

Growth and virulence of mountain pine beetle associated blue-stain fungi, *Ophiostoma clavigerum* and *Ophiostoma montium*

Halvor Solheim and Paal Krokene

Abstract: The mountain pine beetle (*Dendroctonus ponderosae*) is commonly associated with the blue-stain fungi *Ophiostoma clavigerum* and *Ophiostoma montium*. *Ophiostoma clavigerum* is the primary invader of sapwood after beetle infestation and is thought to be the most virulent of the two fungi. Growth of these fungi was studied under oxygen-deficient conditions on malt agar in test tubes and Petri dishes. In addition, growth was studied in phloem and sapwood of young living shore pines (*Pinus contorta* var. *contorta*) and western white pines (*Pinus monticola*) inoculated with fungus in low densities (eight inoculations per tree). In test tubes with limited oxygen *O. clavigerum* grew for a longer time than *O. montium*. Both fungi are fast growing on malt agar (maximum growth 4.4–9.0 mm/day), but *O. clavigerum* grew better at temperatures below 25°C. The rapid growth and the ability to tolerate low oxygen levels may be important adaptations for *O. clavigerum* as the primary invader of fresh sapwood. However, although *O. clavigerum* grew better in the phloem of both tree species, there were no differences between the two fungi in their ability to colonize the sapwood of the inoculated trees.

Key words: blue-stain fungi, *Dendroctonus ponderosae*, growth rate, oxygen deficiency, virulence.

Résumé : Le dendroctone du pin de montagne (*Dendroctonus ponderosae*) est généralement associé à des champignons de la bleuissure, les *Ophiostoma clavigerum* et *Ophiostoma montium*. L'*O. clavigerum* est le premier colonisateur du bois d'aubier après l'infestation par les insectes, et on croit qu'il est le plus virulent des deux champignons. Les auteurs ont étudié la croissance de ces deux champignons sur malt gélosé, sous des conditions de déficience en oxygène, en tubes à essais ainsi que sur plaques de Pétri. De plus ils ont étudié la croissance dans le phloème et l'aubier de jeunes pins tordus (*Pinus contorta* var. *contorta*) et pins blancs de l'ouest vivants (*Pinus monticola*) inoculés avec le champignon à faible densité (huit inoculations par arbre). En tubes à essais et présence d'oxygène réduit, l'*O. clavigerum* pousse plus longtemps que l'*O. montium*. Les deux champignons poussent rapidement sur malt gélosé (croissance maximum de 4,4–9,0 mm/jour), mais l'*O. clavigerum* pousse mieux aux températures inférieures à 25°C. La croissance rapide et la capacité de tolérer de faibles teneurs en oxygène pourraient être des adaptations importantes de l'*O. clavigerum* comme envahisseur primaire de l'aubier frais. Cependant, bien que l'*O. clavigerum* croisse mieux dans le phloème des deux espèces, il n'y a pas de différences entre les deux champignons quant à leur capacité à coloniser l'aubier des arbres inoculés.

Mots clés : champignon de la bleuissure, *Dendroctonus ponderosae*, taux de croissance, déficience en oxygène, virulence.

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Introduction

The blue-stain fungi *Ophiostoma clavigerum* (Robinson-Jeffrey and Davids.) Harrington and *Ophiostoma montium* (Rumbold) von Arx are commonly associated with the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Robinson 1962; Whitney and Farris 1970; Solheim 1995a). This aggressive bark beetle is a major tree killer in northwestern North America, where millions of trees are killed during outbreaks (Raffa 1988). Lodgepole pine, *Pinus contorta* Douglas, and ponderosa pine, *Pinus ponderosa* Lawson, are the principal host species, but outbreaks may also occur in other pines, such as western white pine, *Pinus monticola* Douglas, and sugar pine, *Pinus lambertiana* Douglas (Raffa 1988).

Pathogenic blue-stain fungi associated with the mountain pine beetle assist the beetles in exhausting tree defences and killing trees (Reid et al. 1967; Owen et al. 1987). The relative importance of the beetles and the fungi in killing the trees is still unclear, but a few weeks after attack the fungi have colonized the rays and tracheids in the sapwood and disrupted water transport to the crown (Reid et al. 1967; Ballard et al. 1984). *Ophiostoma clavigerum* is the primary invader of sapwood after beetle infestation (Solheim 1995a), and is thought to be the most virulent of the fungi associated with the mountain pine beetle (Reid et al. 1967; Owen et al. 1987; Yamaoka et al. 1990, 1995). In experimental inoculation studies it has killed ponderosa pine seedlings (Owen et al. 1987) and mature lodgepole pines (Yamaoka et al. 1995). However, *O. montium* may also kill experimentally inoculated trees (Mathre 1964a, 1964b; Basham 1970; Strobel and Sugawara 1986).

Solheim (1991) has suggested that rapid growth and the ability to tolerate low oxygen levels are characteristic of blue-stain fungi that are early invaders of sapwood of beetle-infested trees. In this study we have measured the growth of

H. Solheim¹ and P. Krokene. Norwegian Forest Research Institute, Høgskoleveien 12, N-1432 Ås, Norway.

¹ Author to whom all correspondence should be addressed.
e-mail: halvor.solheim@nisk.no

O. clavigerum and *O. montium* in three different media: (i) malt agar under oxygen-deficient conditions, (ii) malt agar, and (iii) phloem and sapwood of live western white pine and shore pine (*Pinus contorta* var. *contorta* Douglas). Our objectives were to test whether tolerance to low oxygen levels and rapid growth can help explain the reported differences in fungal virulence and ability to colonize live sapwood, and to determine the relative virulence of *O. clavigerum* and *O. montium* in these two hosts of the mountain pine beetle.

Materials and methods

The fungi used in this study were isolated from living lodgepole pine trees infested with mountain pine beetles at Sunday Creek, near Princeton, British Columbia, Canada (Solheim 1995a). Two different isolates of each fungus were used in the inoculation study (*O. clavigerum*: isolates NISK 92-629/113/5 and NISK 92-629/121/5; *O. montium*: isolates NISK 92-628/49/1 and NISK 92-628/59/2). The *O. clavigerum* isolate NISK 92-629/113/5 was lost and replaced with isolate NISK 92-628/57/4 in the growth studies in the laboratory.

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 with an invagination on one side of the tube near the opening, ca. 140 mm from the bottom of the tube. The tubes were arranged horizontally, filled with ca. 15 mL malt agar (2% malt, 1.5% agar) inside the dam, and inoculated with fungi before they were filled with nitrogen gas (99.995% purity, Hydro Gas Norge, Rjukan, Norway) and sealed with air-tight rubber stoppers. Control tubes were plugged with cotton to allow aeration. The tubes were incubated in darkness at 21°C and linear growth was measured every 3–4 days until growth stopped, or until mycelium reached the end of the tube. There were eight replicates per isolate, except for isolate NISK 92-629/121/5 where $n = 4$ for the oxygen-deficient tubes and $n = 2$ for the aerated tubes, and isolate NISK 92-628/49/1 where $n = 5$ for the oxygen-deficient tubes.

Growth at different temperatures

The growth rate of the fungi was tested on malt agar at different temperatures during a 2-week period. Actively growing mycelium was inoculated on malt agar in Petri dishes (diameter 90 mm) and incubated in dark chambers at constant temperatures ranging from 3 to 40°C. Linear growth was recorded for 4–14 days. Six replicates per isolate were used.

Fungal inoculation

Twelve white pine trees (height 7.6 ± 1.1 m (\pm SD)) and 12 shore pine trees (height 11.0 ± 1.4 m) were inoculated with *O. clavigerum* and *O. montium* using a 5-mm cork borer (Wright 1933) on 12 May 1993 at Lens Creek, near Lake Cowichan, Vancouver Island, British Columbia, Canada. Trees were inoculated by removing a bark plug with the cork borer, inserting inoculum, and replacing the bark plug. Inoculum consisted of active mycelium growing on malt agar and sterile malt agar as a control. All trees were inoculated twice with each fungal isolate and twice with the control. The 10 inoculations per tree were evenly distributed in two rings encircling the trunk at 1 and 1.5 m above ground. For each fungus, one isolate was inoculated in the upper ring and the other in the lower ring.

For each tree species, four trees were felled 1, 3, and 5 weeks after inoculation to study tree responses and fungal growth into phloem and sapwood. Two 0.5-m stem sections containing the inoculations were removed from each tree, taken to the laboratory, and stored at 0°C until processed. The outer bark around each inoculation point was removed and maximum length of visible necrotic lesions was measured. The sapwood was split radially through the inoculation points

to measure depth of desiccated sapwood beneath each inoculation. Fungi were reisolated by removing small tissue samples (1–3 mm³) with a sterile scalpel from both phloem and sapwood. These samples were placed on malt agar in Petri dishes and examined for fungal growth over the next 2–3 weeks. One and 3 weeks after inoculation, phloem and sapwood around one inoculation site per isolate were sampled every 2.5 mm from the point of inoculation to determine the extent of hyphal growth relative to zones of necrotic phloem and desiccated sapwood. Phloem necrosis was sampled upwards from the point of inoculation.

Statistical analyses

Data from the growth studies in oxygen-deficient and aerated test tubes and on malt agar in Petri dishes were analyzed using ANOVA. Data from the fungal inoculation study were analyzed using a mixed-effects four-way ANOVA (fungus, duration (week), tree, isolate). The different isolates of each fungus were nested within the fungus treatment and individual trees were nested within week. Within fungus and tree species, weeks were compared using one-way ANOVA. Within week and tree species, fungi were compared using one-way ANOVA with fungal isolate nested within fungus. The following data were subjected to analysis: necrosis length, depth of desiccated sapwood, length of fungal growth into phloem and sapwood, and distance from front of fungal growth to edge of necrosis–occlusion. When necessary, data were transformed ($\log_{10}(y + 1)$) before ANOVA to correct for unequal variances and departures from normality (Montgomery 1991). Where significant treatment effects ($p < 0.05$) were detected with ANOVA, means were separated using the LSD test at $p < 0.05$. ANOVAs were performed with the general linear models (GLM) procedure on SAS (SAS Institute Inc. 1987).

Results

Growth of the two isolates of *O. clavigerum* and *O. montium* differed significantly under oxygen-deficient conditions (Fig. 1; $p < 0.02$, $F = 6.01$, and $p < 0.003$, $F = 9.57$, respectively, for each fungus) and on malt agar in Petri dishes (Fig. 2; $p < 0.00001$, $F = 65.60$, and $p < 0.00001$, $F = 447.95$, respectively). However, for each species the two isolates showed a similar growth pattern. There was no significant difference by tree species between the two isolates of either fungus with respect to growth in phloem or sapwood, or in the extent of host symptoms they caused.

The linear growth of *O. montium* in the oxygen-deficient tubes was completely arrested after 11–18 days (Fig. 1). *Ophiostoma clavigerum* continued to grow until the mycelium reached the end of the tubes in six tubes, whereas growth stopped after 18–21 days in another six tubes. In the aerated control tubes both fungi continued to grow until the mycelium reached the end of the tubes (Fig. 1).

At temperatures below 25°C the growth rate of *O. clavigerum* was up to 5 mm/day faster than that for *O. montium* (Fig. 2). At higher temperatures *O. montium* grew faster than *O. clavigerum* (Fig. 2). The optimum temperature for growth was 22–25°C (7.2–9.0 mm/day) for the two isolates of *O. clavigerum* and 25°C (4.4–5.1 mm/day) for the two isolates of *O. montium*.

Individual trees within each tree species differed significantly in their response to fungal infection, both in the phloem (white pine: $p < 0.001$, $F = 3.49$; shore pine: $p < 0.02$, $F = 3.37$) and in the sapwood (white pine: $p < 0.001$, $F = 3.49$; shore pine: $p < 0.04$, $F = 2.66$). Shore pine had less extensive host symptoms in both phloem and sapwood compared with white pine (Fig. 3).

Fig. 1. Mean linear growth of *Ophiostoma clavigerum* and *O. montium* at 21°C under oxygen-deficient conditions in sealed test tubes filled with nitrogen (open symbols) and in aerated control tubes (solid symbols). For *O. clavigerum* circles denote isolate NISK 92-628/57/4, and squares isolate NISK 92-629/121/5; for *O. montium* circles denote isolate NISK 92-628/49/1, and squares isolate NISK 92-628/59/2 ($n = 2-8$ per isolate).

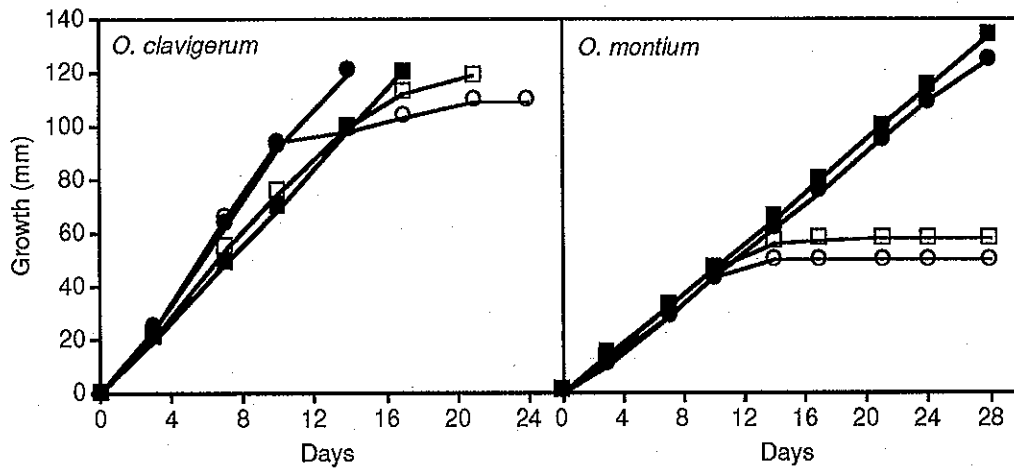
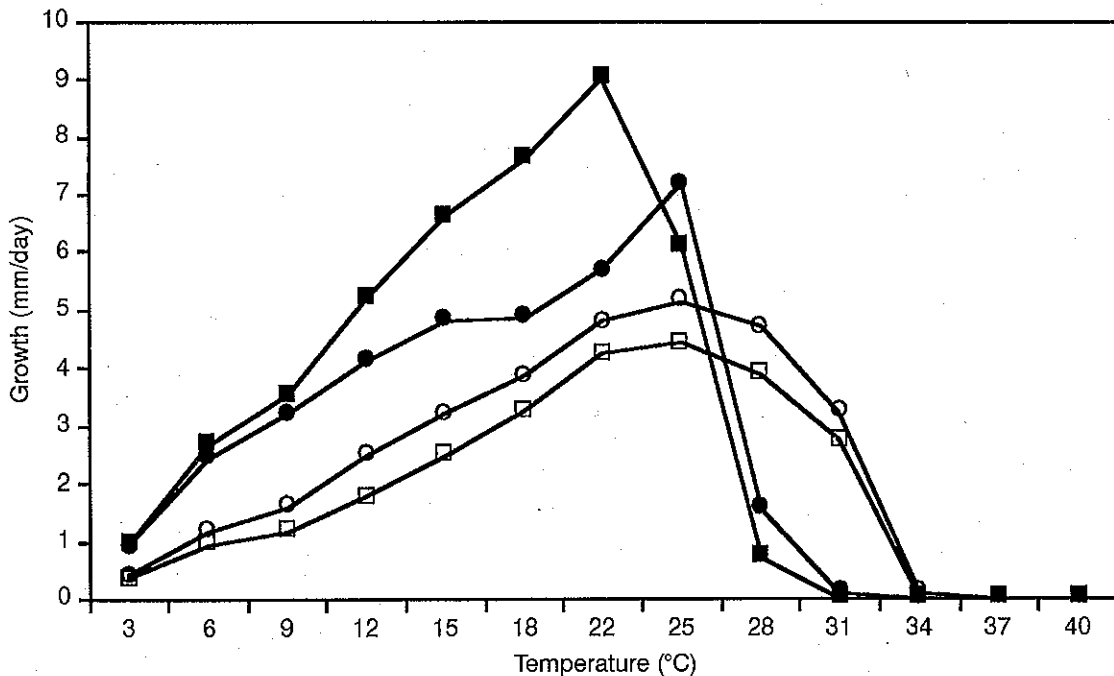


Fig. 2. Mean linear growth on malt agar of two different isolates of *O. clavigerum* and *O. montium* ($n = 6$). ■, *O. clavigerum* isolate NISK 92-628/57/4; ●, *O. clavigerum* isolate NISK 92-629/121/5; □, *O. montium* isolate NISK 92-628/49/1; ○, *O. montium* isolate NISK 92-628/59/2.



In white pine phloem *O. clavigerum* induced significantly longer necroses than *O. montium*, and both fungi induced significantly longer necroses than the sterile agar control (Fig. 3). Only *O. clavigerum* caused significantly longer necrosis at week 5 than at week 1 ($p < 0.0001$, $F = 21.67$). In white pine sapwood both fungi always induced significantly more desiccated sapwood than the control, but there were no significant differences between the two fungi (Fig. 3). For both fungi

there was a steady and significant increase in depth of sapwood desiccation from week 1 to week 5 (*O. clavigerum*: $p < 0.0001$, $F = 67.34$; *O. montium*: $p < 0.0001$, $F = 35.49$).

In shore pine phloem and sapwood there were no significant differences in host symptoms induced by the two fungi (Fig. 3). Both fungi always induced significantly more extensive symptoms than the control, and both fungi induced significantly more extensive symptoms at week 5 than at week 1

Fig. 3. Length of phloem necrosis and depth of sapwood desiccation in white pine and shore pine 1, 3, and 5 weeks after inoculation with *O. clavigerum* (open bars), *O. montium* (hatched bars), and sterile malt agar control (solid bars). Within weeks treatments with the same letter are not significantly different (LSD test at $p < 0.05$ following one-way ANOVA).

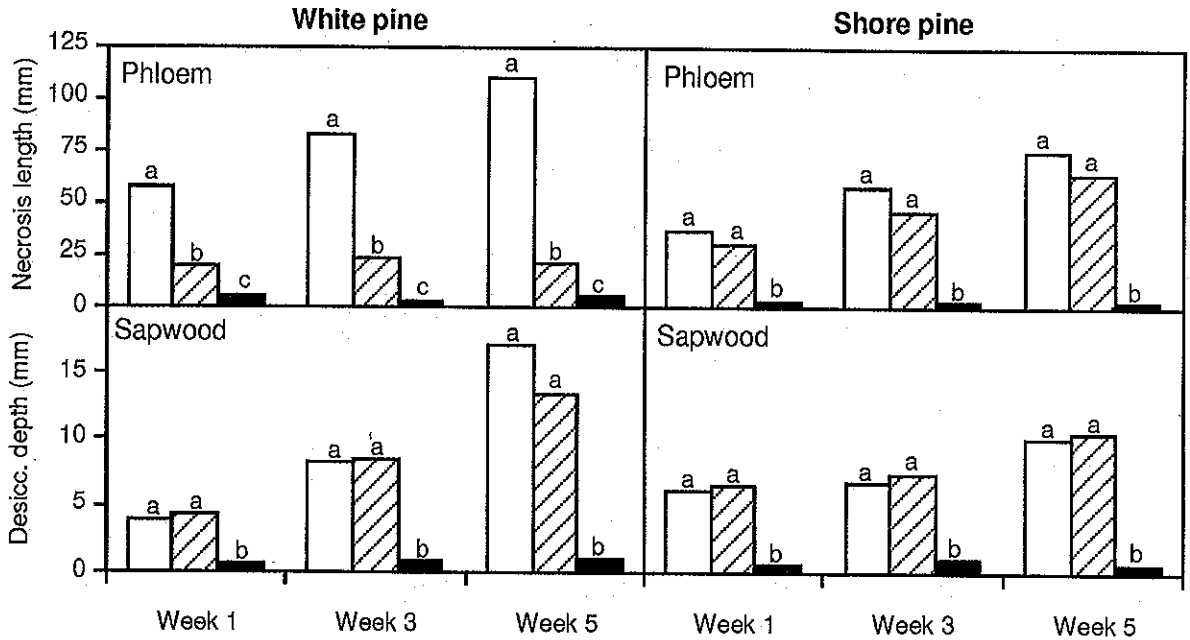
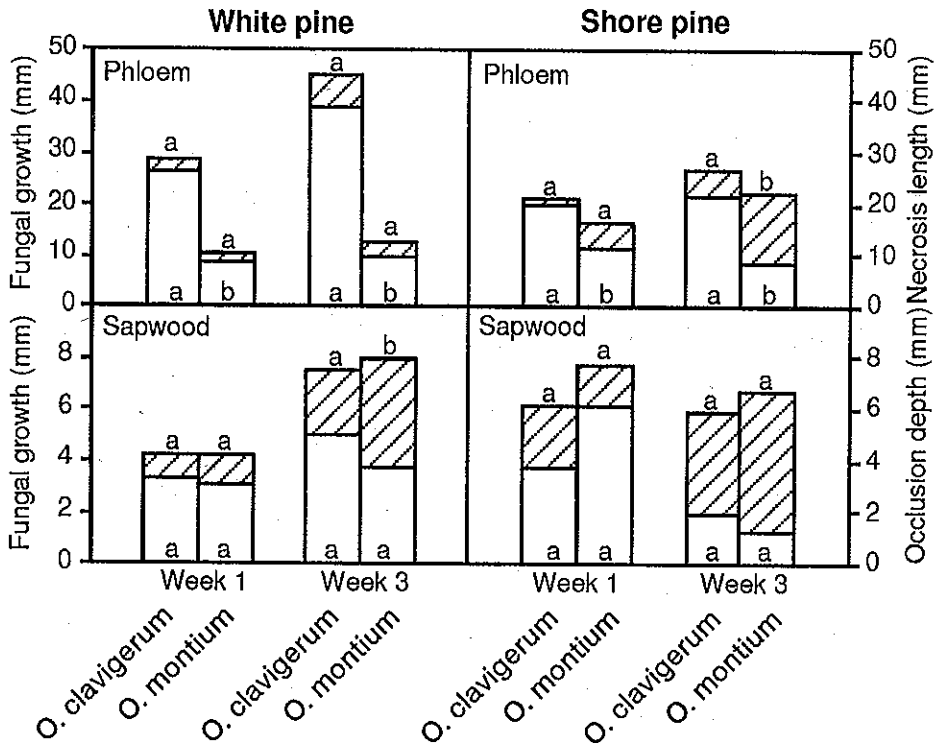


Fig. 4. Growth of *O. clavigerum* and *O. montium* relative to zones of phloem necrosis and sapwood desiccation 1 and 3 weeks after inoculation of white pine and shore pine. The extent of fungal growth from the inoculation point (open bars) and distance from the front of fungal invasion to the front of necrotic phloem or desiccated sapwood (hatched bars) are shown. Within weeks bars with the same letter are not significantly different (LSD test at $p < 0.05$ following one-way ANOVA).



both in phloem (*O. clavigerum*: $p < 0.008$, $F = 6.18$; *O. montium*: $p < 0.0005$, $F = 11.53$) and in sapwood (*O. clavigerum*: $p < 0.0008$, $F = 10.51$; *O. montium*: $p < 0.0006$, $F = 11.04$).

In both tree species, *O. clavigerum* penetrated the phloem

significantly farther than *O. montium* 1 and 3 weeks after inoculation (Fig. 4). There were no significant differences between the fungi in extent of sapwood colonization (Fig. 4). Fungal growth lagged 0.9–13.6 mm behind the front of

phloem necrosis and sapwood desiccation, but there were no significant differences between fungi, except *O. montium* lagged behind *O. clavigerum* in shore pine phloem and in white pine sapwood 3 weeks after inoculation (Fig. 4).

Ophiostoma clavigerum and *O. montium* were always reisolated both in phloem and sapwood.

Discussion

In the nitrogen-filled tubes small amounts of air inevitably got mixed with the nitrogen when the tubes were plugged. As the same procedure was followed with all the tubes, the amount of air should have been roughly the same in all tubes, and probably did not seriously affect our results. This assumption is supported by the low variability found between replicates in our experiments, as well as by earlier experiments (Solheim 1991, 1995b).

In the sealed tubes with only small amounts of oxygen available, *O. clavigerum* was able to grow for a longer time than *O. montium*. This indicates that *O. clavigerum* is able to tolerate a lower oxygen level and so appears to be well equipped to be the primary invader of fresh sapwood. Live sapwood has a high moisture content and low oxygen levels and adaptation to these conditions is probably important for early fungal colonizers. *Ophiostoma clavigerum* is found at the leading edge of fungal penetration into the sapwood of trees mass attacked by the mountain pine beetle (Solheim 1995a).

A relationship between fungal tolerance to low oxygen levels and ability to colonize fresh sapwood has previously been demonstrated for fungi associated with the Eurasian spruce bark beetle, *Ips typographus* L., colonizing Norway spruce, *Picea abies* (L.) Karst. *Ceratocystis polonica* (Siem.) C. Moreau, the most virulent blue-stain fungus associated with the Eurasian spruce bark beetle (Horntvedt et al. 1983; Solheim 1988; Krokene and Solheim 1998), tolerates low oxygen levels better than the other fungi associated with this beetle (Solheim 1991) and is also the primary invader of sapwood of beetle-infested trees (Solheim 1992). Experiments on the fungal associates of the North American spruce beetle, *Dendroctonus rufipennis* (Kirby), also suggest a relationship between tolerance of low oxygen levels and ability to colonize live sapwood (Solheim 1995b).

Rapid growth seems to be another general characteristic of pathogenic blue-stain fungi. Most aggressive bark beetles are associated with a range of different blue-stain fungi, and the most virulent of these associates is always one of the most fast-growing ones. This is true for the fungi associated with the mountain pine beetle (Owen et al. 1987; Six and Paine 1997; this study), the southern pine beetle, *Dendroctonus frontalis* Zimm. (Goldhammer et al. 1989), the western pine beetle, *Dendroctonus brevicomis* LeConte (Owen et al. 1987), the Eurasian spruce bark beetle (Solheim 1991), and the North American spruce beetles (Solheim 1995b). However, many less virulent blue-stain fungi are also fast growing, whereas some pathogenic fungi do not have particularly high growth rates (Käärik 1980; Gibbs 1993). The high growth rate of many pathogenic fungi may be an adaptation to allow rapid colonization of tree tissues before the defences have been mobilized (Owen et al. 1987; Solheim 1991, 1993). Rapid growth may also have evolved through intraspecific competition with other fungi introduced into the tree (Harrington 1993).

Based on host symptoms in the phloem, *O. clavigerum* seems to be more virulent than *O. montium* in white pine. Previously, *O. clavigerum* has been found to be more virulent than *O. montium* in lodgepole pine and ponderosa pine (Reid et al. 1967; Owen et al. 1987; Yamaoka et al. 1990, 1995). Thus, *O. clavigerum* appears to be more virulent than *O. montium* in all the major host trees of the mountain pine beetle. *Ophiostoma montium*, however, appears to be at least moderately virulent. It caused more extensive host symptoms than the control in this study, and in earlier inoculation studies it was able to kill 10- to 20-year-old lodgepole pine, ponderosa pine, and loblolly pine, *Pinus taeda* L. (Mathre 1964a; Basham 1970; Strobel and Sugawara 1986). However, these studies used very destructive inoculation techniques, with complete or almost complete girdling of the trees during inoculation. When the two fungi have been compared in the same study, *O. clavigerum* has always caused high tree mortality, whereas *O. montium* has caused almost no mortality (Owen et al. 1987; Yamaoka et al. 1995).

Despite its higher growth rate and tolerance to low oxygen levels, *O. clavigerum* was not a better sapwood colonizer than *O. montium* in white pine or shore pine. One reason for this may be that the trees received a small number of fungal inoculations, and that trees probably are able to confine fungal growth and resist colonization as long as the inoculation density is below a certain threshold (Christiansen 1985; Christiansen et al. 1987). It may therefore be difficult to assess the virulence of different blue-stain fungi on the basis of results from low-density inoculations (Solheim 1988; Solheim et al. 1993; Ross and Solheim 1996; Krokene and Solheim 1997).

Our results have shown that *O. clavigerum*, the primary invader of sapwood after mountain pine beetle attacks, grows more rapidly on malt agar and better tolerates low oxygen levels than the secondary invader *O. montium*, and thus support the hypothesis that rapid growth and tolerance to low oxygen levels may be important adaptations for fungi that are primary sapwood invaders after bark beetle attacks (Solheim 1991). However, other aspects of fungal growth requirements (e.g., tolerance of host allelochemicals; Cobb et al. 1968; De Groot 1972) are probably important in determining the virulence of different blue-stain fungi.

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