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Effects of growth and virulence of associated blue-stain fungi on host colonization behaviour of the pine shoot beetles *Tomicus minor* and *T. piniperda*

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The pine shoot beetles *Tomicus minor* and *T. piniperda* are common in the Nordic countries. Of these, *T. piniperda* may attack and kill living but severely stressed trees, whereas *T. minor* has never been reported to be individually responsible for killing live trees. Both species are associated with blue-stain fungi: *T. minor* with *Ophiostoma canum* and *T. piniperda* with *Leptographium wingfieldii and Ophiostoma minus*. The growth of these fungi was studied in phloem and sapwood of live Scots pine trees, on malt agar, and on malt agar under oxygen-deficient conditions. *Leptographium wingfieldii* was more virulent (i.e. caused more extensive host symptoms) grew more quickly on malt agar, and was less affected by oxygen-deficient growth conditions than either *O. minus* or *O. canum*. *Ophiostoma canum* was least virulent. In low-density inoculations it induced lesions similar to those induced by sterile control inoculations; it grew very slowly on malt agar and stopped growing after ≈30 mm under oxygen-deficient conditions. *Ophiostoma minus* was intermediate in all respects. The different virulence of the blue-stain fungi associated with the two pine shoot beetles may explain the lower level of aggressiveness in *T. minor*.

Keywords: blue-stain fungi, inoculations, *Leptographium*, *Ophiostoma*, Scots pine, *Tomicus*

Introduction

Most bark beetles breeding in conifers carry mutualistic fungi in mycangia and/or blue-stain fungi in hollows on the body surface (Francke-Grosmann, 1967; Graham, 1967; Levieux *et al*., 1989; Furniss *et al*., 1990). The role of the associated blue-stain fungi has been discussed since Craighead (1928) suggested that they play an important role in the rapid desiccation of living trees attacked by aggressive, tree-killing bark beetles. Krokene & Solheim (1998) suggested that blue-stain fungi associated with aggressive bark beetles are more virulent (i.e. able to cause more severe symptoms in host trees) than those associated with nonaggressive bark beetles. Although many fungi may be associated with aggressive bark beetles, the majority of these are avirulent, with normally only one of these fungal species able to kill trees.

Several fungi are associated with the Eurasian spruce bark beetle *Ips typographus* (Solheim, 1986; Furniss *et al*., 1990). Of these, *Ceratocystis polonica* is the primary invader of attacked trees (Solheim, 1992a; Solheim, 1992b), and is able to kill healthy trees in experimental mass inoculations (Horntvedt *et al*., 1983; Krokene & Solheim, 1998). Based on growth studies of the blue-stain fungi associated with *I. typographus*, Solheim (1991) suggested that rapid growth and the ability to grow under oxygen-deficient conditions are important characteristics for primary sapwood invaders. Studies of other bark beetle/blue–stain fungi associations seem to support this hypothesis (Solheim, 1995; Solheim & Krokene, 1998a; Solheim & Krokene, 1998b).

Scots pine (*Pinus sylvestris*) is attacked by two species of pine shoot beetles in the Nordic countries, *Tomicus minor* and *T. piniperda*. Their shoot-feeding behaviour causes substantial growth losses and is the reason for the economic importance of these beetles (Långström, 1983). Both species breed in Scots pine logs, but *T. piniperda* also may attack and kill severely stressed live trees (Långström & Hellqvist, 1993; Annila *et al*., 1999 and references therein). *Tomicus minor* has been observed to kill trees together with *T. piniperda* (Lagerberg, 1911), but Långström & Hellqvist (1993) concluded that it is usually a secondary colonizer following *T. piniperda*.

Tomicus minor is associated with the blue-stain fungus *Ophiostoma canum* and the ambrosia fungus

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Ambrosiella tingens, which serves as food for the developing larvae (Francke-Grosmann, 1952). The fungal spores are carried in primitive sacs in median suture and lateral folds of the elytra (Francke-Grosmann, 1956). The association between *T. piniperda* and blue-stain fungi is looser, but two species, *Leptographium wingfieldii and Ophiostoma minus* are found associated with this species in varying frequencies (Lieutier *et al.*, 1989; Solheim & Långström, 1991; Gibbs & Inman, 1991). Both fungi are able to kill healthy Scots pine trees in experimental mass inoculations (Långström *et al.*, 1993; Solheim *et al.*, 1993).

This paper describes the growth characteristics of the fungal associates of *T. minor* and *T. piniperda* on malt agar, on malt agar under oxygen-deficient conditions, and in phloem and sapwood of live Scots pine trees.

Materials and methods

Two isolates of each fungus were used for growth tests in the laboratory. The *Leptographium wingfieldii* isolates NISK 88-369/11 and NISK 89-2071/2, and the *Ophiostoma minus* isolates NISK 88-369/20 and NISK 89-2071/1, were all isolated from Scots pine trees infested by *Tomicus piniperda* at Jädraås (89-) or Norrsundet (88-), Sweden (Solheim & Långström, 1991). *Ophiostoma canum* isolates were NISK 1652/2, an old isolate given to NISK by A. Käärik, and NISK 97-33/47, a fresh isolate from Scots pine logs infested by *Tomicus minor* at Hökensås, Sweden. The last-mentioned isolate of each species was used for inoculations in trees. Numbers refer to isolates in the culture collection at the Norwegian Forest Research Institute.

Inoculation of Scots pine

Twenty-six Scots pine trees were selected from a single stand at Hökensås, south-west Sweden on 4 June 1998, and all inoculations were done on the same day. The stand had been severely defoliated by an extensive outbreak of the pine looper, *Bupalus piniaria* in 1996 (Långström *et al.*, 1999). Eighteen trees of similar size (diameter at 1·3 m height: 77 ± 3 mm, mean ± SD) were mass-inoculated with *O. canum*, *O. minus* or *L. wingfieldii* (six trees with each fungus) at a density of 800 inoculations m^{-2} on a 0·6 m band between 1·0 and 1·6 m stem height. Eight slightly larger trees (diameter 90 ± 1 mm) were inoculated three times with each fungus or with sterile agar. The 12 inoculations per tree were evenly distributed within three rings encircling the trunk at ≈1, 1·5 and 2 m height, with each type of inoculum occurring once in each ring. All inoculations were made by removing a bark plug using a 5 mm cork borer, inserting inoculum in the wound, and replacing the bark plug. Inoculum consisted of actively growing mycelium on malt agar (2% Difco malt, 1·5% agar) or malt agar only as a control.

The low-density inoculated trees were harvested on 18–19 August, 11 weeks after inoculation. The outer bark was removed around each inoculation point, and the extent of necrotic phloem was measured above and below the inoculation point. A thin disk (5 mm) was cut at each inoculation ring, and the depth of occluded (desiccated) sapwood beneath each inoculation point was measured. The mass-inoculated trees were harvested on 11 November 1998. The vertical extent of the four uppermost and four lowermost phloem necroses was measured on each tree. Because necroses tended to coalesce within mass-inoculated sections in susceptible trees, their lengths were measured upwards from upper inoculation points, and downwards from lower inoculation points. A thin disk (5 mm) was cut midway through the inoculated stem section, and the areas of heartwood, fresh (water-soaked) and blue-stained sapwood on the upper surface of the disks were outlined, based on translucency. The surface areas were later determined with a computer-connected planimeter (Krokene & Solheim, 1998). The amount of dead and live cambium was measured along the disk circumference. Re-isolations of fungus were made from three inoculation points per mass-inoculated tree, and from all inoculations in the low-density inoculated trees.

Tolerance to oxygen deficiency

The ability of the fungi to grow under oxygen-deficient conditions was tested in modified test tubes (Scheffer, 1935), after a method described by Scheffer (1967). The tubes were so-called dam tubes, which have an invagination on one side ≈10 mm from the opening and ≈140 mm from the end of the tube. The tubes were filled with ≈15 mL malt agar inside the dam, and inoculated with fungi. After 24 h the tubes were filled with nitrogen gas (99·9995 purity; Hydro Gas Norge, Rjukan, Norway), and sealed with airtight rubber stoppers. Control tubes were plugged with plastic caps and sealed with parafilm that allowed aeration. The tubes were incubated in darkness at 21°C, and linear growth was measured every 3 or 4 days until growth stopped or until mycelium reached the end of the tube. Four to eight replicates of each treatment and isolate were used.

Fungal growth at different temperatures

The growth rate of the different fungal isolates was tested on malt agar at different temperatures during a 2 week period. Actively growing mycelium from 1-week-old cultures was incubated on malt agar in Petri dishes (87 mm) in darkness at constant temperatures ranging from 3 to 40°C. Linear growth was measured every 3 or 4 days. Four replicates were used for each isolate.

Statistical analyses

Data were analysed with analysis of variance (ANOVA), using the general linear models (GLM) procedure on SAS

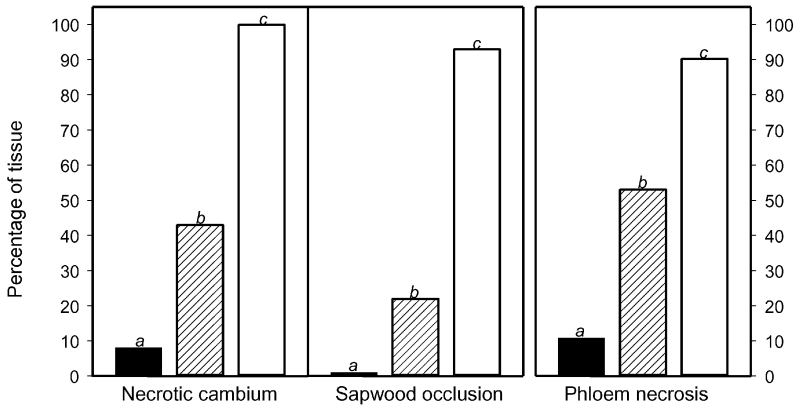

Figure 1 Percentage of necrotic cambium and blue-stained sapwood, and length of phloem necrosis in Scots pine trees 23 weeks after mass-inoculation with *Ophiostoma canum* (black bars); *Ophiostoma minus* (hatched bars); and *Leptographium wingfieldii* (white bars). Bars with different letters were significantly different by the LSD test ($P = 0{\cdot}05$) following ANOVA.

(SAS Institute, 1987). Percentage data were arcsin-transformed, and other data were log-transformed before ANOVA to correct for unequal variances and departures from normality (Montgomery, 1991). Where significant treatment effects ($P < 0{\cdot}05$) were detected with ANOVA, means were separated using the least significant difference (LSD) test at $P < 0{\cdot}05$. Student's *t*-tests were used to compare maximum growth under oxygen-deficient conditions for isolates within species.

Results

The three fungi induced significantly different symptoms, both in the mass-inoculated trees (necrotic cambium, $P < 0{\cdot}0001$; blue-stained sapwood, $P < 0{\cdot}0001$; lesion length, $P < 0{\cdot}0001$) and in the low-density inoculated trees (lesion length, $P = 0{\cdot}0001$; sapwood occlusion, $P = 0{\cdot}0001$). *Leptographium wingfieldii* caused more extensive symptoms in both inoculation experiments (Figs 1 and 2). *Ophiostoma minus* induced more extensive symptoms than *O. canum*. *Ophiostoma canum* caused lesions that were no longer than the control, but caused more occluded sapwood than the control (Fig. 2).

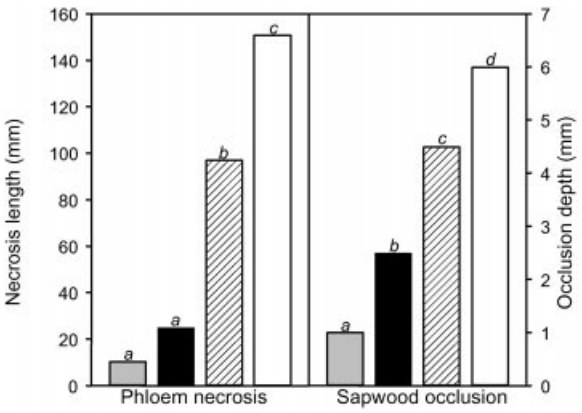

Figure 2 Length of phloem necrosis and depth of occluded sapwood in Scots pine trees 11 weeks after low-density inoculation with sterile agar (grey bars); *Ophiostoma canum* (black bars); *Ophiostoma minus* (hatched bars); and *Leptographium wingfieldii* (white bars). Bars with different letters were significantly different by the LSD test ($P = 0{\cdot}05$) following ANOVA.

Leptographium wingfieldii grew very rapidly on malt agar, with a maximum growth rate of ≈14·5 mm day^{-1} at 25°C for both isolates. The two isolates of *O. canum* grew slowly, with maximum growth rates of ≈3 and 2 mm day^{-1} at 18–21°C, whereas *O. minus* had an intermediate growth rate (maximum growth around 8·5 and 7 mm day^{-1} at 25–28°C for the two isolates) (Fig. 3).

Growth of *L. wingfieldii* was not arrested under oxygen-deficient conditions, except in one tube (isolate 88-369/11), where growth stopped after 94 mm. For isolate 88-369/11 there was no significant difference in growth under aerated and oxygen-deficient conditions [Fig. 4; not significant, two-way ANOVA (growth condition, day)], whereas the other isolate appeared to grow slightly better under oxygen-deficient conditions during the 10 day study period ($P = 0{\cdot}0001$, two-way ANOVA). Growth of the two *Ophiostoma* species was always arrested under oxygen-deficient conditions before the mycelium reached the end of the tube (Fig. 4). The two isolates of *O. minus* grew much further than the *O. canum* isolates before they stopped (≈100 versus 30 mm; $P = 0{\cdot}0001$, one-way ANOVA), and there was no significant difference in maximum growth between isolates within species (*t*-test). The *O. minus* isolates continued to grow for 17–24 days before growth was arrested, compared with 10–14 days for *O. canum*.

In the mass-inoculated trees all three fungi were re-isolated from all six trees. *Leptographium wingfieldii* was re-isolated from all inoculation points; *O. minus* from 94%; and *O. canum* from 44% of the inoculation points. In the low-density inoculated trees, *L. wingfieldii* was re-isolated from 92%; *O. minus* from 83%; and *O. canum* from 46% of the inoculation points.

Discussion

The three species of blue-stain fungi tested in this experiment varied greatly in virulence. *Leptographium wingfieldii* caused extensive symptoms in Scots pine. It produced long lesions in the low-density inoculations,

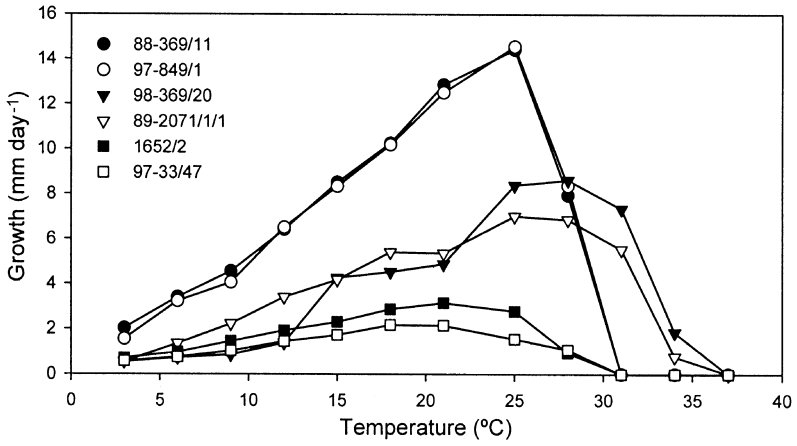

Figure 3 Mean linear growth on malt agar of *Leptographium wingfieldii* (○, ●); *Ophiostoma minus* (△, ▲); and *Ophiostoma canum* (□, ■) at various temperatures. Two isolates of each fungus were tested, and there were four replicates per isolate.

and nearly all the bark was killed and all the sapwood blue-stained after mass inoculations. *Ophiostoma minus* was also virulent, but less so, whereas *O. canum* was hardly virulent at all.

This study confirms the results of earlier inoculation studies with the blue-stain fungi associated with *T. piniperda*, showing that both fungi are virulent and able to kill mass-inoculated Scots pine trees in the absence of the insects, and that *L. wingfieldii* is more virulent than *O. minus* (Lieutier *et al*., 1989; Lieutier *et al*., 1990; Långström *et al*., 1993; Solheim *et al*., 1993). The blue-stain fungi associated with *T. piniperda* therefore may be important for the ability of this bark beetle to kill pine trees. *Tomicus piniperda*, however, is not an aggressive bark beetle in the Nordic countries, but has often been reported to kill severely stressed trees (Lagerberg, 1911; Jørgensen & Bejer-Petersen, 1951; Lekander, 1953; Räisänen *et al*., 1986; Långström & Hellqvist, 1993; Långström *et al*., 1999). In southern Europe *T. piniperda*, together with other bark beetles, has been reported to cause considerable mortality in different pine species (Masutti, 1969; Levieux *et al*., 1985; Piou & Lieutier, 1989; Ferreira & Ferreira, 1990), and in China *T. piniperda* appears to be more aggressive than in Europe (Ye, 1991; Li *et al*., 1993).

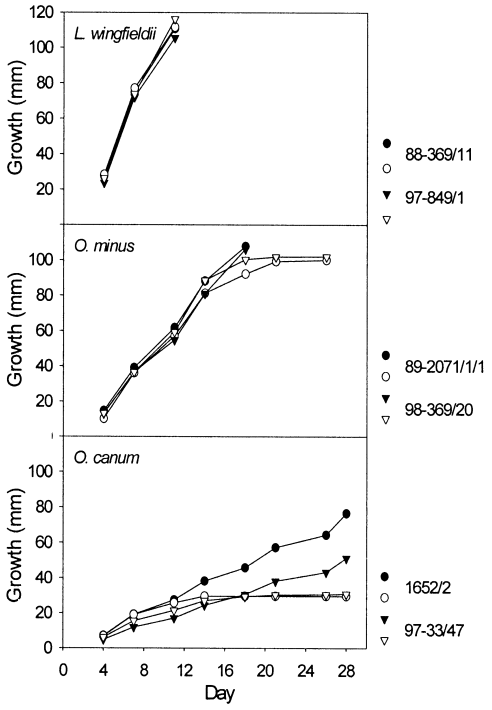

Figure 4 Mean linear growth of two isolates of *Leptographium wingfieldii*, *Ophiostoma minus* and *Ophiostoma canum* at 21°C under oxygen-deficient conditions in sealed test tubes filled with nitrogen (open symbols) and in aerated control tubes (filled symbols). There were four to nine replicates per isolate.

Ophiostoma canum, the major blue-stain fungus associated with *T. minor*, was much less virulent than *L. wingfieldii* or *O. minus*. In the low-density inoculations it did not cause significantly longer lesions than the control, although it caused deeper sapwood occlusion. The difference in the virulence of their fungal associates may be the most important explanation for the different tree-killing abilities of the two pine shoot beetles. This study thus supports the hypothesis that the virulence of associated blue-stain fungi is an important factor determining the aggressiveness of bark beetles (Krokene & Solheim, 1998). Our findings are also consistent with the conclusion by Långström & Hellqvist (1993) that *T. minor* is less aggressive than *T. piniperda*.

Solheim (1991) suggested that rapid growth and the ability to grow under oxygen-deficient conditions might be important for primary invaders of fresh host tissues. This appears to be true for the most important fungal associates of the Eurasian spruce bark beetle *Ips typographus* (Solheim, 1991); the mountain pine beetle *Dendroctonus ponderosae* (Solheim & Krokene, 1998a); the spruce beetle *Dendroctonus rufipennis* (Solheim, 1995); and the Douglas fir beetle *Dendroctonus pseudotsugae* (Solheim & Krokene, 1998b). The same also appears to be true for the fungal associates of the pine shoot beetles tested in this study. The most virulent fungus, *L. wingfieldii*, grew much more quickly than the other fungi, and was hardly affected at all by

the oxygen-deficient conditions in the test tubes. The weakly virulent O. *canum*, on the other hand, grew very slowly, and growth was arrested after only 30 mm under oxygen-deficient conditions.

The use of nitrogen-filled dam tubes is perhaps not the best way to study fungal growth under oxygen-deficient conditions. However, the small amount of air that was mixed with the nitrogen during the plugging process was probably similar for all replicates, and therefore did not appreciably affect the results. This is supported by the low variability between replicates in this study, as well as earlier studies in which this method has been used (Solheim, 1991, Solheim, 1995; Solheim & Krokene, 1998a, Solheim & Krokene, 1998b).

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