *et al.* 1997) and at the Department of Forest Mycology and Pathology, Swedish University of Agricultural Sciences, Uppsala were used to determine the identity of sequences. The criteria used for deciding on the taxon or genus for a given strain was its intra- and interspecific ITS sequence similarity to those present in the databases. Fungal community structures were compared by calculating qualitative (SS) Sorenson similarity indices (Magurran 1988). The occurrence of a given fungus in respective datasets was compared by chi-squared tests, which were calculated from actual numbers of observations (presence/absence data) (Fowler *et al.* 2001).

Results and discussion

Out of 1500 roots used for isolation, 1110 (74.0 %) gave fungal growth, and the remaining 390 (26.0 %) were either colonised by bacteria or remained sterile. As in all cases a single isolate per root was obtained, this part of work yielded a total 1110 of distinct cultures, which were found to represent 87 different taxa. Of those, 77 (88.5 %) were identified at least to genus level. The fungi most frequently isolated were ascomycetes and deuteromycetes: *Fusarium oxysporum*, *Nectria radicicola, Nectria* sp.702, *Trichoderma harzianum, Phialocephala fortinii, Penicillium spinulosum, T. viride and Zalerion varium*.

The results showed that high fungal diversity does exist in decayed roots even within a single root system. Thus, the isolations from three different roots of the same plant had resulted in three similar outcomes only in 17.0 % of seedlings from the nurseries, in 17.5 % of seedlings from the clear-cuts, and 21.7 % of seedlings from abandoned farmland. By contrast, two and three different outcomes were observed in 54.0 % and 29.0 %, 45.8 % and 36.7 %, and 61.7 % and 16.7 % of plants from respective types of terrain.

Amplification of fungal ITS rDNA from 140 root segments was successful for 123 (87.9 %), producing 1 to 4 distinct amplicons in each PCR reaction. Direct sequencing of all amplicons resulted in 160 sequences representing 58 fungal taxa. The fungi most commonly detected by direct sequencing were the ascomycetes *Phialocephala fortinii*, Unidentified sp.NS234A2, *Leptosphaeria* sp.1169, *Nectria radicicola, Nectria* sp.702, *Xenochalara juniperi*, *Fusarium oxysporum* and *Zalerion varium*.

The efficacy of direct sequencing was higher than that of isolation. For example, direct sequencing from 140 root segments yielded 58 taxa, while the isolation from the same number of root samples would count only 27 species as estimated from the species accumulation curves (data not shown). Moreover when sequenced, a single root segment delivered up to 4 sequences of different fungi, when during the isolation similar segment never yielded more than one culture. When pooled, direct sequencing and isolation detected a total of 131 fungal taxa, 92 of which (70.2 %) were identified at least to a genus level. The overlap between the two methods was very low (S_s = 0.19). Only 14 (10.7 %) of the taxa were both sequenced and isolated, 44 (33.6 %) were detected exclusively by sequencing, and 73 (55.7 %) exclusively by isolation. In conclusion, the results showed that pure culture isolations combined with direct sequencing provide complementary data in studies of fungal communities and reveal high abundance of species in roots of declining conifer seedlings.

Acknowledgements

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Remote sensing of forest health

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Abstract

Remote sensing is a promising tool for monitoring forest health. Foliar mass, or correspondingly leaf area index (LAI), together with chlorophyll concentration in the foliage, are two suitable measures of forest health. So far, airborne laser scanning has proven to be very suitable for measuring LAI. The work is in progress, and still in an early phase.

Introduction

Remote sensing technology has been rapidly developing during the last years, and at Skogforsk we are investigating whether and how this tool could be applied for forest health monitoring. We are mainly aiming to develop a monitoring system, which is generally applicable, i.e. it can be used for both abiotic and biotic stress situations. An ideal situation would be if a single monitoring variable could integrate the effects of any kind of stress and damage. The rationale for this is the one used earlier regarding the effects of long-range trans-boundary air pollution on forests. If a general stress factor affects the forests, it is likely to result in a number of different damage types and symptoms, these including both direct effects and indirect effects from pests and diseases. Today, the climate change and the spread of pests and diseases across continents could be regarded as an example of such a stress situation. In addition to integrating the effects across damage types, the advantage of having a general health variable is that it could be used to describe spatial and temporal variation in forest health.

Foliar mass and canopy chlorophyll represent variables that are sensitive to most types of stress and damage. Estimation of defoliation and discolouration degree have been widely used as forest health variables in subjective forest health assessments during the last 20 years both in Europe and North-America. Variations in these two parameters correspond to changes in foliar mass (or leaf area index, LAI) and pigment concentration in the foliage (in particular for chlorophyll). When these two variables are multiplied, we get the canopy chlorophyll, given in mass per ground area, which should be a good candidate variable for forest health monitoring.

Results

So far we have successfully estimated LAI and defoliation using airborne laser scanning (LIDAR). In two studies, one with Scots pine and another with Norway spruce, very strong (R^2=0.95) linear relationships were found between state-of-the-art ground measurements of LAI and airborne laser data, based on the Beer-Lambert law (Fig. 1). The idea is simple: the more foliage there is, the less the laser pulses penetrate through the canopy layer and hit the ground. In a mass-attack of pine sawflies in Solør in southeast Norway in 2005, we demonstrated the ability of this method to map the defoliation (Fig. 2, Solberg *et al.* 2006a). We used the same method to produce a map of LAI with a 10m x10m spatial resolution in a part of the Østmarka forest, near Oslo (Solberg *et al.* 2005). This map gives a good representation of the forest area, and it fits well with the distribution of stand densities and stand ages.

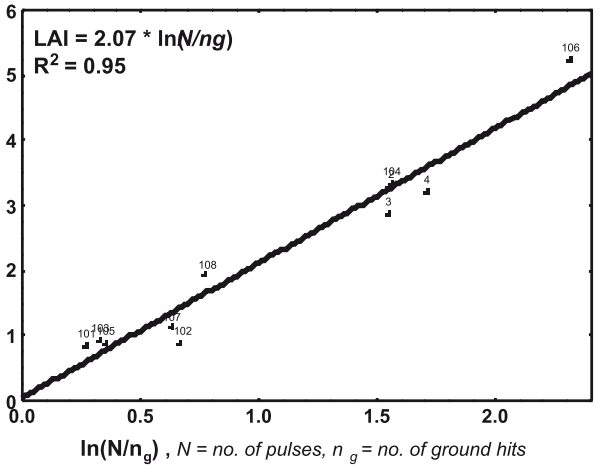

Fig. 1. Linear regression of ground based LAI-2000 measurements against a LIDAR derived variable for eleven 1000 m^2 circular plots of Norway spruce located in Østmarka in Oslo. Accurate geo-referencing of the ground plots was obtained by differential GPS measurements.

The NDVI vegetation index from SPOT satellite data did not correlate well with the LIDAR derived LAI data. This was somewhat surprising, as the NDVI reflects the amount of green biomass. The reason for this was apparently that the NDVI is mostly reflecting the surface characteristics of the vegetation, and it gets saturated at rather low LAI-values, i.e. it is only sensitive to LAI values up to a certain point. Also in young stands the ground vegetation growing between the trees can give a strong NDVI signal, which could easily be mistaken as high LAI values. Anyway, we are searching for other vegetation indexes and other satellites and sensors to try to produce LAI estimates.

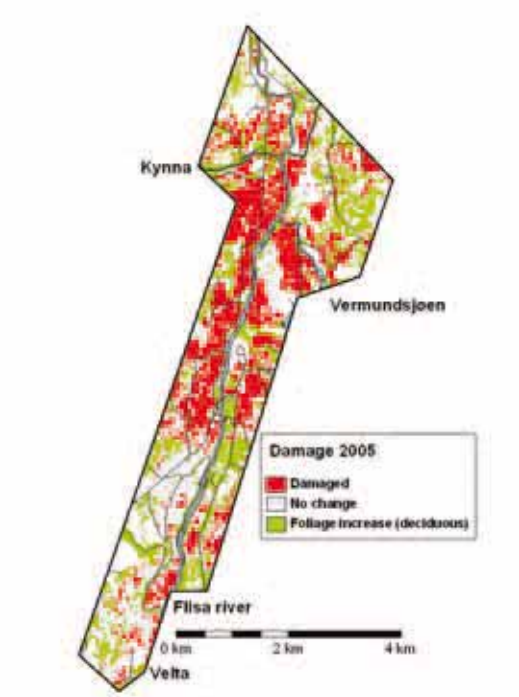

Fig. 2. A map of pine sawfly defoliation during the summer 2005 in Solør in Norway. The colours represent the change in LAI between two flights of laser scanning, performed in May and August.

The approach for remote sensing of foliar mass (and defoliation) presented above is supplemented with another approach for chlorophyll estimation based on airborne, hyper-spectral imagery. The sensor we use here is the Airborne spectral imager (ASI) having 160 bands covering visible and infrared light. This data set has a high spatial resolution (18cmx32cm) allowing modelling of single trees. In order to estimate chlorophyll data from single trees using airborne hyper-spectral data, segments (the outline of horizontal projection of the tree crowns) of single trees were developed from single-tree modelling of the laser data (Solberg *et al.* 2006b). A digital surface model representing the canopy layer was developed (Fig. 3), and single-tree segments were derived from that based on the geometry of the DSM. Foliar chlorophyll concentrations are measured from spruce branches obtained by tree climbing. The results from this work are still preliminary, and not presented here.

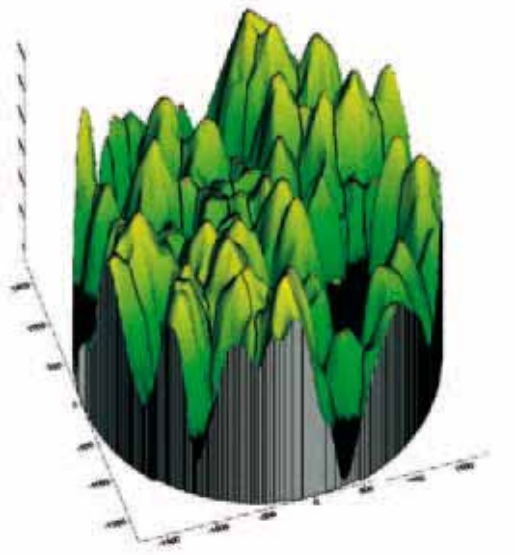

Fig. 3. Digital surface model (DSM) of a 1000 m2 plot.

Finally, multi- or hyper-spectral data may be useful for detecting diseased trees. We have another data set of hyper-spectral data obtained from the ASI airborne sensor. This scene covers a homogeneous stand of about 2000 young spruce trees in a stand heavily attacked by the spruce needle rust *Chrysomyxa abietis*. A preliminary result (Fig. 4) shows the spectral signature of one healthy and one diseased tree from this stand. As expected, the diseased tree has a higher reflectance in the red light area, and a lower reflectance in the near-infrared bands.

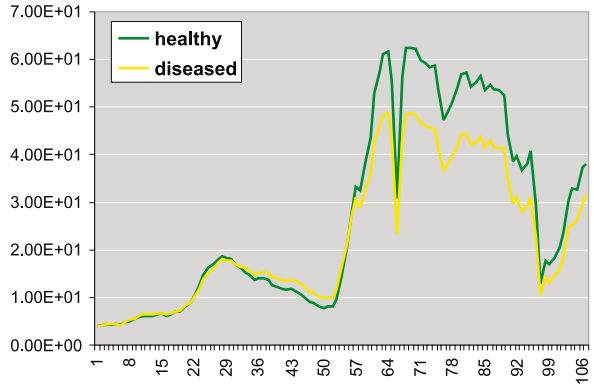

Fig. 4. A spectral signature of two Norway spruce trees in a stand attacked by Chrysomyxa abietis*; showing one healthy tree and one diseased. The band number 1–106 is indicated on the x-axis and goes from 400 nm (left) through visible and NIR-light wavelengths.*

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A collaborative project to better understand Siricid-Fungal symbioses

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Abstract

The Forestry and Agricultural Biotechnology Institute, University of Pretoria and the Department of Forest Mycology and Pathology, Swedish University of Agricultural Biotechnology Institute, Uppsala, Sweden are collaborating on a study of the Siricid-Fungal symbiosis, and its parasites. This project aims to address questions in two general areas, namely (a) the evolution and biology of mutualistic symbiosis and (b) the monitoring and control of wood inhabiting pests and pathogens that threaten biodiversity and forest production in introduced and native environments.

Project background

The symbiosis between woodwasps and fungi (Fig. 1)

A mutualistic symbiosis exists between Siricid woodwasps and *Amylostereum* fungi (Talbot 1977, Martin 1992). The relationship between these organisms is specialised and obligatory species specific, at least for the insects. The principle advantage for the fungus is that it is spread and inoculated into suitable wood substrates during wasp oviposition. In turn, the fungus rots and dries the wood, providing a suitable environment, nutrients and enzymes to the developing insect larvae.

The burrowing activity of the Siricid larvae and fungal white rot of the wood make this insect-fungus symbiosis potentially harmful to its conifer host trees. However, in the northern hemisphere, where the *Siricidae* are native, the insect is of little economic importance, except during times of increased stress due to other factors (Spradbery & Kirk 1978). Here a natural balance exists between the insect-fungus complex, its natural parasites and host trees as long as the trees are generally healthy. These organisms have been studied widely in Europe to understand their fascinating biology.

Amylostereum spp. are Basidiomycetes that are heterothallic and have a tetrapolar nuclear state (Boidin & Lanquetin 1984). Such a mating system increases outcrossing

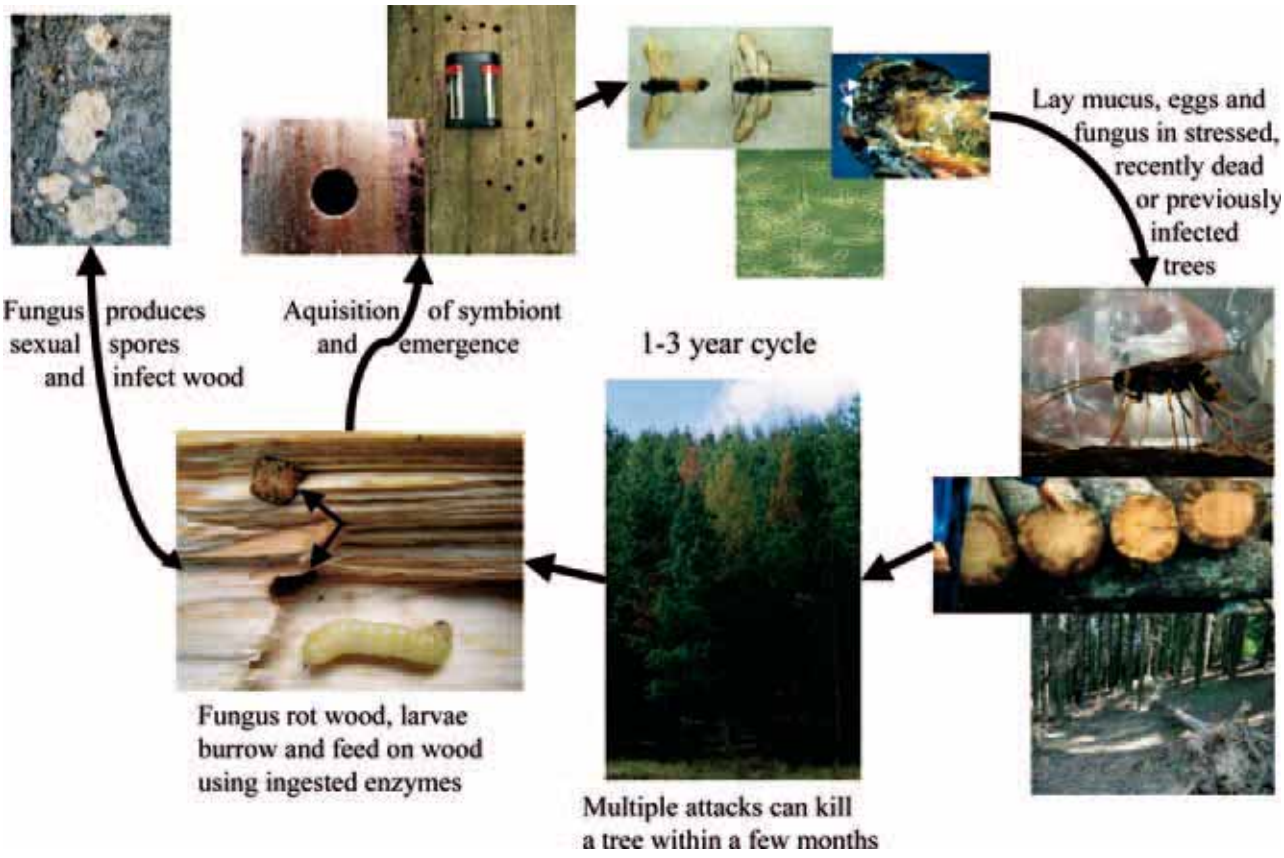

Fig. 1. Life-cycle of Siricid woodwasps and their Amylostereum *symbiotic fungi.*

and thus normally also population diversity. The *Amylostereum* spp. are, however, also spread by woodwasps in the form of asexually produced oidia (thus genetically identical) (Vasiliauskas *et al.* 1998).

In the northern hemisphere clonal lines of *A. areolatum* and *A. chailletii* are preserved over time and occur over large areas as a result of the spread of oidia of by woodwasps (Vasiliauskas *et al.* 1998, Thomsen & Kock 1999, Vasiliasuskas & Stenlid 1999). This situation is even more dramatic in the southern hemisphere where a single vegetative compatibility group (VCG) dominates populations of *A. areolatum* associated with *S. noctilio* (Slippers *et al.* 2001). Isolates from South Africa, Brazil and Uruguay represent the same VCG. This VCG in turn was partially compatible with isolates from New Zealand and Tasmania. These results suggest that the spread of *Sirex* through the southern hemisphere during this century has taken place among the continents and countries of this region, rather than by separate introductions from the northern hemisphere. The results, further, indicate that *A. areolatum* in the southern hemisphere spreads exclusively asexually through its association with *S. noctilio*. No sporocarps of *A. areolatum* have thus far been found in the southern hemisphere.

Woodwasp-fungal symbionts as forest pests and their control

There is an increasing number of exotic pest and pathogen invasions that threaten the world's ecosystems (Bright 1998, Wingfield *et al.* 2001). Many of these introductions have had or are having catastrophic outcomes. The long-term sustainability of native forest and forestry industries will depend on the capacity to effectively deal with such introduced insect pests and pathogens.

Forests in Europe are increasingly at risk from newly introduced pathogens, continued human pressure and alteration of habitat, as well as global weather changes. Evidence of this has been numerous emergences of disease outbreaks or species 'declines' across Europe. Dutch-elm disease and Oak decline in central and southern Europe, *Fraxinus* decline in northern Europe, *Pinus* dieback in various areas in Europe, Ostrya decline in southern Europe, etc. The current amount of freshly dead wood (75 mil m^3) in Sweden following the storm of January 2005 adds to this risk for native forests as many Siricids prefer such material to bread in (Spradbery & Kirk 1978). Significant increases in Siricid populations, coupled with the pressures mentioned above, can hold significant risks for attacks on stored (unharvested) timber and standing trees weakened by other pests (e.g. bark beetles and *Armillaria* root rot). Such a situation exists in parts of Switzerland (Dr. U. Heiniger, pers. comm.).

Sirex noctilio and *A. areolatum* have been introduced into various southern hemisphere countries and, recently, to the USA (where it is currently viewed as a potential threat to forest health) (Slippers *et al.* 2003, Hoebeke *et al.* 2005). In contrast to the native range, these symbiotic organisms have caused extensive mortality in exotic pine plantations in the southern hemisphere (Chou 1991, Madden 1988). Despite the costly efforts to monitor and control the wasp and fungus during the previous century, the pest complex continues to kill significant numbers of trees and spread to previously unaffected areas in Australia, South Africa and South America. In many of these regions this pest complex is considered to be the biggest threat to pine forestry operations.

Sirex noctilio is most effective controlled through biological control agents such as the nematode *Deladenus siricidicola* and some parasitic wasp species, in combination with silvicultural practices aimed at reducing tree stress (Neumann *et al.* 1987, Haugen 1990). The nematode is, however, the main form of control. *Deladenus siricidicola* has a closely co-evolved and integrated life cycle with both the wasp and fungal symbiont (Fig. 2). For this reason, the efficiency of biocontrol programmes is often affected by the specific nematode strain or fungal strain involved. Wasp parasites are currently underused in many countries due to incomplete information from native ranges and weak application strategies.

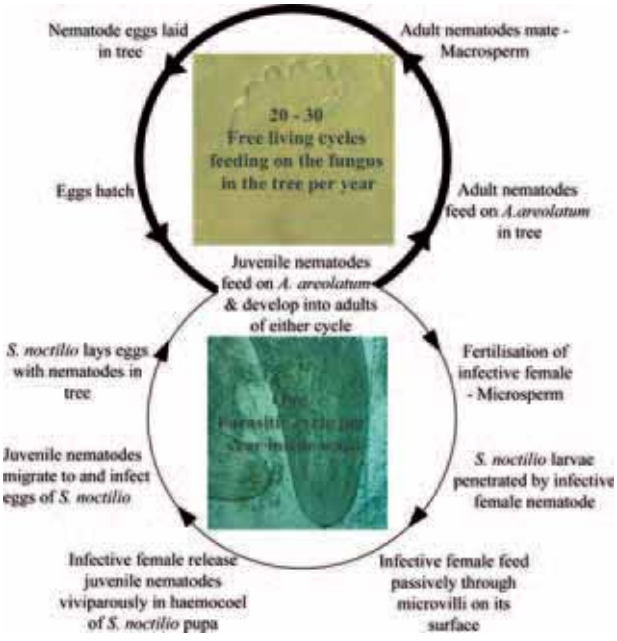

Fig. 2. Bicyclic life cycle of the Sirex *biocontrol nematode,* Deladenus siricidicola*. (Adapted from Bedding 1972, Nematologica)*

General questions addressed in the project

Molecular techniques have only recently been applied to questions pertaining to *Amylostereum* taxonomy, phylogeny and population structures (Vasiliauskas *et al.* 1999, Slippers *et al.* 2000, 2002, Tabata *et al.* 2000). These studies have clarified previous hypotheses that were based on morphological and mating studies, regarding the relationships among *Amylostereum* spp. They have also raised new and challenging questions regarding the identity of the fungal isolates associated with certain woodwasps. From these preliminary observations there appear to be cryptic

speciation that have been overlooked using traditional methods of identification. On a higher taxonomic level, the relationship of *Amylostereum* to other Basidiomycetes is currently unsure due to contradictory literature reports (Slippers *et al.* 2003).

A study of the population structure of *Amylostereum* fungi from many parts of the world, using both VCG's and molecular markers, will give valuable insight into the geographical origin and spread of these fungi, as well as their associated Siricid wasps. Such data have already identified patterns of spread amongst countries in the southern hemisphere and between some local populations in Scandinavia (Vasiliauskas *et al.* 1998, Thomsen & Koch 1999, Vasiliauskas & Stenlid 1999, Slippers *et al.* 2002). Phylogeographic data is, however, lacking for most of natural distribution of Siricids and their fungi. The northern hemisphere origins of southern hemisphere populations of *Sirex* and *Amylostereum* are not known, despite its importance for selection of control agents.

Despite detailed studies of the symbioses between Siricid woodwasps and their fungal symbionts, many fundamental questions remain unanswered. For example, it is thought that vertical transmission (from mother to daughter) predominates. However, the numerous wasp species apparently carrying the same fungal species indicate some level of horizontal transfer of the symbiont between wasp species. The importance of such data is illustrated by the lack of any explanation of the fundamental differences in population structures of *A. areolatum* (highly clonal) and *A. chailetii* (almost indistinguishable from population structures of other basidiomycetes spreading through sexual spores). Furthermore, there is no co-evolutionary or phylogeographic data on which to infer the evolutionary development of the symbiosis. The lack of this information also excludes the comparison of this symbiosis with other symbiotic systems.

Siricid-like wasps are known from the Jurassic period (more than 150 mya) Rasnitsyn 1988). Parallels between the Siricid-fungal symbiosis and other independently derived symbioses are likely to reveal evolutionary factors that are important for the development and stability of such partnerships. Such a co-evolved system also presents important opportunities to study comparative rates of molecular evolution in different symbiotic partners, and non-symbiotic relatives, as well as addressing general questions of the adaptive significance of sex (Herre *et al.* 1999).

The artificial selection during mass rearing of biological control agents in control programmes can lead to severe bottlenecks in populations of these organisms. This will severely reduce population diversity in the control organisms, which will reduce their ability to respond to changes in the environment or host. During the nematode rearing process the accidental selection of less infective strains of *D. siricidicola* has lead to a temporary breakdown of the biological control programme in Australia, resulting in huge damages (Haugen 1990). Despite these dangers, there is currently no data or methods available to study populations, compare strains or track changes in populations of the biological control organisms.

In order to conduct this study, collections of populations of wasps, fungi and biocontrol agents are needed to represent the native occurrence of these organisms, as well as areas where they have been introduced. Collected samples from the southern hemisphere (Argentina, Brazil, Australia, South Africa) and Europe (Austria, Denmark, Great Britain, Italy, Greece, Norway, Sweden, Switzerland) have been made in collaboration with various other researchers and research organization. This material is supplemented from international culture collections and herbaria (Canada, France, Germany, Japan, Russia, USA). As part of collecting efforts, potential attractants and methods have been identified to catch woodwasps. These collections are ongoing.

Conclusion

It is hoped that the project will help unravel the evolutionary causes and consequences of woodwasp-fungal symbiosis. Such basic information will contribute to understanding fungal-insect symbiosis, as well as symbiosis as a general biological theme influencing evolution of organisms. In addition, such data will provide practical assistance to monitoring and controlling programs of introduced population of Siricid woodwasps and their symbiotic fungi. It will also help to characterize patterns of natural and human-mediated spread of these insects. From these data, the project should also contribute to the growing body of knowledge concerning international movement and control of pests and pathogens, to help prevent recurrence of such events.

Acknowledgements

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Alterations of Scots pine needle characteristics after severe weather conditions in south-eastern Estonia

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Abstract

In the spring of 2003 massive deaths of Scots pine trees were registered on drought-sensitive oligotrophic sandy soils in south-east Estonia, together with the stress symptoms on some other tree species. Having at our disposal retrospective long-period data, obtained by the needle trace method (NTM) from three pine stands in south-east Estonia we decided to look into history, searching for seasons, meteorologically resembling the hard seasons of 2002/2003 (severe drought, abrupt winter onset and unusually cold first half of winter), and see how did NTM characteristics respond during last century: 1) to the similar seasons, and 2) to the most severe appropriate seasons. We concluded that none of the mentioned above hard seasons could, separately taken, cause the registered losses but, at the same time, resembling full series of seasons could not be found inside that period, which would be adequately covered by our NTM material.

Introduction

In 2001 a severe outbreak of *Gremmeniella abietina* was registered in stands and plantations of Scots pine (*Pinus sylvestris*) in Sweden (Wulff & Wahlheim 2002) and in eastern Norway (Solheim 2001). In the same year (2001) Scots pine plantations in South Estonia experienced a hard epidemic of *Lophodermium seditiosum* (Hanso & Hanso 2001). In the spring 2002 some concern of a start of a new epidemic of *G. abietina* was expressed in north-eastern part of Estonia (in Sirgala, fig.1), as after a 37-year-long break the perfect stage fruitbodies of the pathogen were found again in Estonia (Hanso & Hanso 2003). *Gremmeniella abietina* teleomorphs had been registered during the first diagnosed epidemic in Estonia, i.e. during the hardest epidemic of the disease in 1964–1965 (Hanso 1969, 1973). During that long break only anamorph (*Brunchorstia*-) stage fruiting of the fungus was observed in forest pathological surveys. Also news about the recent outbreak of *Gremmeniella* in Scandinavia had to be considered seriously as well on the eastern coast of the Baltic Sea.

Fig. 1. Locations of a suspected epicentre of a new G. abietina *epidemic in Sirgala in 2002, the severely damaged young pine plantations in 2003 in Liiva and Loosi, and the pine stands examined by needle trace method (NTM) in Konguta, which served as the source of retrospective long-period NTM material.*

Health condition of the forests of south-eastern Estonia in spring 2003

In the spring 2003 a large-scale death of Scots pine trees in plantations and stands of south-eastern Estonia, especially devastating on drought-sensitive sandy soils (e.g. in Liiva and Loosi, fig. 1), was registered by local forest authorities, who preliminarily attributed the damage to *G. abietina*. After careful diagnostic work (Hanso, unpubl.) it was ascertained that the death of pines was not caused by *G. abietina* or by any other well-known infectious disease.

Concerning health condition of other native forest-forming tree species in south-eastern Estonia in the spring of 2003, Norway spruce (*Picea abies*) had not been seriously affected, but aspen trees (*Populus tremula*) showed abnormal shoot swellings and started to loose their leaves abnormally early, however, without visible fatal results to the trees. Additionally, a sudden death was registered in the stands of some exotic tree species (e.g. *Pseudotsuga* sp.).

In 2002 the weather was very special in Estonia, with a severe and long drought in the summer and autumn followed by an unusually cold winter. A diagram presentation of this weather data (figs. 2 and 3) revealed a third exciting peculiarity of the year 2002, an extremely abrupt autumn in comparison with the long-period mean (fig. 2).

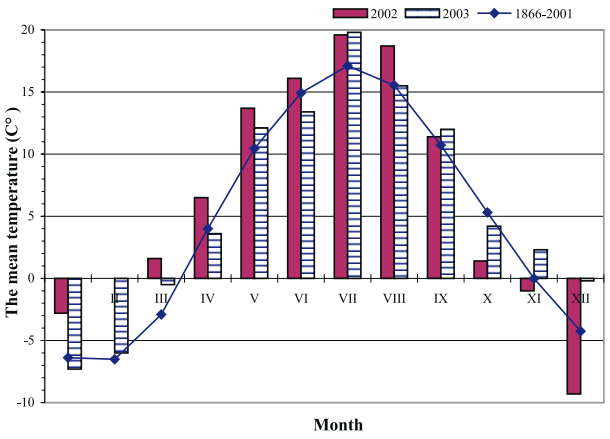

Fig. 2. The mean monthly temperatures in 2002 and 2003 (in columns) together with the long period (1866–2001) mean (curved line)

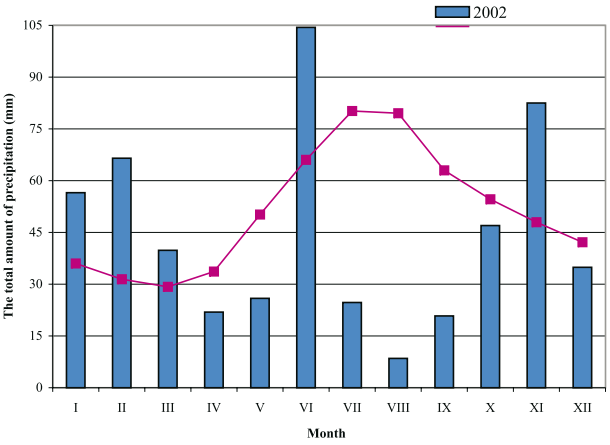

Fig. 3. The sums of monthly precipitation in 2002 (in columns) together with the long period (1866–2001) mean (curved line)

Scots pine seemed to be more stressed than other forest-forming native tree species in south-eastern Estonia in the spring of 2003. Although Scots pine has been classified in Estonia (Laas 1967) as a cold- and drought-resistant tree species, the decisive factor for the annual ring index variation of Scots pine appears to be the temperature of the winter prior to the growing season, but also the mean deficiency in air humidity in June-August has a relatively high correlation with the annual ring index variation (Lõhmus 1992). In other words, Scots pine in Estonia is not indifferent towards hard winters and summer droughts.

Strength of the climate correlations can be increased and the range of extractable parameters extended by including dendrochronology with the different other proxies (McCarroll *et al.* 2003). Long chronologies describing retrospectively different needle characteristics of pines can be drafted using NTM (Needle Trace Method, cf. Kurkela & Jalkanen 1990). Fortunately we had access to long retrospective time-series based on the needle trace method and describing the behaviour of pine in the continuously changing environment of Estonia. This dataset was now used to examine whether the massive death of Scots pine could be explained by climatic factors.

Material and methods

Since we had access to the retrospective long-period (1884–1944 and 1957–1995) NTM material from three Scots pine stands in south-eastern Estonia, analysed by Drenkhan (2002), we decided to look for answers to the recent problems from the past, i.e. to investigate how Scots pine needle characteristics have altered within long period during the first years after seasons with following description:

1. Extreme (dry or cold, respectively) seasons;
2. Seasons meteorologically resembling the summer of 2002 and the winter of 2002/2003, respectively and separately taken (i.e. not in succession).

If the needle characteristics of Scots pine respond to the extreme and long lasting meteorological events (the used meteorological characteristics were the mean air temperature and the sum of precipitation of the appropriate month and/or season), we could attribute the recent massive stress and death of pines in south-eastern Estonia to the meteorological peculiarities of the summer 2002 or the following winter 2002/2003.

It is not known during how many dry summers or how many cold winters within the experimental period the tolerance level of Scots pine was exceeded in such a way that it is reflected in the needle characteristics. Therefore two samples of extreme seasons were chosen from history:

1. 3 years. Alterations in the needle characteristics of pine can be surely caused as well by several other agents in addition to the meteorological extremes and therefore the sample of 3 years is too small.
2. 10 years. If Scots pine would suffer during so many years per century, it would have not been classified to the drought- and cold-resistant tree species.

Therefore the samples consisting of both 3 and 10 declining years were provisionally taken and used to examine closer the correct number of extreme years during which the tolerance level of Scots pine was exceeded.

First we computed mean values of the three different needle characteristics of Scots pines (needle retention, needle age and needle loss, cf. Aalto & Jalkanen 2004) for the period covered by our NTM data and conditionally named «the century». After that these mean values were calculated for the following years within the experimental period:

1. For the three/ten years, which had the highest mean air temperatures of the summer months (from May to September, incl.);

2. For the three/ten years, which had the lowest mean sums of precipitation per the summer months;
3. For the three/ten years, which had the lowest mean air temperatures of the winter months (from previous year December to subsequent year March, incl.).

The possible influence of random agents to the needle characteristics would hopefully be smaller in the ten-year sample. The calendar years selected are shown in Table 1.

Table 1. The definite calendar years, belonging to the different sample sets

Sample sets of the years, regarding							
high mean summer (V-IX) temperature, °C		poor mean summer (V-IX) precipitations, mm			low mean winter (XII-III) temperature, °C		
3 hottest summers	10 hot summers	3 dry summers, similar to 2002	3 driest summers	10 dry summers	3 cold winters, similar to 2002	3 coldest winters	10 cold winters
1934	1901	1913	1901	1901	1902	1893	1888
1936	1920	1964	1939	1913	1909	1940	1893
1937	1932	1976	1976	1939	1912	1942**	1917
2002*	1934	2002*		1941	2002*	1963	1929
	1936			1958			1940
	1937			1964			1942**
	1938			1965			1963
	1939			1971			1970
	1963			1975			1979
	1972			1976			1985
							1987

* The summer 2002 belonged to the 3 most extreme seasons, but was not covered by our NTM data.

** The coldest winter in 1942 was covered by our NTM data but fell out of NTMeng computations for the peculiarity of the program.

By this way we obtained information on the extent (or at least the directions) of alterations in the needle characteristics following severe meteorological conditions of summer or winter. To answer the question «Could the two seasons of 2002/2003, summer and winter separately taken and both clearly deviating from the long-period mean, cause the stress and death of pines in the south-eastern Estonia?» we found three years inside that long period, which resembled the most the summer of 2002 or the winter of 2002/2003, respectively. Then we computed similarly the corresponding mean needle characteristics for this set of years. Comparison of the alterations in needle characteristics among these three samples of years (in short: the extreme, the hard and the similar to 2002/2003 sample) should hopefully give us the answer to the question raised above.

Understanding tree physiology is complicated by the fact that the performance in a given year depends on conditions of previous seasons (James *et al.* 1994). The visible reaction of trees to an unfavourable (i.e. stressing) environment is often temporally delayed, and by the time when the visible symptoms occur (and when the pathologist arrives and becomes involved, cf. Houston 1987), the causative agent may be already absent. Therefore the alterations in needle characteristics were examined one, two and three years after the appropriate pointer year with extreme weather conditions. In the ideal case this 3-year-long period might cover as well a temporal aspect and reveal the peculiarities of the dying apart of the influence of the stressing agent. This period cannot be extended as the retention period of a Scots pine needle set in Estonia rarely exceeds three years (Tullus 1991; Drenkhan & Hanso 2000; Drenkhan *et al.* 2006).

Meteorological data were obtained from the Tartu-Tõravere Meteorological Station, which is situated ca 15 km from the pine stands in Konguta investigated by NTM (fig. 1), from the Institute of Meteorology and Hydrology (Tallinn), from the Võru Meteorological Station and from the data represented in the paper of A. Tarand (2003). NTM data were calculated by a special program NTMeng (Aalto & Jalkanen 2004). Statistical analyses were carried out by MS Excel and statistical program SAS.

Results and discussion

In this investigation the possible influence of the long and hard drought of the summer 2002 and the abnormally cold winter 2002/2003, separately taken, on the alteration of the NTM characteristics were examined. Research work, concerning the influence of abrupt winter onset 2002 on the alterations of NTM characteristics is still in process and the results are not included in this investigation, and only some meteorological data, emphasizing the extremity of the winter onset 2002, are shortly represented.

The cold winter

As mentioned above, the first symptoms of the massive death of pines became visible in the spring of 2003, immediately after the abnormally cold winter. The analysis of the alterations in needle characteristics after three different samples of winters is represented in Fig. 4. After three winters similar to the 2002/2003 winter the mean values of the characteristics needle retention and needle age apparently (although not statistically significantly) increased, while needle loss decreased. After the extreme (the sample of 3 record-cold winters, column 5) or hard (the sample of 10 coldest winters, incl. the full sample of record-cold winters, column 4) winters, the characteristics needle retention and needle age mostly decreased (with several statistically significant differences in mean values), as did also needle loss, although without significant differences. On the basis of the clearly different directions of the alterations of the needle characteristics after the extreme and hard winters, in comparison with the similar to 2002/2003 winters, we conclude that the weather conditions of the winter 2002/2003 are not the reason for the massive pine death in 2003.

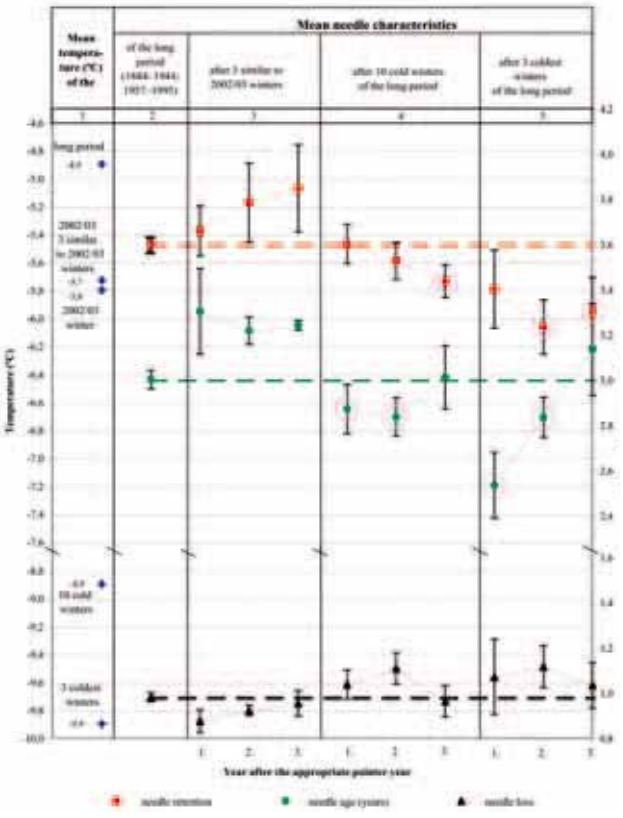

Fig. 4. A comparison of the mean temperature, radial growth and needle characteristics during the long period (1884–1944, 1957–1995) and during the first, second and third year, respectively, after 3 winters similar to 2002/2003, after 10 cold and after 3 coldest winters within the period. Pink circles show statistically significant differences.

The dry summer

The dry summer 2002 preceded the already characterised cold winter. As we have still no access to the computation methods of Palmer drought index, the influence of drought was analysed indirectly on the basis of summer air temperatures and summer precipitation, taken as separately.

The summer 2002 proved to be one of the three hottest summers of the long period. The reason why it is absent from the list of respective sample set of years (Table 1) is, that we had no NTM data for the year 2002. As one can see from the Fig. 5 (column 1), the mean air temperature of the summer months of 2002 was higher than the mean temperature of the sample set of 3 years inside the century with the warmest summer months. Regarding the needle characteristics, only two values were statistically significant, the characteristic needle retention in the first year after the three hottest summers and needle age in the first year after the 10 hot summers of the century. We propose that the weather conditions of summer 2002, though not lethal, could have stressed the trees.

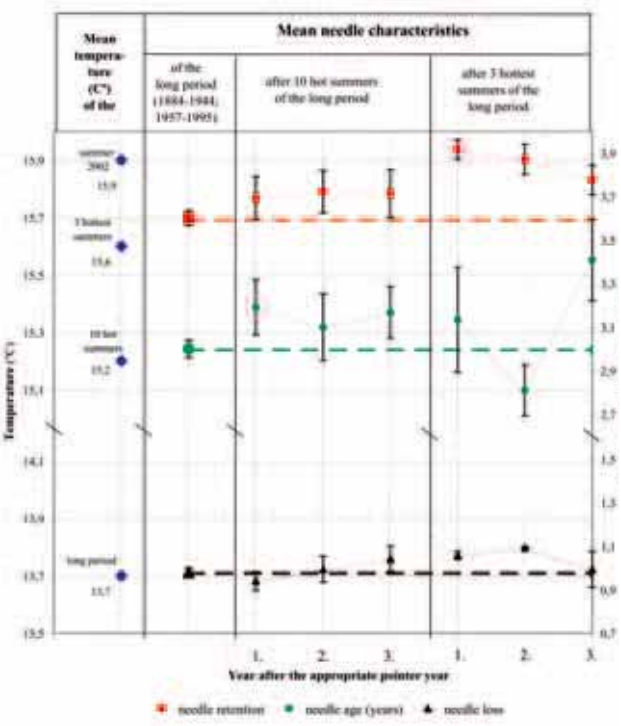

Fig. 5. A comparison of the mean temperature, radial growth and needle characteristics during the long period (1884–1944, 1957–1995) and during the first, second and third year, respectively, after 10 hot summers and after 3 hottest summers within the period. Pink circles show statistically significant differences.

One more figure (not represented in this paper) was constructed by the same way as figures 4 and 5, but concerning the alterations of NTM characteristics after the driest (regarding the sums of precipitation per summer months) and similar to the summer 2002 years. Although none of

the alterations occurred were statistically significant, some directions of the alterations were noteworthy.

The abrupt winter onset

Concerning meteorological peculiarities, the autumn (winter onset) 2002 was the most extreme (Table 2) among the seasons under the investigation. Figure 6 shows the way in which the extremely short autumns inside the long period were computed for three meteorological stations in different counties (towns) of Estonia. Autumn 2002 belonged to the group of extremely abrupt autumns in all three meteorological stations – Tallinn, Tartu and Võru, but in the south-easternmost county of Estonia (Võru) this year (2002) was the absolute record-year (Table 2).

Table 2. Extremely short autumns within the experimental period, and covered by our NTM data (1884-1944 and 1957-1995). Using the temperature datasets of three meteorological stations (Tallinn, Tartu and Võru), the autumn (winter onset) was defined in three different ways, «autumn» extending from August to October, from August to November or from August to December,

Order of the coldest years	August-October			August-November			August-December		
	Tallinn	Tartu	Võru	Tallinn	Tartu	Võru	Tallinn*	Tartu	Võru
1.	1939	1939	1939	1774	1882	**2002**	1788	1876	**2002**
2.	**2002**	**2002**	**2002**	1786	1941	1939	1803	**2002**	1939
3.	1912	1912	1976	**2002**	**2002**	1993	1759/**2002**	1882	1927

*The year 2002 was on the forth place

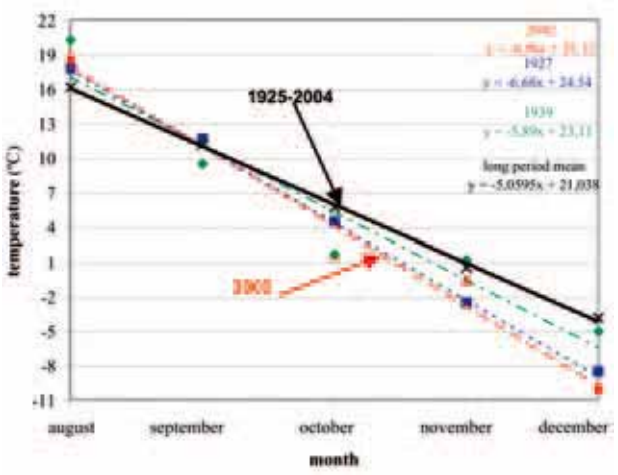

Fig. 6. An example of the computations for finding the most abrupt autumns among the years within the period 1884–1944 and 1957–1995, and calculated on the basis of the fall of mean air temperatures during the appropriate months

The directions of alterations in needle characteristics

Apparently due to the limited NTM material used in this investigation, several data represented in figures as numerical values did not differ statistically. However, if the directions of alteration were similar during all the three years following the pointer (presumably stressing) year, this characteristic direction could be taken more seriously (Table 3).

Table 3. The directions of alterations of the examined NTM characteristics and the radial growth after the hard periods (Pink colour shows statistically significant differences)

Characteristic	Winter	Summer temperature	Summer precipitation
Needle retention	↓	↑	↑
Needle age	↓	Undefinite	Undefinite
Needle loss	↑	↑	Undefinite
Radial increment	↓	↓	↓

Comparing the direction of alterations of NTM characteristics with the direction of alterations in radial increment showed that the former opened much more room for interpretation of pine reactions to the hard environmental conditions than the radial increment, which forms the basis of the dendrochronological method.

Conclusion

The directions and extent of alterations of the NTM characteristics (needle retention, needle age and needle loss) after the abnormally cold winter of 2002/2003, together with hot summer characterised by low precipitation, indicate that the particular unfavourable weather conditions could not act, separately taken, as the reason for the massive stress and death of pines registered in south-eastern counties of Estonia in the spring of 2003. However, although Scots pine is considered to be a cold- and drought-

resistant tree species in Estonia, the sequence of adverse environmental events, which began with the hard epidemic of *Lophodermium* needle cast in 2001, and was followed by the dry summer of 2002 and ended by the abrupt autumn of 2002 and abnormally cold winter of 2002/2003, most probably exceeded the tolerance level of a number of pines, this series of events acting as a hard stress factor and leading to the massive death among pines.

Involvement of NTM data in the diagnostic trial of a complex pathological case was now undertaken for the first time, this approach opening new possibilities for the use of this method in forest science.

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Melampyrum spp. as alternate hosts for *Cronartium flaccidum* in Finland

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Abstract

Distribution and frequency of *Cronartium flaccidum* on *Melampyrum* spp. was studied on Scots pine throughout Finland. Leaves of the alternate hosts were collected, and the frequency of *Cronartium* telia was recorded. Morphological dimensions of fruitbodies and spores were measured, and some telial samples were identified genetically. Telia were observed for the first time on *M. pratense* and *M. nemorosum* in natural forests, and on *M. arvense* in Finland. Telia occurred in 22 % of the *M. sylvaticum*-stands, 3 % of the *M. pratense*-stands, 12 % of the *M. nemorosum*-stands, and in the *M. arvense*-stands investigated. Geographically, telia were lacking on *M. sylvaticum* and *M. pratense* in southern Finland, but they were relatively common on these species in northern Finland, whereas 92 % of the *M. sylvaticum*-stands and 30 % of the *M. pratense*-stands bore plants with telia in the area. The proportions of stands with telia, plants with telia per stand and telia-bearing leaves per plant were greater on *M. sylvaticum* than on the other *Melampyrum* spp.

Introduction

Pine stem rusts, *Cronartium flaccidum* (Alb. & Schwein) G. Winter and *Peridermium pini* (Pers.) Lév cause severe damage on Scots pine (*Pinus sylvestris* L.) in Europe (Gäumann 1959). Genetic analysis suggests that gene flow occurs between these two rusts, and that they, therefore, belong to the same species (Hantula *et al.* 2004). In Finland, *P. pini* is more common than *C. flaccidum* based on population studies conducted with aeciospores (Hantula *et al.* 1998, Kaitera *et al.* 1999). Geographically, *C. flaccidum* has been found locally in northern Finland in the late 1990s (Kaitera & Hantula 1998), but there are several findings of the rust in natural forests in the southern coast of Finland and in the Åland archipelago both on Scots pine and on alternate hosts since the 1800s (Liro 1908, Kaitera & Nuorteva 2003a, b).

Common alternate host genera for *C. flaccidum* in Finland are *Vincetoxicum, Pedicularis, Melampyrum* and *Paeonia* (Liro 1908, Hylander *et al.* 1953, Kaitera *et al.* 1999). In genus *Melampyrum*, the rust has been found on *M. sylvaticum* L. in northern Finland (Kaitera & Hantula 1998, Kaitera 2000), and in artificial inoculations, *M. sylvaticum* L. (Kaitera 1999, Kaitera & Nuorteva 2003a, b), *M. nemorosum* L. (Kaitera & Nuorteva 2003a, b), *M. pratense* L. (Kaitera 1999, Kaitera & Nuorteva 2003b) and *M. arvense* L. (Kaitera & Nuorteva 2003b) have been shown to be susceptible to the rust. According to some old reports (Rennerfelt 1943; Hylander *et al.* 1953), *C. flaccidum* occurs also on *M. arvense* and *M. cristatum* L. in natural forests in Sweden.

In Scandinavia, there are five *Melampyrum* species growing in natural forests (Hultén 1950; Hämet-Ahti *et al.* 1984), which also grow elsewhere in Europe (Hegi 1974). Only two species, *M. pratense* and *M. sylvaticum*, are common and widely-spread in Scandinavia, and thus, may play significant roles as alternate hosts in natural forests. The aim of this study was to clarify the distribution and frequency of *C. flaccidum* on *Melampyrum* spp. in Finland.

Materials and methods

Old leaves of *Melampyrum* spp. were collected systematically throughout Finland in Scots pine stands infected by pine stem rusts in 1998–2002. For a more thorough description of e.g. the data collection, see Kaitera *et al.* (2005). Data of damaged stands collected in private forest owners' land was used as basis for the sample collection. The data included 338 *M. pratense*-, 111 *M. sylvaticum*-, 17 *M. nemorosum*-, one *M. cristatum*- and one *M. arvense*-stand. Geographically, 33 % of stands with *M. pratense* and 25 % of those with *M. sylvaticum* occurred in northern Finland. The corresponding proportions were 46 % and 57 % in southern Finland.

A sample of plants (50 in number) of *M. pratense*, *M. sylvaticum* and *M. nemorosum* were collected per stand close to the infected trees. A sample of similar size of *M. cristatum* and *M. arvense* were checked in the field. The plant leaves were checked for *Cronartium* telia in the field and in the laboratory. The number of telia per leaf and the length and width of fully developed telia, teliospores and urediniospores were measured under microscopes. A few telial samples per host and stand were identified genetically. In about 100 samples, of which 80 % were *M. sylvaticum* leaf samples, DNA was isolated from telia (Vainio *et al.* 1998), the ITS region was amplified using primers ITS1-F and ITS4-B (Gardens & Bruns 1993), and the amplification products were digested. The amplification products from *M. pratense* and *M. nemorosum* were sequenced, and blast searches were made to find most similar sequences in Genbank. For a more thorough descriptions of the used protocols, see Kaitera *et al.* (2005).

Results

Telia occurred in 22 % of the investigated *M. sylvaticum*-stands, and in 3 % of the *M. pratense*-stands, and they located mainly in northern Finland. Ninety-two percent of the *M. sylvaticum*-stands and 30 % of the *M. pratense*-stands included plants carrying telia in northern Finland, while telia were lacking on these alternate hosts in southern Finland. Telia were also found in 12 % of the *M. nemorosum*-

stands, and in the investigated *M. arvense*-stand, but not in the *M. cristatum*-stand. The mean proportion of plants bearing telia per stand was significantly higher for *M. sylvaticum* than for *M. pratense* and *M. nemorosum*. The mean proportion did not differ significantly between site types for either *M. sylvaticum* or *M. pratense*, but was significantly higher in young development classes compared to older ones for *M. sylvaticum*. Variation in the number of leaves bearing telia per plant was highest for *M. sylvaticum*, while 38 % of the infected plants bore telia on 3–13 leaves per plant. Telia occurred less frequently on the rest of the *Melampyrum* spp. The average number of telia per leaf varied between 12.3–16.2 among the *Melampyrum* spp., but it did not differ significantly between *M. sylvaticum* and *M. pratense*. The average width of telia and length of teliospores were significantly greater on *M. pratense*, and the average width of teliospores was greater on *M. arvense* compared to those on the other *Melampyrum* spp. The PCR amplifications of leaves with telia resulted in single amplification products of about 900 bp. After digestion with restriction enzymes followed by gel electrophoresis, the banding pattern for *Cronartium flaccidum* was observed. Based on this pattern, 50–60 % of the samples of *M. sylvaticum*, *M. pratense* and *M. nemorosum* were identified as *C. flaccidum*. The ITS sequences of the samples determined and compared to GenBank gave the highest similarities to *P. pini* and *C. flaccidum*. For a more thorough description of the results, see Kaitera *et al.* (2005).

Discussion and conclusions

The present study confirmed that *Melampyrum* spp. are important alternate hosts for *C. flaccidum* in natural forests in Finland. This is due to the frequencies of *M. sylvaticum* and *M. pratense* bearing telia especially in northern Finland. These findings are also the first ones on *M. pratense*, *M. nemorosum* and *M. arvense* in natural forests, and correspond well with the susceptibility of these species to *C. flaccidum* under inoculation experiments (Kaitera 1999; Kaitera & Nuorteva 2003a, b). The rust is also more common than the aeciospore studies (Hantula *et al.* 1998; Kaitera *et al.* 1999) have suggested. The distribution is, however, strongly concentrated in northern Finland, whereas no telia were found on *M. sylvaticum* or *M. pratense* in southern Finland. Telia were also more common in stands belonging to young development classes compared to older ones on *M. sylvaticum*, which may lead to increasing numbers of epidemics in young pine stands in the future. The high variation in morphological characteristics of telia and different spores corresponds well with the reported dimensions of natural samples in the litterature (Liro 1908; Gäumann 1959; Kaitera & Hantula 1998). The lower dimesions are probably due to the high number of dry, late-summer samples among all studied samples. Molecular analysis of the telial samples of *M. sylvaticum*, *M. pratense* and *M. nemorosum* confirmed that the telia were of *C. flaccidum*. Some samples could not be identified probably due to low numbers of telia in the samples or small numbers of DNA in the teliospores after karyogamy and meiosis.

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Fungal attacks to root systems and crowns of declining *Fraxinus excelsior*

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Abstract

The aim of this study was twofold: 1) to investigate the extent of decay in roots and stems of declining ash; 2) to determine fungal species in damaged roots and shoots, and estimate their potential pathogenicity. In central Lithuania, 33 ash trees showing various degree of decline were felled and their root systems excavated. The positive correlation was detected between severity of the dieback and amount of decayed roots, length of decay within the stems and extent of decay over stump cross-section. A total of 150 isolations from root systems (3 samples from 50 root systems: at 0.5 m, 1 m and 1.5 m away from a stem) yielded 96 isolates representing 28 fungal species. Another 195 fungal isolates with 36 identified species were obtained from sound looking, damaged and heavily damaged shoots. *Armillaria cepistipes* was the fungus, most frequently isolated from root samples, whereas *Giberella avenacea*, *Alternaria alternata* and *Epicoccum nigrum* dominated among crown infecting species. Subsequently, 27 fungal species isolated from decayed roots and 18 species from shoots were tested for pathogenicity against 600 one year-old *Fraxinus excelsior* seedlings.

Introduction

The issue of declining European ash (*Fraxinus excelsior* L.) became important since mid-1990s, when this process was initially observed in Poland and Lithuania. Subsequently conducted studies did not reveal any correlation between tree mortality and geographic location of a stand, forest site type, age of a stand, species composition and edaphic factors (Juodvalkis & Vasiliauskas 2002, Przybyl 2002, Lygis *et al.* 2005). Characteristic symptoms of the disease are gradual crown decline due to necrotic patches on shoots and stems.

However, there were certain differences in pathological process of ash decline in different geographic areas. From Lithuania, for example, heavy root and butt rot of dying and dead trees was reported, cause of which was *Armillaria cepistipes* Velen. (Lygis *et al.* 2005). By contrast, in other countries damage to shoots and branches is thought to be of crucial importance for the decline, and no decay of stem bases and roots was observed (Przybyl 2002, Barklund 2005). In order to acquire more knowledge about pathological process in different parts of a tree, during the present study we investigated: 1) the extent of decay in roots and stems of declining ash and its correlation with the severity of the dieback; 2) fungi that invade roots and shoots of diseased trees and their relative pathogenicity.

Materials and methods

The methodology of this study consists of three basic parts: examination and fungal isolation from root systems and crowns, and pathogenicity tests with the isolated fungi.

Root systems were investigated in three 50–100 year-old *F. excelsior* stands located in south western part of Lithuania, Sakiai forestry district. The trees were of four health categories: 1) slight crown damage (dieback of up to 25 % of shoots); 2) moderate crown damage (up to 50 %); 3) severe damage (up to 75 %); 4) crown death (100 %). A total of 33 trees from all four categories were chosen for further investigation. They were situated at least 20 m from each other. The trees were cut down and the extent of decay in stump, stem base and roots (longitudinal and over cross-section) was estimated. For this, the root systems of cut trees were excavated about 40 cm deep at 1m radius from a stem base. Also, the percentage of decayed roots thicker than 2 cm was calculated. For fungal isolations, 150 wood pieces were taken from roots of 50 moderately damaged trees, – one root per tree, 3 wood samples per root (at 0.5 m, 1 m and 1.5 m distance from stem respectively).

Crowns of declining *F. excelsior* were examined in two sites in Sweden, one near Örebro (central Sweden), and another one near Visby (Gotland). The trees with crown dieback symptoms were cut and branch samples were taken. Depending on symptoms at the shoot base, all shoots were divided in three health categories: sound looking, with initial necroses at the shoot base and with advanced necroses. From the shoot bases, altogether 171 wood samples (58 from first, 58 from second and 55 from third health group, respectively) were taken for fungal isolations.

Pure cultures of fungi were isolated from about 4 x 0.5 cm wood pieces taken from roots, and 2 x 0.5 cm pieces of wood and bark taken from shoots. The pieces were cut out, sterilized in open fire and plated on Petri dishes containing Hagem agar. All samples were incubated at room temperature for two weeks. All obtained fungal pure cultures were grouped depending on mycelial morphology. The representatives of each groups, were selected for molecular identification by ITS sequencing (White *et al.* 1990), similarly as in our previous study (Vasiliauskas *et al.* 2005). Sequence results were checked against available databases – NCBI BLAST database (Altshul *et al.* 1997), and database of the Dept. of Forest Mycology and Pathology at the Swedish University of Agricultural Sciences.

A total of 27 fungal species, isolated from decayed roots and 18 species, isolated from shoots were tested for pathogenicity against 600 one year-old *F. excelsior* seedlings planted under bare root conditions. Pieces of wood 1×1×5 mm in size, autoclaved and pre-colonized with

respective strain, were used as an inocula. Sterile wood pieces were used as control. They were attached with a tape to a 1×5 mm size wound made respectively at the base or at the shoot of a tree. The results will be evaluated after two vegetation seasons.

Results and discussion

The amount of decayed roots varied from 10 to 30 % in trees with slight crown damage, from 20 to 70 % in trees with moderate crown damage, from 30 to 90 % in trees with severe crown damage, and from 80 to 100 % in dead trees. The corresponding values for length of decay in a butt of a stem were 0.1–0.4 m, 0.2–1.5 m, 0.4–1.6 m and 0.4–2.5 m. For extent of decay over stem cross-section the corresponding values for the health categories were 10–20 %, 5–60 %, 30–60 %, and 70–100 %. As a result, there were positive correlations between severity of the dieback and amount of decayed roots (rS = 0.86), length of decay in a butt of a stem (r_S = 0.57), and extent of decay over stump cross-section (r_S = 0.87).

The isolations from roots yielded 96 fungal strains representing 24 species. Mainly the same species of fungi were isolated from roots at different distances from the stem (0.5, 1 and 1.5 m), as in comparisons between the communities Sorensen indices of quantitative similarity (Magurran 1988) were high (S_N = 0.84–0.96). However, general species richness was relatively high and species accumulation curve was not asymptotic, indicating that increased sampling effort in obtained roots would reveal additional species of fungi.

The dominating basidiomycete was *Armillaria* spp. In addition, some other wood-decomposing basidiomycetes, as *Coprinus disseminatus* and *Pholiota carbonaria* were also present. Characteristic ascomycetes were *Nectria* spp., *Xylaria* sp. and *Scytalidium lignicola*. Although mating tests with the isolates of *Armillaria* spp. were not performed in the present study, we suspect species to be *A. cepistipes*, as this species was reported to invade stem bases of declining *F. excelsior* in other parts of Lithuania (Lygis *et al.* 2005). On the other hand, the cited study also demonstrated that the fungus is not the primary cause of *F. excelsior* decline, as its genotypes on examined sites was large and several decades old, when the decline there has been recorded only few years previously (Lygis *et al.* 2005). Moreover, *A. cepistipes* is known as weak opportunistic pathogen, invading trees under stress, weakened by some other factor (Entry *et al.* 1986). Moreover, during earlier extensive field observations sporocarps of the fungus on *Fraxinus* had not been observed (Sokolov 1964), indicating that this tree species is somehow unusual host.

The isolations from shoot bases yielded 195 fungal strains representing 36 species. Mainly the same species of fungi were isolated from crown samples collected at different localities (Örebrö and Visby), as in comparisons between the communities Sorensen indice of quantitative similarity (Magurran 1988) was high (S_N = 0.89). However, general species richness was relatively high and species accumulation curves from both localities were not asymptotic, indicating that increased sampling effort in crowns would reveal additional species of fungi.

Species most commonly isolated were asco- and deuteromycetes: *Alternaria alternata*, *Fusarium* spp., *Epiccocum nigrum*, *Lewia* sp., *Botryosphaeria stevensii*, *Phomopsis* sp., *Phoma glomerata Cladosporium* sp., *Cytospora* spp. and many others. Occasionally, in shoots we recorded the presence of wood decay basidiomycetes – *Coprinus* sp., *Pharenochaete* spp., and one unidentified basidiomycete. As in our work, many similar or related asco- and deuteromycetes were detected in crowns and stems of declining *F. excelsior* during the recent studies in Poland and Lithuania (Przybyl 2002; Lygis *et al.* 2005; Kowalski & Lukomska 2005). However, the question of which of those are primarily responsible for the dieback of crowns, to date remains largely unclear, and we look forward towards the evaluation of the pathogenicity tests.

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Pathological evaluation of declining *Fraxinus excelsior* stands of northern Lithuania, with particular reference to population of *Armillaria cepistipes*

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Abstract

Stem bases of 210 *Fraxinus excelsior* trees of three different health categories were sampled by the means of an increment borer in declining ash stands of northern Lithuania. From this number, 15 sound-looking, 132 declining and 63 dead trees from three discrete plots yielded 352 isolates, representing 75 operative taxonomic units (OTU's). *Armillaria cepistipes* was the most common species (86 isolates from 210 wood samples, or 41.0 %), isolated more frequently and consistently than any other potential tree pathogen. It also showed abundant occurrence on a majority of trees in form of mycelial fans and rhizomorphs, from which 64 and 14 respective isolates of the fungus were obtained. Population structure of *A. cepistipes* revealed the presence of 53–93 genets per hectare, some of which extended up to 30–55 m. The present study led to a hypothesis that saprotrophic behaviour of weakly pathogenic *A. cepistipes* has been shifted to aggressive pathogenic by some predisposing factor (-s) (possibly – water stress) after at least 20–30 years of latent presence in the area.

Introduction

Starting in 1996, decline of European ash (*Fraxinus excelsior* L.) has been a permanent and widespread forest health problem in east-European countries (Juodvalkis & Vasiliauskas 2002, Przybyl 2002, Skuodiene *et al.* 2003). In Lithuania, for example, it affected over 30,000 ha of stands, comprising about 60 % of total ash area (Juodvalkis & Vasiliauskas 2002). The decline was especially destructive in the northern parts of the country.

Reasons for the decline remain largely unknown, although preliminary observations suggest that biotic factors and pathogenic fungi in particular, are the likely cause of the disease (Juodvalkis & Vasiliauskas 2002, Przybyl 2002). Judged by external symptoms on the trees, *Armillaria* root rot was among the most probable reasons of mortality in some parts of Lithuania (Juodvalkis & Vasiliauskas 2002). The main aim of the present study was therefore to identify wood-inhabiting fungi that attack stems of *F. excelsior* in declining stands, focusing the attention on populations of possible disease-causing agents.

Materials and methods

Study sites and fieldwork

The study was carried out during the summer 2001 in declining mixed-aged (20–60-year-old) *F. excelsior* stands located in Biržai Forest Enterprise, Buginiai forest district (northern Lithuania). Mapping, numbering, measurement, and sampling of trees was carried out in three discrete permanent sample plots (about 0.15 ha in size each), each consisting of 70 ash trees that represented three different categories of health condition: i) sound looking or healthy; ii) declining; and iii) dead [according to Innes (1990)]. Isolation of fungi was done from a total of 15 sound-looking, 132 declining, and 63 dead standing ash trees. For every tree, we estimated the diameter at breast height, crown density reduction [or defoliation, determined according to Innes (1990)], the presence of disease signs such as tarry spots or dead bark (scales), and the occurrence at the stem base of distinct basal lesions extending from diseased roots, mycelial fans (underneath the bark) and epiphytic rhizomorphs typical to *Armillaria* spp. [according to Morrison *et al.* (1991)].

Isolation and identification of fungi

The sampling of wood for the mycological investigations was performed as described by Lygis *et al.* (2004a). One wood sample per tree was taken by drilling at the root collar with an increment borer and extracting 4–5-cm-long bore cores. Isolation of pure cultures from the woody pieces was made on Petri dishes containing Hagem agar (Stenlid 1985). When available, pieces of *Armillaria* mycelial fans and rhizomorphs were collected; in a laboratory those were surface sterilized and placed on agar plates for isolation of pure cultures. Fungal operative taxonomic units (OTU's) were defined and identified to species or genus level on the basis of sequence similarities of the ribosomal ITS region (e.g. Lygis *et al.* 2004a, b, Vasiliauskas *et al.* 2004).

Intersterility and somatic incompatibility tests with Armillaria

The precise identification of *Armillaria* species was performed by mating tests on agar plates with representatives of known biological species. Those followed the procedures described by Guillaumin *et al.* (1991). Strains were assigned to species by pairing diploid mycelia with haploid tester strains of four European *Armillaria* species, *A. ostoyae*, *A. gallica*, *A. borealis* and *A. cepistipes* (Korhonen 1978). Somatic incompatibility tests were performed on agar plates to distinguish genetically distinct individuals (genets) of *Armillaria* at each plot (Shaw & Roth 1976). The results of the tests were projected on the constructed map (Fig. 2).

Results

Tree condition and infections by Armillaria

In our investigated stands, about 60 % of ash trees were declining, about 30 % were dead, and only about 10 % looked healthy and were classed as sound-looking. Based on occurrence of mycelial fans characteristic of *Armillaria* underneath the bark (Morrison *et al.* 1991), and the associated distinct typical basal lesions extending from diseased roots, we concluded that 205 out of 210 of the investigated trees (97.6 %) were colonized by *Armillaria* spp. From that number, colonization by the fungus was recorded on 80.0 % of sound-looking, 98.5 % of declining and 100 % of dead ash trees. Moreover, the presence of epiphytic *Armillaria*-like rhizomorphs was recorded at the root collar of every examined tree (above the bark), regardless of tree's condition. No canker or necrotic lesions typical to attacks by other ash pathogenic fungi e.g. *Nectria galligena* or *N. coccinea*, as in Sinclair *et al.* (1987) were observed on the lower part of the stems.

Fungal isolations

Of the 210 wood samples taken, 180 (85.7 %) resulted in fungal growth. A total of 352 isolates were collected and 318 of them (or 90.3 % of the total sample) were identified at least to genus level. They represented 75 distinct OTU's, 60 of which (or 80.0 %) were identified [for reference to the isolated fungal OTU's see Lygis *et al.* (2005)]. *Armillaria* was the most abundant fungus, isolated from 115 trees. Mating tests led to identification of all collected *Armillaria* isolates as *A. cepistipes* Velen. Other 19 OTU's of basidiomycetes were much less common (Lygis *et al.* 2005) and mostly represented widely spread saprotrophic wood decomposers.

Of the 51 isolated OTU's of ascomycetes, several were found quite frequently, although far less often than *A. cepistipes* (Lygis *et al.* 2005). The potential ash pathogens, *Phoma exigua* and *Botryosphaeria stevensii* (anamorph: *Diplodia mutila*) were isolated only in low frequencies irrespectively of tree condition. Other potential ash pathogens included two species of fungi often associated with seedling diseases, *Nectria haematococca* (syn. *Fusarium solani* (Mart.) Sacc.), and *N. radicicola* (syn. *Cylindrocarpon destructans* (Zinssm.) Scholten) (Booth 1971, Domsch & Gams 1972, Sinclair *et al.* 1987). Zygomycetes were isolated at low rates; they were represented only by 4 OTU's (Lygis *et al.* 2005).

Community structure and species richness

The community structure in sound-looking, declining and dead trees differed markedly (Lygis *et al.* 2005). Consequently, Sorensen similarity coefficients (S_s, qualitative) (Krebs 1999) were rather low (Fig. 1). The highest number of OTU's was found in declining (56), followed by dead (36) and sound-looking trees (16). However, this was mainly due to a lower sampling effort in dead and sound-looking trees (Lygis *et al.* 2005). Species accumulation curves (Colwell & Coddington 1994), presented in Figure 1, show that should sampling effort been equal in all three health categories, the differences in species richness between them would be minor. The data indicates also that our sampling efforts did not exhaust the existing diversity of wood-inhabiting fungi.

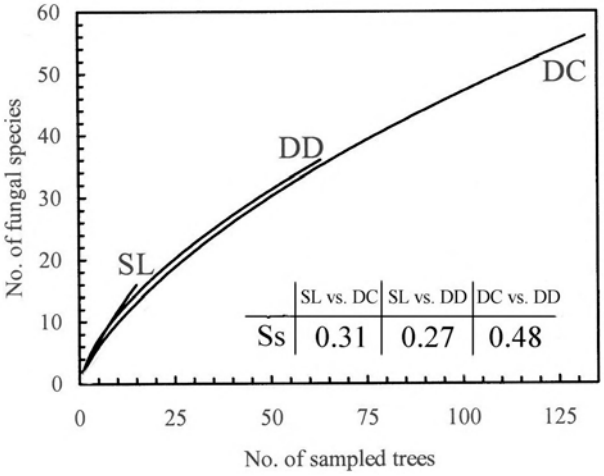

Fig. 1. Increase in species (OTU's) richness in sound-looking (SL), declining (DC), and dead (DD) Fraxinus excelsior *trees as a result of sampling more trees. Species accumulation curves were calculated according to Colwell & Coddington (1994). Qualitative Sorensen similarity coefficients (S_s) are shown between the community structures in SL and DC, between SL and DD, and between DC and DD.*

Population structure of Armillaria cepistipes

Of the 150 isolates of *A. cepistipes*, we identified 8, 13, and 8 genets in three investigated sites respectively (situation on two sites, A and B, is presented in Figure 2), corresponding to 53, 93, and 53 genets per hectare. In all three sites, 11 genets (or 37.9 % of all genets) included only one tree. Genet VII from site A was also found in site B: a forest road built about 20 years ago had seemingly split one large individual into two spatially separated ramets (Fig. 2). Sizes of the genets varied from a single root system to 55 m wide (genet II on the site A, Fig. 2).

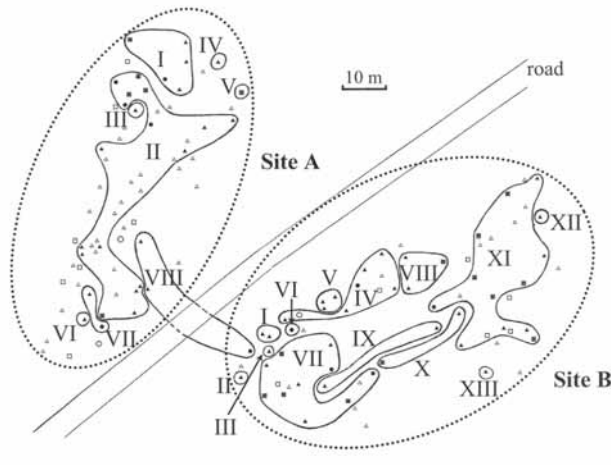

Fig. 2. Distribution of Armillaria cepistipes *genets in two infested sites (A and B) of* Fraxinus excelsior *in northern Lithuania. The small symbols, circles, squares, and triangles, label sound-looking, declining, and dead* F. excelsior *trees, respectively. Black symbols indicate the trees from which* A. cepistipes *has been isolated, while the open ones the trees from which* A. cepistipes *has not been isolated. Limits of genets are encircled by the solid line.*

Discussion

In this study, *A. cepistipes* was found to be the dominant fungus in all tree health categories, as it was most commonly observed and isolated from stem bases of sound-looking, declining and dead trees. This is to a certain extent surprising, since *Fraxinus* seems to be an uncommon host to *Armillaria* (e.g. Sokolov 1964). Moreover, *A. cepistipes* is generally considered to be a weak pathogen, only capable of slow infection of roots of healthy trees (Rishbeth 1982, Guillaumin *et al.* 1985, 1989, Gregory *et al.* 1991, Prospero *et al.* 2004). In our study sites, active decay caused by *A. cepistipes* was consistently recorded on 80.0 % of sound-looking, 98.5 % of declining and 100 % of dead trees, thus the fungus undoubtedly contributed to and accelerated the decline of investigated stands.

On the other hand, it is unlikely that the attacks by *A. cepistipes* were the primary cause of the decline. It is generally accepted that *Armillaria* spp. are opportunistic pathogens able to invade hosts weakened by certain stress factors (Wargo 1977, Singh 1983, Entry *et al.* 1986). However *A. cepistipes* is known to produce abundant rhizomorph networks on the roots of living trees, this characteristic giving it a competitive advantage in a pathogenic colonisation should the tree become stressed or in saprobic colonisation once the host dies (Rishbeth 1985, Redfern & Filip 1991). Increased frequency of dry years and lowered level of a ground water are among the abiotic stress factors that could be involved in ash decline in our geographic area (Juodvalkis & Vasiliauskas 2002, Skuodiene *et al.* 2003), while fungal infection to crowns might be an important biotic factor.

The revealed extensive territorial clonality of *A. cepistipes* (62.1 % of all genets detected colonized more than one host tree) indicates that the fungus was present on the diseased sites for many years before the decline started. According to mycelial growth rates for *Armillaria* in north-temperate forests (Shaw & Roth 1976, Rishbeth 1988, 1991, Smith *et al.* 1992, Legrand *et al.* 1996), the age of the largest *A. cepistipes* genets on our study sites were estimated to be at least 20 years. The forest road that split genet VIII between the sites A and B was also built 20 years ago (Fig. 2). We hypothesize that latent saprotrophic behaviour of *A. cepistipes* has been shifted to the pathogenic by some predisposing factor (-s) after 20–30 years of its presence in the stands, this leading to decline of *F. excelsior*.

Other basidiomycetes isolated during the present work are commonly fruiting on dead wood in northern European forests and are generally considered to have a saprophytic behavior (Lygis *et al.* 2005). It was surprising to find *Bjerkandera adusta* (isolated from intact wood) and *Trametes hirsuta* (isolated from a fresh necrosis) in sound-looking stems of ash, and their possible impact on ash decline cannot be excluded. Even less is known about the role played by the now isolated numerous microfungi (Lygis *et al.* 2005) in the pathological process.

An interesting finding of the present work was also the detection of principally different fungal communities in trees of different health condition growing within the same forest stand (Lygis *et al.* 2005). Although equal sampling effort provided us with rather similar number of OTU's in sound-looking, declining and dead trees (Fig. 1), the shift in the fungal community structure was considerable (Lygis *et al.* 2005), showing that stems of sound-looking, declining and dead ash are inhabited predominantly by different species of fungi. As in our previous study (Lygis *et al.* 2004b), we hypothesize that fungal species in wood of living trees likely change along with changes in tree condition.

Acknowledgements

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Chondrostereum purpureum a potential biocontrol agent of sprouting

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Abstract

In August – October 2003 three biological control experiments were established near Hyytiälä Forestry Field Station of Helsinki University in southern Finland. Water suspension of mycelia of the basidiomycete *Chondrostereum purpureum* was inoculated on stumps just after felling in order to examine the impact of inoculation on tree sprouting. The cut trees were 6–10 years old birch, aspen, willow, rowan or alder. Two plots located in sapling stand and one plot located under an electric power line. In October 2004 the occurrence of sporophores of *C. purpureum* were assessed from the stumps, while the sprouts were counted and measured in August 2005.

Sporophores of *C. purpureum* were found in 24.8 % of inoculated stumps and in 5.0 % of control stumps. This fungus is common in nature and the infections in controls were probably natural. Also dead sprouts were observed, but they were found both in controls and in inoculated stumps. The length of the longest sprout in stump was almost the same in both treatments. The used control methods did not stop sprouting. Three different fungus strains were inoculated in experiment. One of them was the Biochon preparation developed in Netherlands. It seems that in northern conditions more knowledge is needed for developing an effective biocontrol method of sprouting.

Introduction

Chondrostereum purpureum (Fr.) Pouz. has been tested as biocontrol agent of sprouting in Netherlands (De Jong & Scheepens 1982) and Canada (Wall 1990, Pitt *et al.* 1999, Harper *et al.* 1999, Becker *et al.* 1999). It is a wound decay fungus on broadleaved trees and also a pathogen causing silver-leaf disease. In Scandinavia the fungus is common on birch. It infects stumps, cutting waste, timber and wounds in growing trees.

The infection biology of *C. purpureum* on stumps has been studied in New Zeeland (Spiers and Hopcroft 1988). They found that a mycelial inoculum causes bigger lesions than a basidiospore inoculum in Salix. Also the fungus grows better in fresh wounds than old wounds. *C. purpureum* is an out-crossing fungus, and a heterokaryotic condition of mycelia can be checked by the presence of clamp connections, which are not formed in monospore culture.

Two commercial preparations of *C. purpureum* have been developed, Biochon in Netherlands and Myco-Tech^{TM} in Canada. The test results of these have been promising and for example Myco-Tech^{TM} is given 70–100 % efficiency according to commercial information.

The aim of this work was to test preliminarily the efficiency of *Chondrostereum purpureum* as biocontrol agent of sprouting in boreal forest.

Material and Methods

The field experiments were established in southern Finland at Ruovesi and Orivesi locating in surroundings of Hyytiälä Forestry Field Station. Three experiments were established in autumn 2003 (Fig. 1). The young trees were felled with brush cutter/clear cut saw in 10x10 m plots and the stumps were painted immediately with inoculum. In control plots the stumps were open for natural inoculation without treatments. Two experiments were located in spruce sapling stand and one experiment under an electric line. The age of felled trees was 6–10 years.

Fig. 1. Experimental design.

Three fungal strains were used; Biochon, Orivesi and 2.65. The Biochon is a commercial preparation from Netherlands, the Orivesi strain was isolated from a birch stump without sprouts, and the strain 2.65 originated from FFRI collections and has been isolated by Anna-Maija Hallaksela.

The appearing of sporophores in stumps was inventoried in October 2004. The sprouts were counted and measured in August 2005.

Results

Chondrostereum purpureum inoculations increased clearly the sporophore production in stumps (Fig. 2). In inoculated birch stumps the sporophore frequency varied between

27–43 % in August –October inoculations. In control stumps the sporophore frequency was 5 % (Table 1). Sporophores were found also in alder, rowan, aspen and willows.

Fig. 2. Sporophores of Chondrostereum purpureum *on birch stump. Birch stump was inoculated in May and the photo was taken in October. Photo: Henna Penttinen*

Table 1. The percent of birch stumps with sporophores one year after inoculation.

Inoculation time	Sporophores, %
August 2003	43 %
September 2003	34 %
October 2003	27 %

Table 2. Number of sprouts, number of dead sprouts and length of the longest sprout in three experimental sites. Treatments; inoculation and control.

Plot	Sprouts/ stump	Dead sprouts	Length of the longest sprout, cm
Ruovesi 1 inoc.	3.47	1.22	76
Ruovesi 1 control	4.19	0.75	90
Ruovesi 2 inoc.	1.5	0.4	83
Ruovesi 2 control	2.0	0.69	109
Orivesi inoc.	2.59	0.87	137
Orivesi control	2.63	0.69	130

The depth of fungus growth was not systematically measured. At least in the few cut stumps examined it seemed that the whole stump was decayed, but no isolations were made.

The inoculation in this experiment did not stop sprouting during the first two years (Table 2). It could have a mild effect, but not enough for commercial purposes. Some sprouts were dying during the second season, but dying sprouts were observed also in control plots.

Discussion

This experiment shows the possible light effect of biocontrol treatment with *C. purpureum* on sprouting, but probably a longer incubation time is needed to verify the now presented data. For developing of a more effective control method with *Chondrostereum purpureum*, there are still several possibilities. The sporophore frequency was not 100 % in this experiment, which raises suspicion that the used inoculation method was not the best one. At least the Biochon preparation was contaminated with bacteria. Anyway, Biochon produced sporophores clearly more than controls.

In this experiment the inoculations were made from August to October. It seemed that the production of sporophores was decreasing along with delayed inoculation time. So testing also other inoculation times could be important.

Three strains of *C. purpureum* were now used in this experiment. Pitt *et al.* (1999) concluded that the fungal isolate used could be an important source behind variation in treatment efficiency. The screening of a large number of isolates would seem necessary to find the most suitable fungal strains for biocontrol of sprouting. The process how *Chondrostereum purpureum* is stopping the sprouting is not known very well either and the roles of e.g. fungal enzymes and toxins should be examined.

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Vitality of Norway spruce fine roots in stands infected by *Heterobasidion annosum*

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Abstract

Normally, infection by *Heterobasidion annosum* does not affect the fine roots of Norway spruce. Thus, mycorrhizas may be found with rot-affected conifers. The objective of the given study was to compare the morphological indices and mycorrhization of fine roots for rot-infected and healthy Norway spruce trees. The root samples were collected on 14 plots. In 6 of the plots *H. annsoum* was established. The plots were either on mineral soils or peaty soils.

The major morphological indices of fine roots (such as root length, volume, number of root tips) were found to be substantially higher (α=0,05) for the plots with only healthy Norway spruce trees. Twisted, irregularly thickened mycorrhizas of bunch-like distribution were dominant for the plots with *H. annosum* infected Norway spruce trees.

Introduction

In Latvia, a considerable proportion of Norway spruce [*Picea abies* (L) Karsten] stands suffer from root rot. It has been found that in 60–130 year-old Norway spruce stands of the Dm *Hylocomiosa* and Vr *Oxalidosa* site type the proportion of stems with rot may exceed 80 % (Šica, Huhna, unpublished data). Mycorrhiza (symbiotic association between roots and fungi) is known to enhance the vitality of woody plants, and also enhance their resistance to various diseases (Schönhar 1990). However, a number of researchers believe that rot-suffering conifers may also show healthy, well-developed mycorrhizas. The objective of the present study was to find out how *Heterobasidion annosum* (Fr.) Bres. s. lat. affects the root mycorrhization in Norway spruce and to compare the vitality and morphological indices of fine roots between healthy and *H. annosum* infected Norway spruce stands.

Material and methods

Sample plots

The experimental material was collected in the forest districts of Kandava, Mūsa, Smiltene, Cesvaine, and Madona, and also in the forests of the Forest Research Station (FRS) (Kalsnava and Škēde) as well as in the Trei Forest District of the Riga Forest Agency (Fig. 1).

Altogether 14 stands were now inventoried, of which 6 were characterized by the occurrence of root rot. The sites under study were arbitrarily divided into two groups: Norway spruce stands on mineral soils and spruce stands on peaty soils. The stands on mineral soils represented the following forest site types: As *Myrtillosa mel*. (6 sites); Dm *Hylocomiosa* (4 sites); Kp *Oxalidosa* turf. Mel. (4 sites). The age of the Norway spruce stands studied was 44–96 years.

Fig. 1. Location of sample plots. Healthy stands (sircles), and H. annosum *infected stands (squares).*

Field work

In stands with rot the presence of infection was determined following the availability of macroscopic traits: fungal fruit bodies; rotten stems fallen down; thinning of tree crowns, etc. In clear-cut areas, the presence of rot was determined by inspecting the stumps for patches of rotten wood.

On each sample plot some 10–20 samples of wood containing rot-causing agents were collected by using a sterile Pressler's borer with the sample taken at the height of root collar. The samples were placed in sterile test tubes and taken to the laboratory for storage in refrigerator until further processing. In stands with rot samples of fruit bodies of *H. annosum* were also collected and taken to the laboratory and kept in paper envelopes at the room temperature.

To describe soil horizons and to collect soil samples for chemical analyses a trench revealing the soil profile was dug on each sample site. The chemical analyses were done at the Soil Laboratory of the Latvian Forest Research Institute «Silava». Larger soil samples (20×10×10 cm) were also taken to obtain the material for identifying the dominant mycorrhiza types (Agerer 1987–1991). The root samples were collected next to spruce stems, using a four millimetre high and 100-cm^3-sized metallic cylinder. On each sample plot 25 root samples were taken. The samples around 3–4 stems were taken at random from the topsoil layer within the tree crown projection. For identifying the mycorrhiza species the root samples were fixed in ethyl alcohol.

Laboratory work

At the laboratory the root samples were carefully rinsed. The typological structure of mycorrhiza (mainly the colour) and the vitality (using 5 vitality classes) were studied by using the Leica MZ-7.5 microscope (magnification 6.5–50×). Then the root samples were scanned by calibrated scanner STD-1600+, using the software Win RHIZO 2002 C (Regent instrument^{R}). Scanning was done with the resolution ability 500 dpi [Standard 8 bit; grey tones (256)]. Fourteen classes were introduced for comparing the root diameter: 0–0.1 mm; 0.1–0.2 mm; 0.2–0.3 mm; 0.3–0.4 mm; 0.4–0.5 mm; 0.5–0.6 mm; 0.6–0.8 mm; 0.8–1.0 mm; 1.0–1.2 mm; 1.2–1.6 mm; 1.6–1.8 mm; 1.8–2.2 mm; 2.2–2.6 mm; and >2.6 mm. Win RHIZO 2002 C was employed for the mathematical processing of scanned images. For further processing the data were transferred to the MS Excel, using XL RHIZO V2003a; t-criterion and analysis of variance were used for data treatment.

Five vitality classes were used to describe root vitality:

I Mycorrhizas well developed and show typical ramification; the root bark is sound.
II Mycorrhizas slightly damaged; mycorrhiza frequency is lower.
III Damaged mycorrhizas found; twisted mycorrhizas having mantle of no uniform thickness predominate.
IV Mycorrhizas heavily damaged; living mycorrhizas rare.
V Fine roots heavily damaged; no living mycorrhizas are found.

Results and discussion

Assessment of root morphological indices

The mean length of roots of healthy spruce trees growing on mineral soils was 238.5±12.8 cm, while for trees in rot-infected stands this length was111.7+7.5 cm. (Table 1). According to the analysis of variance these differences were significant (Table 2).

Table 1. Mean values of the root parameters examined in Norway spruce stands.

Root length, cm	Root volume, cm^3	Number of root tips	Root weight, g
Healthy trees on mineral soils			
238.5±12.8	0.55±0.03	1392±84	0.21±0.11
Trees with rot on mineral soils			
111.7±7.5	0.33±0.02	685±52	0.12±0.009
Healthy trees on peaty soils			
228.0±15.6	0.43±0.03	1331±108	0.16±0.01
Trees with rot on peaty soils			
87.4±20.5	0.12±0.03	536±134	0.05±0.01

Table 2. Analysis of variance: the impact of the *H. annosum* infection on root lenght

Variance	Sum of deviation squares	Degrees of freedom	Mean square	F	P
Factor	1064268.4	1	16458	64.66	< 0.0001
Residual	4377776.4	266			
Total	5442044.8	267			

The impact of the factor is described by η=l9.6 %. Thus, a considerable proportion of the factor under analysis, i. e. the differences in root length for healthy and rot-infected stands, remains unexplained. These differences may be attributed to soil heterogeneity, i. e. the impact of diverse biotic and abiotic factors on root development. The root volume and root weight, too, showed higher values for healthy spruces, and these differences were highly significant (P<0.0001). The number of root tips, which to a great extent characterizes the total number of mycorrhizas, is a significant indicator for the vitality of fine roots. In healthy trees (n=149) the average number of root tips was 1392+84, while 685±52 root tips were scored in diseased trees (n=l19).

When examining root length in the different root diameter classes (Fig. 2), it was found that for the diameter classes in the range 0.10–0.20 mm -0.30–0.40 mm, which represent typical mean diameters for mycorrhizal roots, the differences in root length between healthy and diseased trees were significant (P<0001).

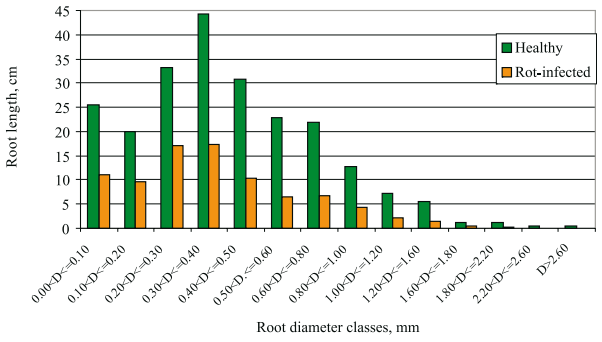

Fig. 2. Distribution of roots into diameter classes (samples from mineral soils).

For the samples originating from peaty soils, too, indices such as the mean root length, root volume, the number of root tips, and the root weight were significantly higher for healthy than for diseased trees. For healthy trees the number of root tips was 1331±108, while in diseased trees 536+134 were scored on average (P=0.001). Also for the other parameters significantly higher values were obtained in healthy trees than in diseased trees (P < 0.0001).

When comparing the distribution of root length within different root diameter classes for peaty soils (Fig. 3), it

was found that, similarly as in mineral soils, the root length up to the diameter class 1.80–2.20 mm was significantly higher for the samples coming from healthy stands than for diseased stands.

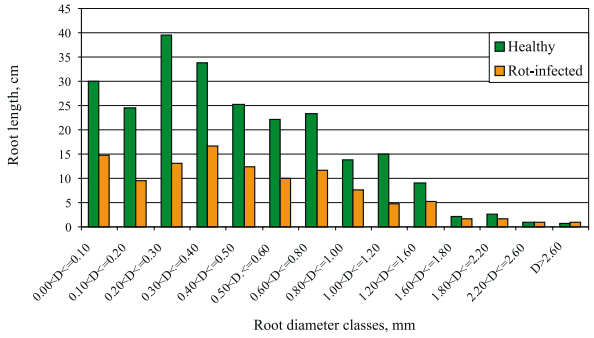

Fig. 3. Distribution of roots into diameter classes (samples from peaty soils).

Comparison of mycorrhiza typological structure and vitality between H. annosum *infected and healthy spruce stands*

Root vitality and the frequency of mycorrhiza types were compared for the samples analysed (Table 3). The mycorrhiza vitality for diseased trees in mineral soils was described by the coefficient 3.2, with this indicator for healthy trees being 2.9 (a lower value of the coefficient points to a higher percent of roots of higher vitality classes). For healthy and diseased stands on mineral soils it was difficult to identify the dominant mycorrhiza types. On Sample Plot 6 with diseased trees light-coloured mycorrhizas (*Piceirhiza* sp.) were found in 50 % of the samples. However, it is probably due to the presence of grey alder and other deciduous trees in the stand.

Table 3. Mycorrhiza frequency (%) and vitality for the root samples analysed (average of 25 samples)

Sample plots	Mycorrhiza type								
	Light-coloured	**Dark**	**Light yellow**	***C.geophilum***	**With external hyphae**	***A.byssoides***	***P.involutus***	***Piceirhiza sp.***	**Vitality**
Healthy trees on mineral soils									
1	12.5	50	8	50	46	46	-	-	2.6
2	76	12	4	-	20	12	-	-	3.1
3	32	-	-	8	48	36	4	4	3.0
4	38	11.5	11.5	58	-	15	8	8	3.0
5	64	16	-	92	64	-	32	32	3.0
Diseased on mineral soils									
6	21	12.5	50	21	42	33	-	-	2.9
7	4	39	39	4	4	4	-	-	3.0
8	54	8	12.5	7.5	-	25	8	21	3.0
9	58	4	-	71	-	5	-	17	3.6
10	20	20	4	-	-	28	-	8	3.6
Healthy trees on peaty soils									
11	16	80	16	40	-	64	-	-	2.3
12	72	-	16	3.0	16	-	12	36	2.9
13	8	-	-	-	-	27	23	19	3.0
Diseased trees on peaty soils									
14	5	11.5	-	-	-	5	21	21	3.3

When comparing soils with a higher proportion of mineral fraction (sample plots 1, 4, 5 compared with sample plots 6, 7, 8) more *Cenococcum geophilum* Fr. was found on the roots of healthy spruce trees than on diseased ones. For healthy trees the mycorrhizal fungus *Paxillus involutus* (Batsch.) Fr. was found in 3 out of 5 sample plots, while for diseased trees on one plot only out of 5 plots. As already mentioned, for diseased trees on peaty soils the material is insufficient for assessing differences between diseased and healthy trees.

Mycorrhiza ramification and morphological traits are also essential for characterising the mycorrhiza vitality. Mycorrhizas showing external hyphae and rhizomorphs were quite often associated with healthy spruce. The mycorrhizal fungi *Amphinema byssoides* (Pers.) J. Erikss., *Piceirhiza* sp., *Cortinarius* sp. and *Piloderma* sp. were also found quite frequently. Clusters of dark (predominantly *Piceirhiza* sp.) and light-brown mycorrhizas were also encountered.

Mycorrhiza ramification and distribution are regarded as typical for the respective species. The mycorrhiza on the roots of diseased spruce showed bunch-like projections and also a lot of damaged mycorrhizas, protruded, twisted and atypically swelled. Meyer (1985) also points out that in *H. annosum* infected spruce trees, the mycorrhizal mantle is poorly developed. There were also lots of heavily damaged roots, which pertain to vitality class 4. On sample site 6 the fine roots were heavily damaged (vitality class 3–4). However, on sample plots 7 and 8, where there is a mixture of grey alder, a good deal of vital mycorrhizal clusters was found. This suggests that the deciduous have a positive effect on the development of mycorrhiza in spruce. The literature, too, suggests that a mixture of deciduous species suppresses the root pathogen in spruce (Piri *et al.* 1990). Yet, it must be pointed out that there are also opposite opinions regarding the role of deciduous in suppressing the spread of *H. annosum* (Werner 1973).

On the sample plots of peaty soils, diseased spruce trees were found in one case only. Also the literature sources indicate that *H. annosum* infection is less common in peaty soils than in mineral soils (Redfern 1997). This is explained by soil acidity. It has been found that on mineral soil plot with healthy spruce trees the soil pH at the depth of 5 cm is 3.6 with the same index on diseased plots being 4.6. At the depth of 20 cm the same indices are 3.9 and 4.8, respectively. No differences in soil acidity have been found for the depth of 40 cm.

In future there is a need to analyse also other factors, which affect the development of mycorrhiza.

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Genetic linkage of growth rate and intersterility genes in *Heterobasidion* s.l.

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A genetic linkage map of the basidiomycete *Heterobasidion annosum* (Fr.) Bref. s. lat. was constructed from a compatible mating between isolates from the North American S and P intersterility groups. In a population consisting of 102 progeny isolates, 358 AFLP (amplified fragment length polymorphism) markers were scored. The linkage analysis generated 19 large linkage groups covering 1468 cM and several smaller. Segregation of three intersterility genes were analysed through mating tests with tester strains. The loci for the two intersterility genes S and P were successfully located in the map. Quantitative trait loci (QTL) for mycelial growth rate were identified and positioned on the genetic linkage map. The mycelial growth rate among 84 progeny isolates were analysed in two different temperature regimes 12 and 24×C on malt extract agar plats. The assay identified three QTL positioned on linkage groups 1, 17 and 19 with peak LOD values of 3.18, 2.93 and 4.80 at low temperature. At high temperature corresponding QTL on the same linkage groups, with peak LOD values of 1.34, 2.76 and 2.19, were identified. The QTL for the low temperature regime explained 20.9 %, 18.1 % and 24.0 % of the variation in mycelial growth rate, respectively. The broad-sense heritability was estimated to 0.97 and 0.95 for growth rate at low and high temperature respectively. Two of the QTL for mycelial growth rate are tightly linked to the intersterility genes S and P, which control mating between closely related species and intersterility groups of *H. annosum* s.l.. Localisation of intersterility genes and QTL for mycelial growth rate form the basis for map based cloning and identification of the corresponding genes.

Diversity of viruses inhabiting *Gremmeniella abietina* in Finland

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Gremmeniella abietina (Lagerb.) Morelet is the causative agent of *Scleroderris* canker of conifers. We have observed that isolates of this fungus host viruses belonging to four different families. Mitoviruses and Totiviruses occur in both types A and B of *G. abietina*, but the related viruses hosted by the two types are genetically distant. Partitiviruses have been observed only in type A and endornaviruses in type B. A single isolate of *G. abietina* type A was shown to host viruses of three different families: Totiviridae, Partitiviridae and Mitoviridae. There was some fluctuation in the relative frequencies of the three viruses in single sites during two successive years (2003 and 2004).

Effects of winter hardening and winter temperature shifts on *Pinus sylvestris-Gremmeniella abietina* plant-pathogen interactions

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The pathogenic ascomycete *Gremmeniella abietina* (Lagerb.) Morelet causes shoot dieback in several genera of conifers, in Sweden mainly on *Pinus* species. The fungus is favoured by cold, wet summers and mild winters. *Gremmeniella abietina* infects the top shoots of its host in summer, and stays as a latent infection until winter, when it starts to grow in the inner bark and into the wood. It has been shown that *G. abietina* needs at least 44 conducive days of mild winter weather with temperatures near zero ºC in order to be able to break latency.

Two experiments were conducted. In the first experiment 750 two-year-old *Pinus sylvestris* L. seedlings were pre-treated in three separate regimes (two winter-hardening regimes and one constant regime resembling Swedish autumn conditions) and subsequently inoculated with *G. abietina* mycelia in order to examine the relationship between the process of winter-hardening in the host and the growth of *G. abietina* within the host tissue during autumn and winter.

Seedlings winter-hardened outdoors showed a significantly higher degree of disease incidence than seedlings winter-hardened in a phytotron climate chamber. Instead the latter showed about the same disease incidence as the seedlings pre-treated in the constant regime. However, all the plants that had visible necroses, showed the same disease severity, regardless of which pre-treatment they had been subjected to. This implies that the winter-hardening process itself doesn't predispose the host tree for *G. abietina* infection. Nor does it lead to severer infections. Instead, weather data indicated that the host may become prone to infection either when subjected to sudden large temperature shifts during winter or when its dormancy.

The second experiment looked at the effect of large temperature shifts during winter on the growth of *G. abietina* within the host tissue. Two-year-old seedlings of *P. sylvestris* were winter- and cold hardened in the phytotron and subsequently subjected to large temperature shifts, where after they were inoculated with *G. abietina* mycelia. Preliminary data analysis suggests that the effect of temperature shifts is minor. If anything, this kind of temperature stress may actually strengthen the host's ability to hamper the growth of *G. abietina* in the inner bark.

Gremmeniella infection on pine seedlings planted after felling of severely *Gremmeniella* infected forest

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During 1999 and 2001 the most severe *Gremmeniella* epidemic ever appeared in Sweden. Big forest areas needed to be clear cut in advance followed by replanting. In this investigation we wanted to find out to what extent newly planted seedlings became infected and also if remaining twigs and branches support new infections. Seedlings were planted on clear cut areas felled in 2001 in the most affected areas of Sweden. They were planted in 2002, 2003 and 2004 and infection was controlled the year after planting.

Seedling planted in 2002, the year after felling, were infected between 50 to 90 % the following year showing that it is unsuitable to replant already the year after felling due to sever *Gremmeniella* infections. The infection decreased for seedling planted two and three years after felling but at this time there was a big variation between different areas. The infection was not influenced very much if twigs and branches were left on the clear cut areas. Seedling planted in the adjacent diseased forest became much more infected than seedlings planted on the clear cut areas. The different result will be discussed.

Susceptibility of Scots pine provenances to shoot diseases

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Nine Scots pine (*Pinus sylvestris* L) provenances, two from Estonia and seven from Finland represent an area of about 1200 kilometers in south-north latitude. The seeds were collected from natural stands and were seeded and planted to three growing sites at the beginning of 1991. The conditions between the sites differed most in temperature and in the length of growing season and on the day-length in growing season of cource.

In the northernmost site, in Rovaniemi Hietaperä all the seedlings of southernmost provenance from Estonia, Saaremaa, died. There were a lot of injuries caused by *Gremmeniella abietina* (Lagerb.) Morelet (*Scleroderris* canker) in the provenances which originated south from growing site. Only the three northernmost provenances, from Muonio Ylitornio and Suomussalmi could succeed rather well without injuries caused by *Scleroderris* canker or frost.

Generally all the Scots pine provenances succeeded best in Suonenjoki, which locates almost in the middle of the south-north latitude of the origins used in the trials. There were injuries caused by autumn frost only in two southernmost provenances.

Pine weevil, *Hylobius abietis* L., caused most damages in the southernmost site, Estonia, Konguta. The northernmost provenances, especially from Muonio and Suomussalmi did not suucced because they were unadaptable to a long and warm growing period. In every growing site those provenances, which origin were closest to the growing site, succeeded and grew best.

Recent disease problems in Swedish forests

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Conspicuous damages on ash and juniper are appearing and so far for unclear reasons. Also conspicuous resin top disease on Scots pine *(Pinus sylvestris*), in the very north of Sweden.

Ash shoot dieback was noticed at least since 2002. Weather conditions seem to have been conducive for the development of damages. Since 2004 it is occurring all over the area of natural distribution of ash, *Fraxinus excelsior*. Trees of all ages are affected and the shoot dieback in many cases leads to death of trees. Shoots seems to be killed during the winter season, but also new shoots die during the summer. The cause is not yet identified. The extent of the problem is much greater than seen earlier. Problems with ash are also reported from Lithuania and Poland.

For the second year junipers, *Juniperus communis*, show more damages than normal. Many junipers are killed and different types of symptoms are occurring. Attacks caused by *Stigmina juniperina* on needles and *Gymnosporangium cornutum* on shoots are frequent, but weather damage is also common probably frost damage.

In Norrbotten resin top rust disease caused by *Cronartium flaccidum* has struck unusually hard in Scots pine, stands 5–30 years old. More than 50 % of the trees are attacked in some stands and many young trees are already killed.

QTL mapping of pathogenicity in *Heterobasidion annosum sensu lato*

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The basidiomycete *Heterobasidion annosum* (Fr.) Bref. *s. lat.* is the most devastating fungal pathogen on conifers in the world. Its intersterility groups S and P are named after host preference (spruce and pine). Using a mapping population of 102 single spore isolates, originating from a compatible mating between North American isolates of the P and S groups, a genetic linkage map of the *H. annosum* genome was constructed. The map consists of 39 linkage groups and spans 2252 cM in total. The average distance between two markers is 6.0 cM.

To map QTLs for pathogenicity to methods were used to estimate pathogenicity. First, 29 two weeks old *Pinus silvestris* L. seedlings were grown in homogenized mycelia for 25 days. Every third day the number of dead seedlings were estimated. The virulence was determined as the regression value of the disease increase rate for each isolate. The data suggested a QTL on linkage group 11 with a LOD of 3.09, explaining 16.4 % of the variation in virulence.

Second, for each fungal isolate ten plants of one year old *P. silvestris* was infected with a fungal infested wooden plug in a wound in the cambium. After four weeks the necrosis was measured upstem and downstem from the cambial wound. The virulence was determined as mean necrosis length for each isolate. The data suggested two QTLs, one on linkage groups 15 and one on group 20, with peak LOD values of 3.29 and 4.24, explaining 15.8 % and 18.2 % of the variation in virulence, respectively.

Using map based cloning these QTLs will be identified and characterised in future studies.

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Gene expression during the switch from saprotrophic to pathogenic phases of growth in the root and butt rot fungus *Heterobasidion annosum*

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The tree pathogen *Heterobasidion annosum* (Fr.) Bref. *s. lat.* can prevail in dead roots and spread from dead tissue to living trees. We therefore examined whether a shift in gene expression occurs during the switch from saprotrophic to pathogenic growth. We used a macro-array differential gene analysis to identify genes that are either induced or suppressed during either stages of growth of the fungus. Macro-arrays containing a selected number of clones from cDNA library of *H. annosum* s. s. and *H. parviporum* Niemelä & Korhonen representing a functionally diverse range of genes were investigated. Dead pine seedlings were inoculated with *H. annosum* and transferred to water agar plates containing living pine seedlings, the hyphae were then sampled from various stages of interaction before and after contact with the pine host. Total RNA will be isolated, reverse transcribed into cDNA to be used as probes for differential screening of the macro-array membranes. Signal intensity values for differentially expressed genes will be documented with Quantity one (Bio-RAD) and the data will be statistically analysed to identify significantly up and down-regulated genes.

Progressive patterns of distribution of the genets of *Heterobasidion parviporum* in a Norway spruce stand

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The study was carried out in a Norway spruce [*Picea abies* (L.) Karsten] stand in the Ruotsinkylä Research Area, 30 km north of Helsinki. The site has previously been covered by Norway spruce forest affected by *Heterobasidion* root rot. The present spruce stand was established naturally under the spruce overstory. The overstory trees were removed in 1951. In 1952, the site was supplementary planted with alders [*Alnus glutinosa* (L.) Gaertner and *A. incana* (L.) Moench]. In the spring of 1993, shortly after the first thinning carried out in the winter 1992, all the standing trees, thinning stumps and old stumps of the previous tree generation on the study plot (20 x 50 m in size) were mapped and sampled for *Heterobasidion*. At that time the approximately age of the spruces was 43 years. In 2005, 13 years after the first thinning, the trees on the study plot were resampled again in order to obtain detailed information about the persistence and spatial distribution of *Heterobasidion* genets on the site over a period of several decades. In 1993, seven *Heterobasidion* genets were isolated from old stumps of the previous tree generation. These old genets had infected 15 spruces of the subsequent tree stand (i.e. 83.3 % of all infected spruces). In 2005, 13 years after the first thinning, three of the seven old genets had died out. No new genets were established after the first thinning. However, five new trees of the residual stand were infected by the old genets, most likely from the thinning stumps of infected trees. At the age of 56 years, 16.1 % of the spruces of the present stand generation were infected by *Heterobasidion*. Half of them were infected before thinning and half after thinning. As a result of thinning the mean size of the *Heterobasidion* genets had decreased from 3.0 (1993) to 1.7 trees (2005). All the *Heterobasidion* genets isolated on the study plot proved to be *H. parviporum* Niemelä & Korhonen.

Agrobacterium mediated gfp-tagging of *Heterobasidion annosum*

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The green fluorescent protein (GFP) is a powerful tool that can be used in microscopy when studying interspecific hyphal interactions and in functional studies of candidate genes. In our study (Samils *et al.* 2006) we developed a transformation system based on co-cultivation of *Agrobacterium tumefaciens* and germinating spores of a homokaryotic North American P isolate of the root-rot pathogen *Heterobasidion annosum* (Fr.) Bref. We used two different constructs with the *A. tumefaciens*, the first construct is pJF4–5 where gfp is controlled by an *Aspergillus* gpd promoter, and the second is pCD61 where the gfp gene is controlled by an ubiquitin promoter. In both constructs a hygromycin resistance gene, used as a selectable marker is controlled by the trp C promoter. *A. tumefaciens* transfers parts of its Ti-plasmid, the T-DNA into the DNA of the recipient. This occurs naturally in wounds of dicotyledonous plants that are being infected by the *A. tumefaciens*. The virulence genes of the bacteria are induced by compounds like acetosyringone that are excreted by the target plant. Therefore this compound is added to the fungal transformation process to mimic the bacterial-plant infection. To verify a successful transformation, studies in UV-microscopy and PCR-reactions were performed.

We recovered 120 hygromycin resistant colonies from two individual transformation experiments performed at pH 5.6 and 20 °C. Stable GFP fluorescence was detected in seven sub isolates transformed with pJF4–5 and 11 sub isolates transformed with the pCD61. All but one sub isolate grow well and produced conidia on media with or without hygromycin. These 18 sub isolates proved to be mitotically stable and expressing GFP activity after 18 months post transformation. Further molecular analyzes are underway.

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