Variation in pathogenicity of a mountain pine beetle–associated blue-stain fungus, *Grosmannia clavigera*, on young lodgepole pine in British Columbia

Alex Plattner, Jae-Jin Kim, Scott DiGuistini, and Colette Breuil

Abstract: *Grosmannia clavigera* is the most pathogenic blue-staining fungal associate of the mountain pine beetle (*Dendroctonus ponderosae*). In contrast to its importance as a primary invader of lodgepole pine (*Pinus contorta*) sapwood, intraspecific variability in pathogenicity of *G. clavigera* on lodgepole pine, the predominant host of mountain pine beetles in British Columbia, has not been investigated in detail. The present work reports on pathogenicity indicators induced by five *G. clavigera* isolates inoculated into lodgepole pines and growth characteristics of the isolates on artificial media. Fungi were inoculated at 200 inoculations/m^2 into young lodgepole pine trees. Phloem lesion length, sapwood occlusion area, and sapwood moisture content were measured after 7 or 48 weeks. Three isolates produced long lesions, occluded larger areas, and reduced more the moisture content after 48 weeks compared with the remaining two isolates. Isolate ATCC 18086 induced the strongest pathogenic symptoms after 7 weeks and grew the fastest up to 22.5 °C but grew the slowest at 27.5 °C. In a low-oxygen environment, most isolates grew faster than under ambient conditions. Significant intraspecific variation was observed among *G. clavigera* isolates for all parameters tested.

Key words: Grosmannia clavigera, *Ophiostoma clavigerum*, mountain pine beetle, blue-stain fungus, intraspecific variation, pathogenicity.

Résumé : *Grosmannia clavigera* est le plus pathogénique des champignons du bleuissement associés au dendroctone du pin ponderosa (*Dendroctonus ponderosae*). Contrairement à son importance en tant qu'envahisseur primaire de l'aubier du pin tordu latifolié (*Pinus contorta*), la variabilité intraspécifique de la pathogénicité de *G. clavigera* envers ce dernier, hôte principal du dendroctone du pin ponderosa en Colombie-Britannique, n'a pas été étudiée en détail. Les présents travaux font état d'indicateurs de pathogénicité produits par cinq isolats de *G. clavigera* appliqués sur des pins tordus latifoliés ainsi que des caractéristiques de croissance de ces isolats cultivés en milieu artificiel. De jeunes pins tordus latifoliés ont été inoculés avec le champignon à raison de 200 inoculations/m^2. Au bout de 7 ou 48 semaines, la longueur des lésions subies par le phloème, la surface d'occlusion de l'aubier ainsi que son degré d'humidité ont été mesurés. Au bout de 48 semaines, comparativement aux deux autres, trois des cinq isolats avaient produit de longues lésions, occlus de plus larges surfaces et avaient davantage réduit le degré d'humidité. Au bout de 7 semaines, l'isolat ATCC 18086 avait provoqué les symptômes pathogènes les plus aigus. Son taux de croissance avait également été le plus rapide, et ce, jusqu'à 22,5 °C, pour devenir le plus lent à 27,5 °C. La plupart des isolats ont crû plus rapidement dans un environnement faible en oxygène que dans les conditions de milieu ambiant. Une variation intraspécifique significative a été observée chez les isolats de *G. clavigera*, et ce, relativement à tous les paramètres analysés.

Mots-clés : Grosmannia clavigera, *Ophiostoma clavigerum*, dendroctone du pin ponderosa, champignon du bleuissement, variation intraspécifique, pathogénicité.

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Introduction

The sap-stain fungus *Grosmannia clavigera* (Robinson-Jeffrey & Davidson) Zipfel, de Beer, and Wingfield (previously known as *Ophiostoma clavigerum* (Robinson-Jeffrey and Davidson) Harrington) is the most pathogenic fungus associated with the mountain pine beetle (MPB, *Dendroctonus ponderosae* Hopkins) (Lee et al. 2006a; Reid et al. 1967). The MPB and its pathogenic fungal associates are able to kill many pine species (Lee et al. 2006a; Mathre 1964; Owen et al. 1987; Rice et al. 2007a, 2007b; Strobel and Sugawara 1986; Yamaoka et al. 1990, 1995). In British Columbia alone, the MPB and its fungal associates have destroyed over 9.2×10^6 ha of mature pure and mixed lodgepole pine (*Pinus contorta* Dougl. ex Loud.) stands (B.C. Ministry of Forests and Range 2007). *Grosmannia clavigera* encounters low-oxygen conditions after it is introduced into the water-saturated environment of host pines. As the fungal associates grow in and stain the sapwood of infected trees, they impede water and mineral transport in these tissues (Amman 1978). The reduction in viable sapwood decreases the tree's ability to synthesize and mobilize secondary compounds. This reduces the speed at which a tree can deliver defense compounds to the infection site, which benefits the MPB (Christiansen et al. 1987; Huber et al. 2004). Variation in a tree's susceptibility to fungal colonization and variation in fungal pathogenicity are believed to be important determining factors for epidemic dynamics.

Infected trees respond to fungi and beetles through defenses that consist of preformed (primary) and induced (secondary) resins. Host trees attempt to flush out MPB and its fungal associates through the flow of preformed resins, whereas induced resins reduce the suitability of host tissues for growth and development of MPB fungi and beetle brood (Huber et al. 2004; Raffa and Berryman 1982; Shrimpton and Whitney 1967). The induced resin response involves the de novo synthesis of secondary compounds, which requires energy and nutrients (Christiansen et al. 1987). Lodgepole pine trees that resist MPB attack produce a strong induced response, whereas susceptible trees show a weaker response (Reid et al. 1967; Shrimpton 1978). Younger trees tend to produce stronger resin responses than older trees (Christiansen et al. 1987; Raffa and Berryman 1982, 1983; Shrimpton 1973). During an MPB attack, beetles land on trees and release aggregation pheromones to attract conspecifics. As attacking beetles accumulate, a lodgepole pine's ability to respond with preformed or induced resins decreases until the tree can no longer secrete additional resin (Raffa and Berryman 1982, 1983). The threshold density for a successful MPB attack is determined by the total defensive capabilities of a tree (Christiansen et al. 1987) but is likely influenced by the spread of pathogenic fungi as the MPB builds its gallery.

Fungal pathogens can overcome tree defenses, cause disease, and eventually kill a host tree. Characterizing pathogenic variation over large geographic areas in a forest epidemic is important to understand epidemic dynamics and to identify resistant trees. This task is a challenging one, because pathogenicity tests involve inoculating many trees at various densities with fungi. Usually fungal inoculations are performed manually with metal cork borers (Lieutier et al. 2004; Yamaoka et al. 1995). Pathogenicity tests using ophiostomatoid fungi have been carried out for short (2–3 months) and long (1 year) incubation times. Researchers using short incubation times tend to measure lesion length and (or) occlusion area since these symptoms develop relatively quickly (Bois and Lieutier 2000; Krokene et al. 1999; Lieutier et al. 2004; Reid et al. 1967; Rice et al. 2007a, 2007b; Solheim 1995; Solheim and Krokene 1998; Yamaoka et al. 1998). For longer incubation times, researchers tend also to measure moisture content and tree death since these symptoms take longer to develop (Kim et al. 2005; Lee et al. 2006a; Yamaoka et al. 1995). Shorter incubation periods are comparable to the length of time that colonizing beetles and fungi spend in trees before temperatures drop in the fall season, reducing activity and growth. Longer incubation periods allow more time for symptoms to develop and permit assessment of the over-wintering survival of the fungi being studied.

Variation in pathogenicity is poorly characterized for many fungal forest pathogens. Among MPB-associated fungi, only one study examined intraspecific variability (Rice et al. 2007a). That study focused on the ability of *G. clavigera* and *Ophiostoma montium* (Rumbold) von Arx to induce lesions after a short incubation time in mature jack pine (*Pinus banksiana* Lamb.), jack pine × lodgepole pine hybrids, and lodgepole pine. Jack pine is a major component of boreal forests in Canada and is now threatened by MPB. Rice et al. (2007a) reported that differences in lesion length caused by three isolates were not significant on lodgepole pine or jack pine, whereas one of the three isolates induced smaller lesions on hybrid pine. Thus, intraspecific variability was not observed on mature lodgepole pine.

In contrast to previous disease epidemics, in which most of the MPB-infested trees were more than 80 years old, in the current BC epidemic, young tree plantations between 20 and 55 years old have been heavily attacked (MacLaughlan and Brooks 2007). In the present study, we characterized the pathogenicity of five isolates of *G. clavigera* inoculated into young lodgepole pine trees. We measured three pathogenic indicators and collected data over both short and long fungal growth periods (7 and 48 weeks). We compared pathogenic symptom development trends in young versus mature lodgepole pines by assessing lesion length development in older trees over a short time period (7 weeks) for two of the isolates. Finally, we characterized the growth rates of the five isolates in a low-oxygen environment and over a range of temperatures using artificial media to determine intraspecific variation in a more controlled environment than living trees.

Materials and methods

Fungal isolates: sources, maintenance and growth tests on artificial medium

Five isolates of *G. clavigera* were used for pathogenicity testing (Table 1). ATCC 18086 is the type specimen of *G. clavigera* and was isolated from ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.) during an MPB outbreak in 1965 (Robinson-Jeffrey and Davidson 1968). The other four isolates were obtained from lodgepole pine trees infested

Table 1. *Grosmannia clavigera* isolates used in this study.

Isolate	Geographic location	Date isolated	Host of origin
ATCC 18086	Cache Creek, B.C.	1965	*Pinus ponderosa* infested by *Dendroctonus ponderosae*
KW 1407	Kamloops, B.C.	2001	*Pinus contorta* infested by *Dendroctonus ponderosae*
B5	Banff, Alberta	2003	*Pinus contorta* infested by *Dendroctonus ponderosae*
B20	Banff, Alberta	2003	*Pinus contorta* infested by *Dendroctonus ponderosae*
H55	Houston, B.C.	2003	*Pinus contorta* infested by *Dendroctonus ponderosae*

with MPB during the early 2000s. Isolate KW 1407 has been used to measure pathogenicity indicators in older lodgepole pine trees (Lee et al. 2006a), whereas isolates B5, B20, and H55 have been used in a population genetic study using amplified fragment length polymorphism (Lee et al. 2006b). The authors found that the majority of isolates (>100) belonged to genetic group 1, whereas only nine isolates belonged to genetic group 2. Isolates H55 and B20 were part of genetic group 1, and B5 was part of group 2 (Lee et al. 2006b). Isolates B5 and B20 were obtained from trees harvested near Banff, Alberta, and isolate H55 was obtained from a tree near Houston, British Columbia. Thus, a small amount of genetic and geographic variation was present in the isolates used for pathogenicity testing.

Isolates were stored at –80 °C in 20% glycerol. For field inoculation, 5 mm plugs were taken from colonies that had been grown on 2% Oxoid malt extract agar (OMEA) containing 33 g Oxoid malt extract agar and 10 g technical agar No. 3 in 1 L distilled H_2O (Oxoid Ltd., Hants, England) for 5–7 days at 20 °C. For growth at different temperatures and in low-oxygen environments, plugs were taken from cultures that had been placed at 4 °C for 1–3 months.

The growth rate of each fungal isolate was tested at 4, 10, 15, 20, 22.5, 25, 27.5, 30, and 37 °C. Mycelial plugs, 5 mm in diameter, were placed on 2% OMEA in 9 cm diameter Petri dishes. Two radial measurements per dish were taken daily for 5 days. Each isolate was plated in triplicate, and the experiment was repeated once. For statistical analysis, only growth rates after 4 days were used because this was usually the last day of growth before some fungal isolates reached the edges of dishes. To measure growth in low-oxygen environments, two small pieces of Tygon tubing were placed on the edges of Petri dishes to allow for air circulation. Dishes were placed in 2 L glass bells that were then sealed with silicone grease (Dow Corning Corp., Midland, Mich.). Air in the glass bells was replaced with pure nitrogen (99.99% N_2; Praxair, Mississauga, Ont.) that was circulated for ≥1 min prior to sealing. The glass bells were sealed for 4 days at 20 °C, after which two growth measurements per dish were taken. In control Petri dishes, no nitrogen was circulated into the glass bells. Each isolate was plated once per experiment. The experiment was repeated three times.

Study sites and inoculation of lodgepole pine trees

Young lodgepole pine trees were selected from the 1986 Dardanelles Lake Plantation, located northeast of Merritt, British Columbia, off highway 5A near the Stump Lake Ranch turnoff. The site slopes gently downward from the northwest. Trees were spaced 1.0 m from each other in straight lines. In 2005, trees were selected at random from four tree families, two of which had much higher 10 year diameter at breast height (DBH) growth than the other two tree families. Trees were inoculated between 4 and 7 July 2005 and felled on 9 June 2006 after approximately 48 weeks. In 2006, trees were all located within a 15 tree × 15 tree grid on the flatter, southeastern side of the test site to minimize microsite variation because no differences in tree family background were detected the year before (data not shown). Trees from 2006 were inoculated on 6 and 7 July 2006 and felled on 23 and 24 August 2006. All young trees during both years were inoculated with all five *G. clavigera* isolates.

Forty-three 20-year-old lodgepole pine trees were inoculated in the summer of 2005, and thirty-six 21-year-old lodgepole pine trees were inoculated in the summer of 2006. The DBH of trees inoculated in 2005 ranged from 4.5 to 14.6 cm, whereas the DBH of trees inoculated in 2006 ranged from 6.7 to 16.6 cm. None of the trees used in field tests or in the plantation showed signs of MPB attack. However, the area around the plantation, consisting predominantly of older lodgepole pines, was heavily infested with MPB.

Old lodgepole pine trees were selected from Ketchan Mountain, located about 35 km south of Merritt, British Columbia. Ten trees with diameter >18 cm at DBH from a 200 m × 200 m stand were randomly selected and inoculated with either isolate B5 or KW 1407. In this stand, the DBH of trees ranged from 19.1 to 31.8 cm. Trees with a DBH of ≥18 cm were on average 145 years old (J.A. McLean, Forest Sciences Centre, University of British Columbia, Vancouver, B.C., personal communication, 2006). Trees were inoculated on 24 June 2006, and lesions were measured on 8 August 2006, nearly 7 weeks after inoculation. Old trees were not harvested. No controls were used in this experiment because the aim was to compare relative lesion size between (i) isolates B5 and KW 1407 and (ii) young and old trees. Control lesions on mature trees from this site used in other experiments have shown minimal (<10 mm) lesion development (A. Plattner, J.-J. Kim, and S. DiGuistini, personal observations).

To inoculate all trees, a 0.5 cm cork borer was used to remove sections of bark from trees as described by Yamaoka et al. (1995). Agar plugs of 0.5 cm in diameter from 2% OMEA without (control) or with fungal hyphae were removed from Petri dishes and inserted into the opening with the mycelium facing the cambium. The bark section was replaced to seal the opening. In the area to be inoculated, all nonliving branches were trimmed using a branch cutter and the bark was smoothed using a rough brush. No noticeable resin secretion occurred from the removal of these

branches. For young trees at Dardanelles Lake, depending on their circumference, there were between 4 and 7 rows of inoculations, and each row contained between 4 and 10 plugs. Rows were separated vertically by 10–18 cm, depending on the spacing of the whorls of branches on the lower bole of the tree. Plug inoculations were spaced, on average, 3 cm apart. This is approximately equal to an inoculation density of 200 plugs/m^2, although the exact area of inoculation differed slightly between trees. Allowances had to be made when the presence of tree branches or stumps interfered with plug inoculation. After all inoculations were made, each ring of inoculations was covered with duct tape. For older trees at Ketchan Mountain, each tree received only one row of inoculations because of time constraints; however, because of the much larger DBH, young trees (38.9 ± 7.7) received about 1.5 times the number of plugs compared with old trees (23.8 ± 4.5).

Measurement of pathogenicity indicators

Phloem lesions were measured after peeling the bark off trees in the field with a chisel and blade. Lesions were measured from the top of the inoculation point to the darkened visible leading edge of the lesion. Only the uppermost inoculation points were used to measure lesion length because many lesions overlapped with one another.

Felled tree bolts of young lodgepole pine trees measuring approximately 1.3 m long were transported back to the laboratory and stored at –4 °C for 1–3 days before indicators of pathogenicity were analyzed. Ends of bolts were sealed with silicone. Three disks of approximately 3–6 cm in width were cut from the middle of each bolt. Two disks were sectioned at points where rows of fungal inoculations were present. One of these disks was used to measure occlusion area, and the other was used to measure moisture content. Disks were also used to reisolate fungi. The third disk was used in case one of the first two disks was damaged.

Disks for assessing sapwood occlusion area were soaked overnight in the dark in 1% TTC (2,3,5-triphenyltetrazolium chloride) (Sigma-Aldrich, Oakville, Ont.). Wood tissues that stained red were considered to be alive (Towill and Mazur 1975), whereas tissues remaining white were occluded and indicated fungal growth in the area. The following day, all disks were digitally photographed. The percentage of sapwood occlusion area was calculated as

$$[1] \qquad \text{Percent occlusion area} = \left(\frac{\text{TA} - \text{HWA} - \text{RA}}{\text{TA} - \text{HWA}}\right) \times 100$$

where TA is the total area, HWA is the heartwood area and RA is the red area. Areas were calculated using the imaging software in ImageJ (Rasband 2006).

Sapwood moisture content was measured by removing two pieces of tissue that did not include bark or heartwood area from a disk. These pieces were weighed, placed for 24 h at 105 °C, and weighed again. Moisture content was calculated as

$$[2] \qquad \text{Percent moisture content} = \left(\frac{\text{WM} - \text{DM}}{\text{DM}}\right) \times 100$$

where WM is wet mass and DM is dry mass.

For the trees from 2005 and 2006, three random wood samples were taken near inoculation points with or without fungi and were plated onto 2% OMEA for reisolating the fungi.

Statistical analyses

Comparisons among means were performed using an ANOVA (GLM procedures) with SAS software (SAS Institute Inc., Cary, N.C.) followed by a Bonferroni correction for multiple pairwise comparisons. Data from obvious outliers were removed before the analysis. These included cases where fungi failed to germinate on plugs inoculated into trees or where the presence of tree branches prevented the spread of fungal-induced lesions. Residual variances almost always followed a normal distribution; however, when variances did not satisfy tests of normality, data sets were power transformed according to Kuehl (1994) to improve the fit of the model. Arithmetic means and standard deviations are still reported even if power transformed means were used for tests of significance. All field measurements were nested within tree and tree family (in the first field season) or just tree (in the second field season). For temperature-related growth rates, comparisons were made at the individual temperature level. Mean growth rates at different temperature levels were not compared. Values for temperature-related growth rates and oxygen-deficient growth rates were nested within Petri dish number and replicate number. For Figs. 1 and 2, values are standardized as follows:

$$[3] \qquad \text{Standardized value} = \left(\frac{\text{Mean of pathogenic indicator}}{\text{Highest mean indicator value}}\right) \times 100$$

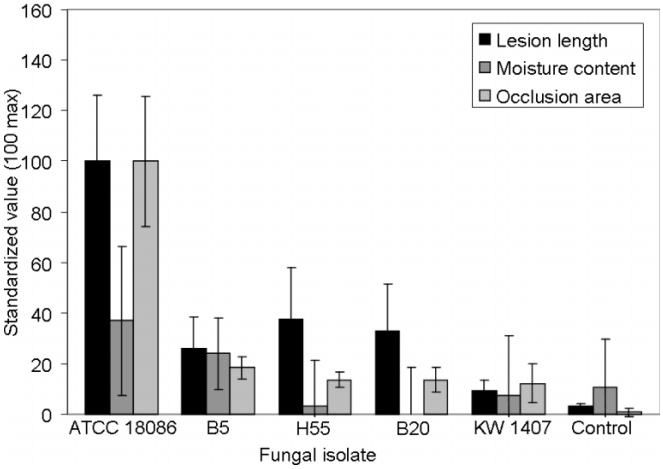
where the numerator is the mean value of a pathogenic indicator and the denominator is the highest mean indicator value from the five different fungal isolates and one agar control mean. For moisture content (because lower moisture content instead of a higher one indicates a more pathogenic isolate), the ratio was then subtracted from 100. Hence, on each graph, the most pathogenic isolate will have a lesion length and an occlusion area value of 100 and a moisture content value of 0. Standard deviations were standardized in the same manner to keep the linear relationship between mean values and standard deviations.

Results

Measurement of pathogenicity indicators at 7 weeks in young trees

For young trees harvested 7 weeks after fungal inoculations, isolate ATCC 18086 induced significantly longer lesions and larger occluded areas than all of the other isolates (Fig. 1). This isolate also induced a greater decrease in the sapwood moisture content than the other isolates, but the differences were not statistically significant (Table 2). For all other isolates, lesion length, sapwood occlusion area, and moisture content were not significantly different from each other. For lesion length and occlusion area, controls produced significantly lower measures compared with all isolates except for the lesion lengths of isolate KW 1407.

Fig. 1. Standardized values after 7 weeks of incubation with *Grosmannia clavigera* inoculated at 200 holes/m^2 in young lodgepole pine trees for phloem lesion length, sapwood moisture content, and sapwood occlusion area for five fungal isolates and agar controls. The maximum value was defined as the largest mean value for each pathogenic indicator among the five isolates according to eq. 3. Error bars are standardized standard deviations of pathogenic indicator means. Statistical groupings are given in Table 2.



For moisture content, none of the isolates nor controls was significantly different from one another. For isolate ATCC 18086, lesion length, occlusion area, and moisture content were 57.3 ± 14.9 mm (mean ± SD), 27.8% ± 7.2%, and 88.1%, respectively. For the other four isolates, the values were 15.2 ± 10.3 mm, 3.7% ± 1.4%, and 127.7% ± 29.2%, respectively. For controls, the values were 1.9 ± 0.6 mm, 0.2% ± 0.5%, and 125.3% ± 27.2%, respectively.

Measurements of pathogenicity indicators at 48 weeks in young trees

In young trees harvested 48 weeks after inoculation, isolates ATCC 18086, B5, and H55 consistently produced longer lesions, larger occlusion areas, and lower moisture content than isolates KW 1407 and B20 (Fig. 2, Table 2). Lesion length, occlusion area and moisture content were 201.1 ± 103.0 mm, 90.0% ± 15.2% and 39.5% ± 13.0%, respectively. Isolates KW 1407 and B20 induced less pronounced symptoms. Lesion length, occlusion area, and moisture content were 44.6 ± 26.6 mm, 4.0% ± 4.1% and 143.7% ± 21.3%, respectively. Agar controls had lower values for lesion length (5.0 ± 1.4 mm) and occlusion area (1.0% ± 1.9%) and higher moisture content (181.7% ± 25.4%).

After 7- and 48-week incubation periods, all isolates were successfully reisolated near inoculation points from tree disks in both years.

Measurements of pathogenicity indicators at 7 weeks in old trees

In older lodgepole pine trees 7 weeks postinoculation, isolate B5 induced significantly longer lesions than isolate KW 1407 (Fig. 3). The lesion length from all five trees inoculated with isolate B5 was 48.9 ± 29.1 mm, whereas the lesion length for all five trees inoculated with isolate KW 1407 was 10.46 ± 10.8 mm. For isolate B5, lesion lengths ranged from 30.2 ± 19.3 mm to 75.7 ± 26.3 mm per tree. For isolate KW 1407, lesion lengths ranged from 6.9 ± 5.3 mm to 16.2 ± 16.9 mm. Significant variation for isolate B5 was observed among trees.

Growth rates of *G. clavigera* at varying temperatures and in a low oxygen environment 4 days after inoculation

Isolate ATCC 18086 grew significantly faster than all of the other isolates from 10 to 22.5 °C. However, at 15 °C, there was no significant difference between the growth rates of isolates KW 1407 and ATCC 18086. At 25.0 °C, there were no differences in growth rates between all fungal isolates. At 27.5 °C, isolate ATCC 18086 grew the slowest, although it was not significantly different from isolate KW 1407 (Fig. 4). The other isolates had similar growth rates at 10, 22.5, 25, and 27.5 °C. At 15 °C and 20.0 °C, there was some variation among the growth rates of isolates. Isolates did not grow or grew poorly (<4 mm after 4 days) at 4, 30, and 37 °C; thus, these temperatures were not used for statistical comparisons. When isolates were incubated at 4 or 30 °C for 4 days and transferred to room temperature (~22 °C), normal growth resumed. Isolates incubated at 37 °C did not grow at all even after being transferred to room temperature.

Under low-oxygen conditions, all isolates except ATCC 18086 grew significantly faster than under normal oxygen conditions (Fig. 5). In both the low-oxygen and normal environments at 20 °C, isolate ATCC 18086 grew significantly faster than all of the other isolates. The growth rates of the other isolates did not differ significantly from each other.

Notes from the first field season

During the first field season, all trees used for field testing originated from four tree families, two of which had very high 10-year DBH growth rates, and two of which had lower than normal 10-year DBH growth rates. Trees with a high 10-year DBH are more likely to resist MPB attack than trees with a low 10-year DBH. Tree family background was initially used as a covariate, but there was no significant difference in lesion length, moisture content, or occlusion area among trees from high 10-year DBH growth rates or low 10-year DBH growth rates that had been inoculated with the same fungal isolate (data not shown). Tree family background was ignored for the second field season. Instead, trees were selected from a smaller area to minimize microsite differences among trees. During the first season, to accommodate tree family background, trees were selected up to 1 km apart, and they were either above, on, or at the bottom of a slope. All trees from the second field season were located at the bottom of the slope. DBH was also used as a covariate for both the first and second field seasons, but DBH was not found to be related to any of the three pathogenic symptoms measured (results not shown; all $p > 0.70$).

Discussion

This study addressed a concern related to large-scale ecological issues in the current MPB epidemic, namely heavy MPB attack on young lodgepole pine trees. The results de-

Table 2. Lesion length, sapwood moisture content, and sapwood occlusion area induced by *Grosmannia clavigera* in 20- to 21-year-old lodgepole pine after either 7 or 48 weeks of incubation.

	G. clavigera isolate					
	ATCC 18086	B5	H55	B20	KW 1407	Control
7 weeks incubation						
Phloem lesion length (mm)						
Mean ± SD	57.3±14.9 a	15.0±7.1 b	21.6±11.6 b	19.0±10.5 b	5.3±2.4 bc	1.9±0.6 c
No. of trees	6	6	6	6	6	6
No. of measurements	39	36	39	34	38	34
Sapwood moisture content (%)						
Mean ± SD	88.1±41.3 a	106.2±19.8 a	135.4±25.4 a	139.9±26.0 a	129.7±33.2 a	125.3±27.2 a
No. of trees	6	6	6	6	6	6
No. of measurements	12	12	12	12	12	12
Sapwood occlusion area (%)						
Mean ± SD	27.8±7.2 a	5.1±1.2 b	3.8±0.8 b	3.8±1.4 b	3.4±2.1 b	0.2±0.5 c
No. of trees	6	6	6	6	6	6
No. of measurements	12	12	12	12	12	12
48 weeks incubation						
Phloem lesion length (mm)						
Mean ± SD	193.6±85.4 a	200.0±103.0 a	207.8±116.0 a	45.4±30.9 b	43.2±18.3 b	6.0±3.5 c
No. of trees	7	7	8	8	5	6
No. of measurements	35	35	45	49	37	37
Sapwood moisture content (%)						
Mean ± SD	38.8±13.5 a	40.1±14.4 a	39.7±12.3 a	143.3±22.3 b	144.5±20.7 b	181.7±25.4 c
No. of trees	8	6	8	8	5	6
No. of measurements	16	12	16	16	9	12
Sapwood occlusion area (%)						
Mean ± SD	89.1±15.4 a	94.0±8.5 a	87.5±19.3 a	4.3±3.2 b	3.6±5.3 b	1.0±1.9 b
No. of trees	8	7	8	8	6	6
No. of measurements	16	14	16	16	12	12

Note: Means and SDs within a row with the same letter are not significantly different from one another at the 0.05 level.

scribed are the first to characterize intraspecific variability in the pathogenicity of a MPB fungal associate towards young lodgepole pine. For *G. clavigera*, we found statistically significant variation for all pathogenic symptoms measured, except for moisture content after 7 weeks.

Only one other study has examined intraspecific variation on lodgepole pine (Rice et al. 2007a). Our results differed from those of Rice et al. (2007a), which indicated that no intraspecific variation was present on mature lodgepole pine and jack pine that had been inoculated with three isolates of *G. clavigera*. The differences in results may be due to differences in objectives and methods. Rice et al. (2007a) inoculated older trees (~50 years in age), which are currently the major class of trees threatened by the MPB in Alberta. To characterize the ability of *G. clavigera* and *Ophiostoma montium* to colonize mature pines from three species, they used six isolates (three *G. clavigera* isolates and three *O. montium* isolates), had a positive and negative control on each tree, and measured only lesion development. As noted above, we worked with younger lodgepole pine trees. We reduced the risk of cross contamination and interactions among fungal isolates by inoculating a single fungal isolate per tree. We used a high inoculation density (200 holes/m^2) and measured three pathogenicity indicators. Some authors have suggested a beneficial link between corroborating results from lesion length to other pathogenic metrics (Langstrom et al. 1993; Solheim 1988).

Using large numbers of isolates to examine intraspecific variability has resulted in pathogenicity metrics being distributed across a range of values (Lieutier et al. 2004; Sallé et al. 2005). However, characterizing such variability requires an appropriate experimental design, such as using a large number of isolates and hosts, and this may not always be feasible for field work. In the work reported here, pathogenicity metrics after 48 weeks were not distributed across a range but, rather, appeared as either pathogenic or less pathogenic. Sapwood occlusion area was either >85% or <5%, phloem lesion length was either approximately 200 or 45 mm, and sapwood moisture content was either approximately 40% or 145%. Isolates ATCC 18086, B5, and H55 caused greater pathogenic symptom development and were most likely to be capable of killing young trees after a long incubation period at the density tested. In contrast, isolate KW 1407 and B20 caused weaker pathogenic symptom development. They were usually contained in the reaction zone or spread minimally in the phloem or sapwood. However, only five isolates of *G. clavigera* were tested. It is possible that increasing the number of isolates would result in some isolates producing intermediate values for pathogenicity indicators.

Fig. 2. Standardized values after 48 weeks of incubation with *Grosmannia clavigera* inoculated at 200 holes/m^2 in young lodgepole pine trees for phloem lesion length, sapwood moisture content, and sapwood occlusion area for five fungal isolates and agar controls. The maximum value was defined as the largest mean value for each pathogenic indicator among the five isolates according to eq. 3. Error bars are standardized standard deviations of pathogenic indicator means. Statistical groupings are given in Table 2.

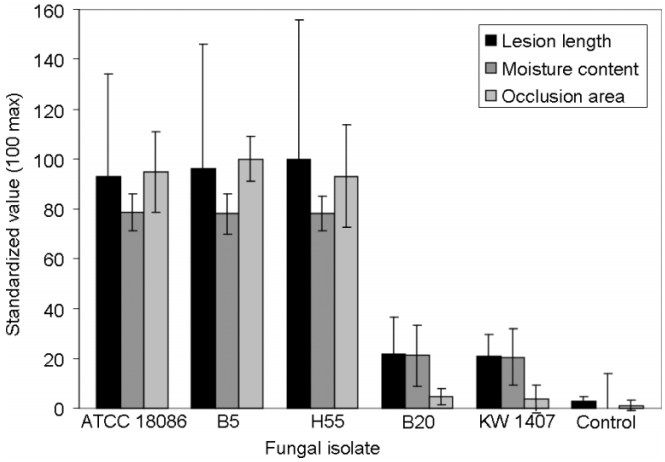
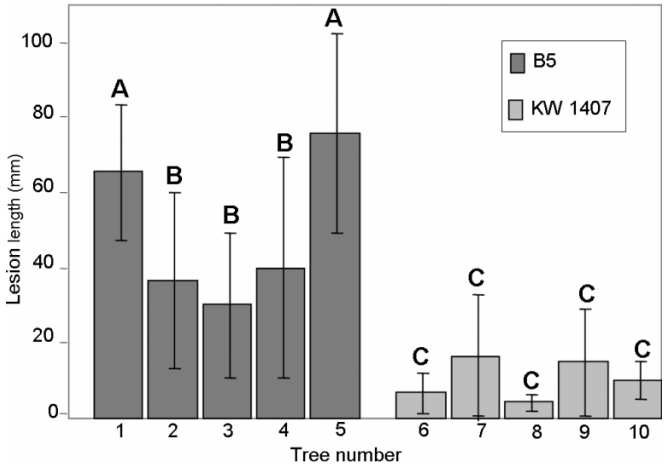


Fig. 3. Mean phloem lesion length in ~145 year old lodgepole pines inoculated with two isolates of *Grosmannia clavigera* at 200 holes/m^2 after 7 weeks. Results from five individual trees are shown because of the large amount of variation in lesion length between trees. Tree age, health, amount of sunlight received, and diameter at breast height (DBH) were more variable compared with young trees from the plantation and likely account for a large amount of the variation seen among individual trees. Lesion lengths induced by isolate B5 were always significantly longer than lesions induced by isolate KW 1407. Bars with the same letter are not significantly different at the 0.05 level according to a multiple means comparison using a Bonferroni correction. Error bars are SDs. Trees received 18–30 holes/tree, depending on DBH.



Variation in intraspecific pathogenicity has been reported for other species of bark beetle associated ophiostomatoid fungi. Of these, the most extensively studied are *Ceratocystis polonica* (Siemaszko) Moreau and *Leptographium wingfieldii* Morelet. The former is a fungal associate of *Ips typographus* L. that kills Norway spruce (*Picea abies* (L.) Karst.) trees. The latter is a fungal associate of the pine shoot beetle (*Tomicus piniperda* L.) that affects Scots pine (*Pinus sylvestris* L.) and other pines and has been found recently in North America (Hausner et al. 2005; Krokene and Solheim 2001; Lieutier et al. 2004). Although *L. wingfieldii* killed healthy trees when artificially inoculated at a density of 400 inoculations/m^2, its ecological role in beetle-attacked

Fig. 4. Total growth rates of five isolates of *Grosmannia clavigera* grown from 10 to 27.5 °C on 2% Oxoid malt extract agar after 4 days. Within each temperature, mean growth values with the same letter are not statistically different at the 0.05 level. Error bars are SDs. No comparisons were made between fungal isolates at different temperatures. Little to no growth was observed at 4, 30, and 37 °C. For the experiment, triplicates of each fungal isolate were used at each temperature, and the entire experiment was repeated once.

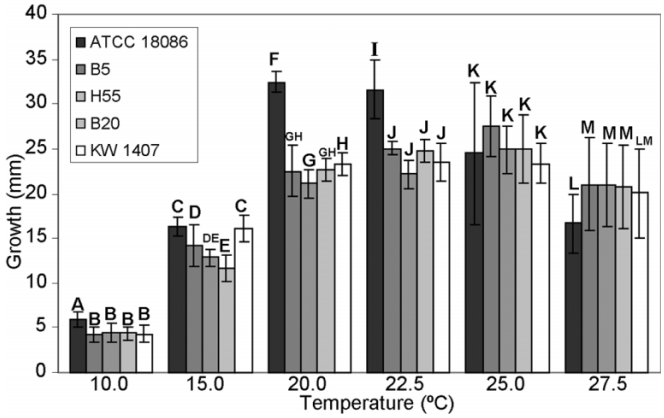
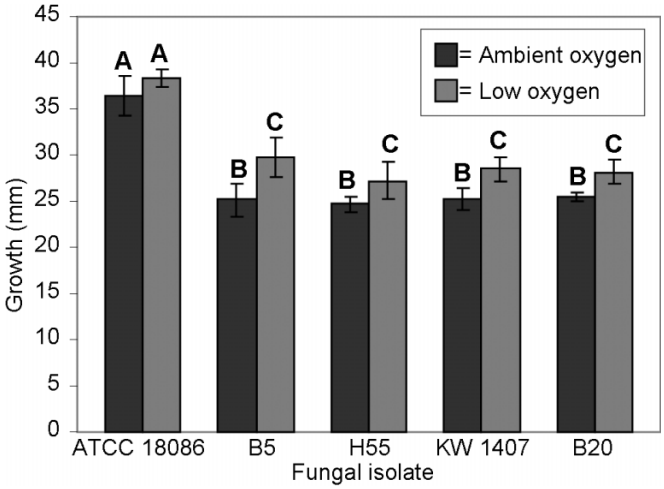


Fig. 5. Total growth rates of five isolates of *Grosmannia clavigera* grown at 20 °C on 2% Oxoid malt extract agar in ambient and low-oxygen environments after 4 days. Bars with the same letter are not significantly different at the 0.05 level. Error bars are SDs. Because of space constraints in glass bells, each fungal isolate was only plated once per experiment. The entire experiment was done in triplicate.



trees is still unresolved. Previous studies have reported a range of variation when examining intraspecific variation. When Krokene and Solheim (2001) inoculated six isolates of *C. polonica* into Norway spruce, lesion length and occlusion area allowed isolates to be characterized as nonpathogenic, of intermediate pathogenicity, or pathogenic. Lieutier et al. (2004) worked with *L. wingfieldii* isolates over 2 years of study. In the first year, using six isolates, the occlusion area covered approximately 0%, 25%, or 50% of sapwood area, but the differences were not statistically significant because of the small sample size. In the second year, using 15 isolates, lesion length and occlusion area varied over a large range of values (Lieutier et al. 2004). Using more isolates resulted in pathogenic metrics having a broader distribution as opposed to being pathogenic or less pathogenic.

Patterns of pathogenicity symptoms differed between short and long incubation periods of *G. clavigera* in younger trees. After 7 weeks, isolate ATCC 18086, but not H55 and B5, consistently appeared to be the most pathogenic, inducing longer lesions and occluding more sapwood area. For sapwood moisture content, isolates and controls did not differ significantly. Isolate ATCC 18086 induced lower moisture content, but this was not statistically significant. Moisture content was the least sensitive of the metrics tested. More time is likely needed for the sapwood colonizing fungi to affect water-conducting cells and moisture content. In previous studies where moisture content was measured, data were recorded after approximately 1 year, providing more time for the fungi to impede water transport and reduce moisture content (Kim et al. 2005; Lee et al. 2006a). However, trees used in previous studies were older than the trees used in the present study.

Using younger trees appeared to affect the rate of pathogenic symptom development. Younger trees tend to respond more vigorously to fungal invasion than older trees (Christiansen et al. 1987; Raffa and Berryman 1982, 1983; Shrimpton 1973). Overcoming a stronger resin response likely requires more time. Thus, 7 weeks postinoculation, lesion lengths were about three times longer in older trees than in younger trees, indicating that lesions developed faster in older trees. Consistent with the pattern observed in younger trees after 48 weeks, isolate B5 induced significantly longer lesions in older trees than isolate KW 1407. This trend was evident but not statistically significant in younger trees after 7 weeks. The more rapid development of pathogenic indicators in older trees is consistent with results from the literature. Shrimptom (1973) reported that trees aged 41–60 years were about three times more resistant to inoculations with blue-stain fungi than trees aged 111–140 years. In other studies, lesions were longer and sapwood area was nearly totally occluded after 6–8 weeks for 50- to 150-year-old lodgepole pines (Reid et al. 1967; Rice et al. 2007a; Solheim 1995). Older trees are more likely to have fewer energy reserves that can be mobilized in defense responses (Christiansen et al. 1987). For future field experiments, researchers should be aware of these differences and allow for a longer time for symptoms to develop in younger trees. It also suggests that younger trees are better able to defend themselves against MPB attack and that a larger number of MPB are needed to colonize young trees. Unfortunately, the number of MPB present in British Columbia is now sufficiently high to allow for the successful attack of young lodgepole pine trees. However, the ability of MPB to overwinter in younger trees with thinner bark is unknown. It is possible that younger trees are a sink for MPB rather than a source if fewer new adults emerge from colonized trees compared with the number of beetles required to successfully colonize the tree.

Intraspecific variability in ophiostomatoid fungi has also been examined in vitro, where conditions are more controlled. Sallé et al. (2005) observed intraspecific variation for two ophiostomatoid species in their ability to tolerate resveratrol. For MPB-related fungi, Solheim and Krokene (1998) found some visual variation among isolates in their ability to grow in oxygen-deficient conditions or at different temperatures; however, only two isolates of *G. clavigera* and *O. montium* were used, and differences between isolates were not compared statistically. A low-oxygen environment is typical of the phloem and sapwood of healthy trees before bark beetle attack. Our results on *G. clavigera* are consistent with previous work. This primary sapwood invader grows well in a low-oxygen environment, giving it an advantage when colonizing host trees (Solheim and Krokene 1998). Intraspecific growth rate variability of *G. clavigera* was observed in this environment. This is possibly influenced by the more rapid growth rate of isolate ATCC 18086 at 20 °C rather than this isolate tolerating a low-oxygen environment to a greater extent than other isolates. The intraspecific variation in growth rates over a range of temperatures suggests that at least some of the isolates are well suited to accompany the MPB as it expand its geographic range.

Isolates from the current epidemic are more likely to be representative of the range of pathogenicity in current populations of *G. clavigera*. Thus, isolates H55 and B5 were more pathogenic than isolates KW 1407 or B20. Differences were not observed in young trees after a short incubation time likely because not enough time was given for symptoms to develop. In older lodgepole pine, isolate B5 induced longer lesions than isolate KW 1407. In the study by Rice et al. (2007a) on older hybrid pine, isolate B5 induced longer lesions than B20.

The technique used in the current work for measuring lesion length differs from studies where only a single row of inoculations is performed on each tree. In these studies, the total lesion length above and below the inoculation point is often measured as opposed to measuring one-half of the lesion from the inoculation point to the top of the lesion. This may lead to lesion length inconsistency when data are reported in the literature.

Overall, isolate ATCC 18086 was the most pathogenic isolate, whereas isolate KW 1407 was the least pathogenic. However, when inoculated at a density of 800 inoculations/m^2, isolate KW 1407 was capable of killing mature lodgepole pine trees (Lee et al. 2006a), demonstrating its pathogenicity. This indicates that some isolates of *G. clavigera* may be more pathogenic than previously reported. If MPB transmits more virulent isolates, then fewer beetles may be needed to successfully colonize trees. However, isolate ATCC 18086 was recovered during a previous MPB epidemic and from a different host tree. No isolates

tested from the current epidemic so far were as virulent as ATCC 18086.

Isolates of lower pathogenicity could be useful when managing small urban or plantation areas, where individual trees are of importance. Raffa and Berryman (1983) showed that low- to medium-density inoculations of *G. clavigera* prior to beetle attack improve a tree's ability to respond to mass attack. A similar enhanced defense reaction was reported for Norway spruce inoculated with *Ceratocystis polonica* (Krokene et al. 1999) and for Scots pine trees inoculated with either *Leptographium wingfieldii* or *Ophiostoma canum* (Münch) H. Sydow & P. Sydow (Krokene et al. 2000). Using isolates of lower pathogenicity may stimulate and enhance the tree's defensive capabilities against future MPB invasion without largely affecting overall tree productivity and health. Intraspecific variation may also be beneficial in research aiming to link genetic factors with pathogenicity. For example, differences in expression profiles between highly pathogenic and less pathogenic isolates may help to identify fungal genes associated with pathogenicity.

The current study has demonstrated that five isolates of *G. clavigera* inoculated into lodgepole pine varied in their ability to induce pathogenic responses. Intraspecific variation was detected for all parameters tested, both in the field and in the laboratory. Isolate KW 1407 was less pathogenic than other *G. clavigera* isolates and may be a good candidate for future tests aiming to enhance a tree's defensive capabilities. Isolate ATCC 18086 was highly pathogenic and would be a good candidate for future studies on gene expression involved in pathogenicity. The development of pathogenicity indicators appears to take longer in younger trees than in older trees.

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