Interaction between the bluestain fungal associates of mountain pine, and pine engraver beetles, (*Dendroctonus ponderosae* and *Ips pini*, Coleoptera: Scolytidae) and their effects on the beetles

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ABSTRACT

We investigated the potential antagonism between the fungal associates of the pine engraver and mountain pine beetles. We measured and compared their rates of growth in bolts of lodgepole pine and in living trees: *Ophiostoma ips* from *Ips pini* against *O. clavigerum* from *Dendroctonus ponderosae*. We measured the length of lesions shown by discolored xylem, but we found both fungi outside of visibly stained areas, which showed that mere staining is not a reliable indicator of fungal growth. There were no significant differences in brood development or survival between the two beetle spp., when bolts were inoculated with either fungal associate.

Key words: Coleoptera:Scolytidae, bluestain fungi, *Pinus contorta*, Princeton, Williams Lake, B.C.

INTRODUCTION

The mountain pine beetle, *Dendroctonus ponderosae* Hopk., is one of the most destructive insect pests of mature pines in British Columbia (B.C.) (Unger 1993). In 1993, an average year for bark beetle activity, losses to the mountain pine beetle amounted to 4.8 million trees on nearly 45,000 hectares (Wood and Van Sickle 1992). Forest management for timber and other resources is often greatly disrupted by mountain pine beetle outbreaks. Current short-term management techniques to reduce timber losses from mountain pine beetle attack include sanitation logging, single-tree treatments, and the use of trap trees baited with pheromones (Unger 1993, Safranyik 1995).

A novel biological control technique is to bait trees attacked by mountain pine beetles with the aggregation pheromone of the pine engraver beetle, *Ips pini* Say. This approach is based on observations that secondary attacks by pine engraver beetles at very high densities often results in high mortality of mountain pine beetle broods (Andrews 1987, Humphreys and Ferris 1987, Rankin and Borden 1991). The reasons for this mortality are unknown, but Rankin and Borden (1991) speculated that the fungal associate of the pine engraver may have a direct antagonistic effect on brood development of the mountain pine beetle or on its fungal associate.

Ophiostoma ips is the bluestain fungus most closely associated with the pine engraver (Mathre 1964, Raffa and Smalley 1988, Furniss *et al.* 1995). Both *O. clavigerum* and *O. ips* are associated with the mountain pine beetle (Robinson 1962, Reid *et al.* 1967, Whitney 1971) although *O. clavigerum* is frequently the only bluestain fungus recovered (H.S. Whitney personal communication). Antagonism between the two fungi has been demonstrated by the reduced pathogenicity of *O. clavigerum* to seedlings of ponderosa

pine, *P. ponderosa* Laws, when inoculated in combination with *O. ips* (Owen *et al.* 1987). Similar antagonism was also demonstrated when other bluestain fungi were inoculated in combination compared with separate inoculations (Owen *et al.* 1987; Parmeter *et al.* 1989, 1992; Nevill *et al.* 1995). Our own investigations show that *O. ips* appears to inhibit the growth of *O. clavigerum* when grown on malt extract agar (unpublished data).

Because an antagonistic effect on brood survival has been shown to occur between the southern pine beetle, *Dendroctonus frontalis* Zimmerman, and its fungal associate, *Ophiostoma minus* [(Hedgc.) H.&P. Syd. = *Ceratocystis minor* (Hedgc.) Hunt] (Barras 1971, Franklin 1970) and between the California five-spined ips, *Ips paraconfusus* Lanier, and its fungal associate, *O. ips* (Rumb.), as well as other bluestain fungi (Fox *et al.* 1992) we investigated potential antagonism of *O. ips* and *O. clavigerum* (Robins. Jeff. & Davids) to brood development of the mountain pine beetle and the pine engraver.

The objectives of this study were to investigate the antagonism between the respective fungal associates of the mountain pine beetle and the pine engraver and to determine whether this antagonism has an effect on mountain pine beetle brood survival and development

MATERIALS AND METHODS

The mountain pine beetle fungal associate, *O. clavigerum,* was taken from a mountain pine beetle egg gallery in lodgepole pine collected at Saturday Creek near Princeton, B.C. The pine engraver associate, *O. ips*, was obtained from the bodies of adult *I. pini* beetles collected from duff at Sunday Creek also near Princeton. Both fungi were maintained on 1.5% malt extract agar (MEA) at room temperature, 20° C.

a. Bioassays of antagonism of fungal associates in lodgepole pine bolts. To investigate antagonism between the two fungi, 20- to 30-cm dbh lodgepole pine growing at Sunday Creek were felled and cut into 45 cm bolts. The exposed ends of the bolts were dipped in melted paraffin to prevent drying. The bolts were stored at 5°C and used at two week intervals

Inoculum consisted of the respective fungi grown on MEA at room temperature for 2 - 4 weeks.

Each bolt set vertically received six, randomly assigned, inoculations evenly spaced around the circumference of the bolt. The treatments were: 1) inoculation with sterile malt extract agar (MEA) - control, 2) MEA colonized by *O. clavigerum,* 3) MEA colonized by *O. ips*, 4) combination inoculations of *O. clavigerum* and *O. ips* , *O. clavigerum* placed first, 5) combination inoculations of *O. clavigerum* and *O. ips* , *O. ips* placed first, and 6) single inoculations of *O. clavigerum* and *O. ips* separated along the vertical axis by 5 cm. Treatments were assigned to five bolts (replicates) and repeated four separate times (blocks), at two week intervals, for a total of 20 bolts.

The inoculation sites were prepared by smoothing the bark with a drawknife at six places around the circumference of the bolt approximately at midpoint. The bark was sprayed with 95% ethanol and flamed, and a hole was made with a flame-sterilized leather punch 5 mm in diameter. Using an ethanol-dipped and flamed spatula, a 5-mm plug of colonized or sterile MEA was placed into the hole (two plugs for combination inoculations), a sterile cotton roll was dipped in distilled water placed over the agar, and the area was sealed with duct tape. Single inoculations of *O. clavigerum* and *O. ips* separated by 5 cm were made by establishing two points along the vertical axis of the bolt 5 cm apart. One end of the bolt was marked with a red felt pen with the *O. clavigerum* inoculation closest to the pen mark "above" the *O. ips* inoculation.

The bolts were examined 3 weeks after inoculation by removing the bark with a drawknife sprayed with 95% ethanol and flamed. The evidence of colonization by the fungi (e.g. blue/black stain) was measured above and below the inoculation point. Colonization of the xylem was confirmed by removing disks with a flame-sterilized 5 mm dia leather punch at 3.0 cm intervals to 12.0 cm above and below the inoculation point. The disks were placed on to MEA amended with 5% cycloheximide (McCall and Merrill 1980) and incubated 10 days at 20°C. For inoculations of *O. clavigerum* and *O. ips* 5 cm apart, wood samples were taken at 3.0 cm intervals to 12.0 cm above the *O. clavigerum* inoculation point and below the *O. ips* inoculation point. One sample was also taken between the inoculation points

Lesion lengths for each treatment, as measured by visible staining in the wood, were compared by a 2-way analysis of variance, blocking for treatment and replicate, using the SAS Proc-GLM procedure (SAS Institute 1989). When significant differences were indicated by the *F*-test within the ANOVA, means were separated using the Student Neuman-Kuels test at $p < 0.05$.

b. Bioassays of antagonism fungal associates in living lodgepole pines. For inoculation, ten 20- to 30-cm dbh lodgepole pine trees were selected in two stands, five at Saturday Creek and five at Sunday Creek near Princeton, B.C. Both sites are in the IDFd biogeoclimatic zone (Mitchell and Green 1981).

Each tree received the same six inoculation treatments as described for the bolts. Treatments were assigned to five trees (replicates) at each stand for a total of 10 trees. Inoculations were made in early August to correspond with mountain pine beetle flight (Unger 1993, Safranyik 1995).

The inoculation sites were prepared by smoothing the bark with a drawknife at six places approximately equidistant around the tree at breast height. The bark was sprayed with 95% ethanol and flamed, and a hole was made with a flame-sterilized leather punch 5 mm in diameter. Using an ethanol-dipped and flamed spatula, a 5-mm plug of colonized or sterile MEA was placed into the hole (two plugs for dual inoculation), a sterile cotton roll dipped in distilled water placed over the agar, and the area sealed with duct tape.

The trees were felled after 8 weeks. The bolts were examined as above with the exception that wood disks were taken at 5.0-cm intervals to 25.0 cm above and below the inoculation point. For inoculations of *O. clavigerum* and *O. ips* spaced 5 cm apart, wood disks were taken at 5.0-cm intervals to 25.0 cm above the *O. clavigerum* inoculation point and below the *O. ips* inoculation point. One sample was also taken between the inoculation points.

Lesion lengths for each treatment, as measured by visible staining in the wood, were compared by a 2-way analysis of variance, blocking for treatment and replicate, using the SAS Proc-GLM procedure (SAS Institute 1989). When significant differences were indicated by the *F*-test within the ANOVA, means were separated using the Student Neuman-Kuels test at $p < 0.05$.

c. Bioassays of brood establishment. Pine engraver beetles were collected from duff beneath trees attacked by them near Sunday Creek, and from bolts cut from lodgepole pine logs attacked by them near Williams Lake B.C. Mountain pine beetles were collected from bolts taken from beetle-killed trees at Sunday Creek.

To establish brood, living lodgepole pine trees of 20- to 30-cm dbh were felled and cut into 45 cm bolts. The bolts were split in half and the exposed side and ends of the bolts were dipped in melted paraffin to prevent drying.

Adult mountain pine and pine engraver beetles were caged separately on lodgepole pine bolts inoculated with either *O. clavigerum*, *O. ips* or sterile agar as a control. The three inoculation treatments were replicated seven times with the mountain pine beetles for a total of 21 bolts. Treatments with the pine engravers were replicated six times for a total of 18 bolts. Inoculation of the bolts was similar to "*a*" above except that the bolts were inoculated at three sites that were 5 cm apart and 15 cm from one end of the bolt.

Beetle attack was induced immediately after inoculation by carefully cutting the bark to the cambium with a 5-mm-diameter cork borer so that the resulting bark disk was not removed. A 2-mm hole was made with a hand drill into the centre of the bark disk and either a single pine engraver male or mountain pine beetle female was caged on the bolt with a gelatin capsule. Beetle attack points were 2.5 cm from the end of the bolt and 12.5 cm from the inoculation sites. Twenty-four hours after successful attack (e.g., frass appearing at an entrance hole) two female pine engraver beetles or a single male mountain pine beetle were caged with their respective species onto the bolts using gelatin capsules as described. Once both sexes had entered the bolts, the bolts were placed in nylon mesh bags and stored on end with the beetle entrance point nearest the floor.

Emerging beetles were counted over 10 weeks after which the bolts were examined for remaining brood. Success of mountain pine beetle brood development was measured by length of adult female gallery and numbers of emerging beetles whereas success of pine engraver development was measured only by numbers of emerging beetles. Length of mountain pine beetle egg galleries and numbers of both species of bark beetles emerging from the inoculated bolts were compared by analysis of variance using the SAS Proc-GLM procedure. When significant differences were indicated by the F-test within the ANOVA, means were separated using the Student Neuman-Kuels test at $p < 0.05$.

RESULTS

a. Bioassays of antagonism of fungal associates in lodgepole pine bolts. Although lesion length, as measured by visible staining, was significantly different among inoculation treatments and among blocks, lesion length was not a reliable indicator of fungal growth. In almost all instances the fungi could be recovered from the wood up to 12.0 cm above and below the inoculation point - at least 2.5 cm beyond visible staining (Table 1). Differences of staining lengths between blocks appeared to be related to bolt age as staining lengths were longest in the bolts inoculated immediately after the trees were harvested and decreased with the period of time the bolts were stored.

Characteristics of *O. clavigerum* growing in culture included appressed to effuse growth, colorless at first but rapidly becoming dark brown to grayish black; color of the colony from the reverse side to the Petri plate was black; mycelial growth of 10-day-old colonies was superficial and immersed. Both mononematous and synnematous conidiophores and conidia resembling the description of *O. clavigerum* by Upadhyay (1981) were observed. Although perithecia were not seen, the distinctive, almost club-shaped conidiospores differentiated this fungus from *O. ips*.

Characteristics of *O. ips* growing in culture included appressed to effuse growth, colorless at first becoming pale yellow to light brown; color of the colony from the reverse side of the Petri plate was pale yellow; mycelial growth of 10-day-old colonies was superficial and immersed. Conidiophores mononematous 1.5-4.5 X 1-2 mm, pale brown at base becoming hyaline. Perithecia were not observed, but the round to oval (prolate) conidiospores differentiated this fungus from *O. clavigerum*.

Table 1.

Mean lesion length and recovery of *Ophiostoma clavigera* (*O.c.*) and *O. ips* (*O.i.*) from inoculated living lodgepole pine trees.

		Distance from inoculation point and percent of bolts from which fungi were collected											
	Mean lesion		3.0 cm			6.0 cm			9.0 cm			12.0 cm	
Inoculation Treatment	Length (cm) ±S.E.	O.i. only	O.c. only	Both	O.i. only	O.c. only	Both	O.i. only	O.c. only	Both	O.i. only	O.c. only	Both
O.c. only	7.5 ± 0.8 bc [5]		100.0			100.0			100.0			100.0	
O.i. only	9.7 ± 0.6 a	100.0			100.0			100.0			100.0		
O.i./O.c. [1]	7.6 ± 0.5 bc			100.0			100.0			100.0			100.0
O.c./O.i. [2]	9.5 ± 0.7 a			100.0			100.0			100.0			100.0
O.c.-O.i. [3]	6.3 ± 0.5 c			100.0		13.3	87.7		60.0	40.0		100.0	
O.c.- O.i. [4]	8.6 ± 0.7 ab			100.0			100.0			100.0			100.0

Table 2.

Mean lesion length and recovery of *Ophiostoma clavigera* (*O.c.*) and *O. ips* (*O.i.*) from inoculated lodgepole pine trees.

		Distance from inoculation point and percent of bolts from which fungi were collected											
	Mean lesion		5.0 cm			10.0 cm			15.0 cm			20.0 cm	
Inoculation Treatment	Length (cm) ±S.E.	O.i. only	O.c. only	Both	O.i. only	O.c. only	Both	O.i. only	O.c. only	Both	O.i. only	O.c. only	Both
O.c. only	11.1±0.9 ab [5]		100.0			10.0							
O.i. only	13.2±0.7 a	100.0			50.0			20.0					
O.i./O.c. [1]	9.7±0.8 b		70.0	30.0		10.0							
O.c./O.i. [2]	12.5±1.0 ab			100.0			70.0	20.0			10		
O.c.-O.i [3]	10.5±1.1 ab		100.0										
O.c.- O.i. [4]	12.4± 0.7 ab	100.0			80.0			20.0					

[1] combined inoculation, *O. c.* placed first. [2] combined inoculation, *O.i.* placed first. [3] single inoculations of *O.c* and *O. i.* 5 cm apart, collected below *O.c* inoculation point.

[4] single inoculations of *O.c.* and *O.i.* 5 cm apart, collected above *O.i.* inoculation point.

[5] treatments followed by the same letter are not significantly different ($p > 0.05$, GLM procedure and Student Neuman-Keuls test).

Fungal growth characteristics of combination inoculations of *O. ips* and *O. clavigerum* on MEA, whether *O. clavigerum* or *O. ips* was placed first, most closely resembled those of *O. ips*. However, the distinctive conidiophores and conidia of *O. clavigerum* could be recovered together with those of *O. ips* up to 12.0 cm from the inoculation point in all but 3 of 20 inoculations.

Samples taken from wood of single inoculations separated by 5 cm revealed that both fungi could be recovered to 12.0 cm below the *O. ips* inoculation in all instances. Both fungi could also be isolated from all samples taken between the inoculation points although all colonies closely resembled those of *O. ips*. Fungal growth at 3.0 and 6.0 cm above the *O. clavigerum* inoculation point produced distinct colonies of *O. ips* or *O. clavigerum* which contained conidiospores of both fungi. However, at 9.0 cm above the *O. clavigerum* inoculation point conidiospores of both fungi were recovered from only 40% of the samples and for the remainder only conidiospores of *O. clavigerum* were observed. At 12.0 cm, only distinctive colonies of *O. clavigerum* and conidiophores of that fungus were observed.

b. Bioassays of antagonism fungal associates in living lodgepole pines. Although lesions in living trees in response to either single or combined inoculations of *O. clavigerum* or *O. ips* were similar to those in the bolts, lesion lengths were significantly different only between two treatments. In addition, the fungi were only consistently recovered from stained wood or wood disks taken 5.0 cm from the inoculation point (Table 2). At 10 cm from the inoculation point, recovery dropped to 50-80% of wood disks for single and combination inoculations in which *O. ips* had been placed first. Recovery from inoculations in which *O. clavigerum* had been placed first was poor. Otherwise, fungal growth patterns were similar to those described for the bolts.

Characteristics of combination inoculations in which *O. ips* was placed first resembled typical colony growth for single inoculations of that fungus although both fungi were recovered from wood disks taken at 10 cm. However, at 15 and 20 cm only conidiospores typical of *O. ips* were seen. Combined inoculations in which *O. clavigerum* was placed first either showed growth typical of single inoculations of that fungus or had a portion of the Petri plate with growth typical of *O. ips*. In portions with growth typical of *O. clavigerum* only conidiospores of that fungus could be recovered while in portions with growth typical of *O. ips* conidiospores of both fungi were observed.

For single inoculations separated by 5 cm, both fungi could be recovered from samples taken between the inoculation points, but in contrast to the bolts, distinct colonies of both fungi were observed. At 5.0 cm from the *O. ips* or the *O. clavigerum* inoculation points only distinct colonies and conidiophores of the respective fungi were seen.

c. Bioassays of brood establishment. Analysis showed no significant effect of inoculation with either fungus on adult gallery length or numbers of emerging adult mountain pine beetles (Table 3). Similarly, fungal inoculations had no significant effect on numbers of emerging pine engraver beetles.

Mountain pine beetle broods were established in five of seven control bolts inoculated with sterile agar, four of seven bolts inoculated with *O. ips* and four of seven bolts inoculated *O. clavigerum*. In one of the control bolts, the adult beetle exited after creating a 7-cm egg gallery and in a second bolt the beetle created a 2-cm egg gallery before exiting. There was no obvious reason why either beetle exited (e.g.: no visible staining or decay). Dissection of the bolts inoculated with either fungus showed that the adult beetles exited before encountering stained areas in all but one instance.

Table 3

Mean Gallery length (cm) and number of adult beetles emerging from lodgepole pine bolts inoculated with the fungal associates of the mountain pine beetle and the pine engraver.

	Dendroctonus ponderosa		*Ips pini*	
	Gallery	No. adults	Gallery	No. adults
Inoculation	**length (cm) ± S.E.**	**± S.E.**	**length (cm) ± S.E.**	**± S.E.**
Control	45.8 ± 12.0 [1]	37.4 ± 11.8 [1]	nm [2]	24.3 ± 5.9 [1]
O. ips	54.1 ± 10.6	36.5 ± 9.4	nm	25.0 ± 12.7
O. clavigera	48.5 ± 8.5	25.0 ± 4.5	nm	33.5 ± 16.2

[1] no treatments were significantly different $p > 0.05$ GLM procedure and Student Neuman-Keuls test).
[2] not measured

Larval galleries in bolts inoculated with either fungus were indistinguishable from controls and larvae created typical galleries at right angles from the adult gallery. These galleries did not deviate if the inoculated fungus was encountered.

Pine engraver broods were established in all six of the control bolts and in four of the bolts inoculated with either *O. clavigerum or O. ips*. Adult beetles which exited without ovipositing did so before encountering areas stained by the inoculated fungus. In all instances where oviposition took place larvae developed in the stained areas.

DISCUSSION

Because of the negative effect on brood survival of the southern pine beetle and the California five-spine ips of their respective fungal associates we thought it prudent to test whether this effect could be observed with the mountain pine beetle and the pine engraver beetle. However, we found no significant reduction of brood development and survival of the mountain pine beetle or pine engraver after inoculation with *O. clavigerum* or *O. ips*. This was not entirely unexpected because both *O. clavigerum* and *O. ips* are recorded as associates of the mountain pine beetle (Robinson 1962, Reid *et al.* 1967, Whitney 1971). Therefore, we conclude that introduction of foreign fungi by the pine engraver does not explain the decreased success of mountain pine beetle brood when both insects attack the same host.

While longitudinal growth of the fungi was recorded up to 20 cm from the inoculation point we were not concerned about interference between the different treatments from horizontal growth around the bolts. Both Parmeter *et al.* (1992) and Nevill *et al.* (1995) have shown that horizontal growth of blue stain around the bolts is minimal within the inoculation period used in this study.

We found little or no antagonism between *O. clavigerum* and *O. ips* when inoculated together either in the bolts or in living trees. Although in both cases there were significant differences in lesion lengths, the length of lesions produced by single inoculations were not significantly different from those lengths produced by combination inoculations in which the respective fungus was placed first. For example, the average lesion length of single inoculations of *O. ips* was not significantly different from combination inoculations in which *O. ips* was placed first (Table 1,2). Moreover, lesion length is generally not a good indication of the growth of blue stain fungi in the sapwood and these fungi can often be recovered in apparently clear wood beyond the lesions (Owen *et al.* 1987; Parmeter *et al.* 1989, 1992; Nevill *et al.* 1995).

It is not entirely clear which is the more aggressive fungus. Owen *et al.* (1987) found *O. clavigerum* to be more virulent than *O. ips* to ponderosa pine. Solheim (1995) observed that when the two fungi were found together in beetle-attacked trees, *O.*

clavigerum was always at the leading edge of fungal colonization. From the combination inoculations in living lodgepole pine trees our results showed that when inoculated first, *O. clavigerum* was recovered more frequently alone than when *O. ips* was inoculated first. However, when *O. ips* was inoculated first, it was found alone at further distances from the inoculation point than *O. clavigerum*. Thus, for combined inoculations the recovery of the fungi could be interpreted as either a positional effect or as antagonism.

As previous studies have shown that combined inoculations of blue stain fungi may demonstrate both antagonism and non-antagonism (Owen *et al.* 1987, Parmeter *et al.* 1989, 1992; Nevill *et al.* 1995) we decided to determine whether prior colonization by the fungi would demonstrate any effect by inoculating them 5 cm apart. In living trees, both fungi could be readily recovered from wood disks taken between the inoculation points, but only the inoculated fungus could be recovered from wood disks distal from the inoculation. This was not entirely the case with the bolts where both fungi could be recovered between the two inoculation points and distal from the *O. ips* inoculation point to 12 cm. Both fungi could also be recovered distal from the *O. clavigerum* inoculation point to 3 cm, but starting at 6 cm *O. clavigerum* was progressively isolated alone up to 12 cm where it was the only fungus recovered. Both sets of observations refute our earlier results when growing the two fungi together on MEA. Thus, when growing in its host environment, *O. clavigerum* appears to be the more aggressive of the two fungi as suggested by Solheim (1995).

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