Scandinavian Journal of Forest Research
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713711862

Comparison of two methods to assess the virulence of the mountain pine beetle associate, **Grosmannia clavigera**, to **Pinus contorta**

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Online Publication Date: 01 April 2008
To cite this Article: Kim, Jae-Jin, Plattner, Alex, Lim, Young Woon and Breuil, Colette (2008) 'Comparison of two methods to assess the virulence of the mountain pine beetle associate, **Grosmannia clavigera**, to **Pinus contorta**', Scandinavian Journal of Forest Research, 23:2, 98 - 104
To link to this article: DOI: 10.1080/02827580701743153
URL: http://dx.doi.org/10.1080/02827580701743153

Scandinavian Journal of Forest Research, 2008; 23: 98–104

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ORIGINAL ARTICLE

Comparison of two methods to assess the virulence of the mountain pine beetle associate, *Grosmannia clavigera*, to *Pinus contorta*

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Abstract

Characterization of the virulence of bark beetle-vectored fungi is important for assessing potential impacts of beetle outbreaks. Massive inoculation of trees with a cork borer appears to give the most accurate estimate of fungal virulence, but cork borer inoculation is time and labor intensive. In October 2003, 18 *Pinus contorta* var. *latifolia* were inoculated with a beetle-associated fungus, *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel et al., at densities of 200 and 800 cork borer holes m^{-2}. In July 2004 nine trees were inoculated using bark flap inoculations. The fungal-induced moisture content reduction, sapwood occlusion area and needle discoloration were similar for the 800 cork borer holes m^{-2} density and for bark flap inoculations, while pathogenicity symptoms induced by the 200 cork borer holes m^{-2} were less intense. Bark flap inoculations were three times faster to perform than high-density cork borer inoculations, but differences in incubation time and yearly weather variation highlight the need for further studies. The bark flap method may be an efficient alternative to using massive inoculation densities when testing the ability of specific fungi to kill hosts, while the cork borer method may be a better method to assess pathogenic symptoms or the aggressiveness of specific fungi.

Keywords: *Bark flap method, blue stain, cork borer, fungus, Grosmannia clavigera, mountain pine beetle.*

Introduction

British Columbia (BC) is experiencing the largest outbreak of mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (MPB) in recorded history, with 9.2 million ha of pure and mixed stands of lodgepole pine (*Pinus contorta* var. *latifolia* Engelm.) already attacked by the MPB as of 2007 (Ministry of Forests and Range, 2007). The MPB is found in close association with *Grosmannia clavigera* (Robinson-Jeffrey and Davidson) Zipfel et al., *Ophiostoma montium* (Rumbold) von Arx and *Leptographium longiclavatum* Lee Kim and Breuil, all of which are pathogenic, sap-staining ophiostomatoid fungi (Mathre, 1964; Reid et al., 1967; Basham, 1970; Strobel & Sugawara, 1986; Owen et al., 1987; Yamaoka et al., 1990, 1995; Lee et al., 2006*a*, 2006*b*; Zipfel et al., 2006). *Grosmannia clavigera* has been studied the most extensively among all the fungal associates of the MPB because it is considered to be more aggressive than either *O. montium* (Reid et al., 1967; Owen et al., 1987; Yamaoka et al., 1990, 1995; Solheim, 1995; Solheim & Krokene, 1998) or *L. longiclavatum* (Lee et al., 2006).

Sapwood moisture content, sapwood occlusion area and needle discoloration are pathogenicity indicators that have been used to assess the virulence of sap-staining ophiostomatoid fungi (Yamaoka et al., 1990, 1995; Krokene & Solheim, 1997; Bois & Lieutier, 2000; Solheim et al., 2001; Fernandez et al., 2004; Lieutier et al., 2004; Lee et al., 2006*a*). Sap-staining fungi grow inside the sapwood and phloem of trees and impede water and nutrient transport (Amman et al., 1990; Langstrom et al., 1993; Paine et al., 1997), leading to a reduction in sapwood moisture content and the occlusion of host cells. As adult beetles construct vertical galleries to lay eggs, they disseminate fungal spores and mycelia, resulting in narrow, vertical inoculations of the fungi

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(Received 29 May 2007; accepted 7 October 2007)

ISSN 0282-7581 print/ISSN 1651-1891 online
DOI: 10.1080/02827580701743153

at the surface of the sapwood. Further growth of fungi in the tree also results in foliage discoloration, as needles turn from green to yellow and then red owing to continued moisture loss (Amman et al., 1990). Since bark beetles carry many fungal species, it is difficult at first to assess which species are pathogenic and thus responsible for participating in tree decline.

Rapid characterization of the virulence of the many fungal species associated with bark beetles is important at the onset of new epidemics. Most pathogenicity tests involving ophiostomatoid fungi have been conducted using a cork borer method (Christiansen et al., 1985*a, b*; Horntvedt, 1988; Yamaoka et al., 1990, 1998; Solheim et al., 1993, 2001; Lanstrom et al., 1993; Krokene & Solheim, 1997, 2001; Bois & Lieutier, 2000; Fernandez et al., 2004; Lieutier et al., 2004; Lee et al., 2006*a*). This method is time and labour intensive since a high density of holes is generated manually and then inoculated with fungi. Testing threshold densities, the density of inoculations necessary to result in tree death (Christiansen et al., 1987), typically requires a fungal inoculation level up to 800 holes m^{-2} (Solheim et al., 1993, 2001; Bois & Lieutier, 2000; Lee et al., 2006*a*). Testing enough trees at high inoculation levels requires a large amount of time and labour, and may not be feasible when financial or time resources are limited. However, it is important to deliver massive inoculations of fungi when testing pathogenic symptoms, since increasing levels of inoculation tend to result in stronger pathogenic symptom development and are likely to simulate decreases in tree defense resources facing massive beetle attacks (Christiansen, 1985*a*, *b*; Solheim et al., 1993; Krokene & Solheim, 1998). An alternative to the cork borer method is the bark flap inoculation method, where flaps of bark are peeled back from trees and inoculated with fungi. Two studies have used this technique to demonstrate the virulence of *G. clavigera* (Yamaoka et al., 1995) and *O. montium* (Strobel & Sugawara, 1986) to lodgepole pine. Although the cork borer and the bark flap inoculation have been used for assessing fungal virulence, the efficiency of the two methods has not been compared.

The aim of this study was to compare the pathogenic effects of fungal inoculation when performed at a density of 200 or 800 cork borer holes m^{-2} and using bark flap inoculations. Measures of virulence included reductions in sapwood moisture content, percentage occluded sapwood and needle discolouration. Results are discussed with regard to the time needed to perform each technique.

Materials and methods

Growth of inoculum

The fungus *G. clavigera*, strain SL-KW 1407 (= DAOM 234193) was grown on 2% Oxoid malt extract agar (OMEA, 33 g Oxoid malt extract agar, 10 g technical Agar #3, 1 liter distilled H_2O; Oxoid, Hants, UK) for 1 week before being inoculated into trees (Lee et al., 2006*a*). This strain was isolated in Kamloops, BC, in 2001, from lodgepole pine sapwood that had been attacked by the MPB.

Fungal inoculation of lodgepole pine

In both years, all trees used for testing fungal virulence were healthy before inoculations. The field site was located near Kamloops, BC. Between 29 September and 1 October 2003, 12 lodgepole pines were inoculated with *G. clavigera* and six with sterile agar. Half of the trees were inoculated at a density of 200 cork borer holes m^{-2}, and the other half were inoculated at a density of 800 cork borer holes m^{-2}. The field site was inaccessible earlier in the year because of forest fires in the region. Trees from 2003 had a mean diameter at breast height (dbh) of 18.8 (14.5–26) cm and an average age of 116 (98–130) years. Starting at dbh, a 5 mm metal cork borer was used to remove sections of wood from the outer bark down to the cambium in a 60 cm wide band encircling the tree. Bark pieces were replaced over holes after inoculum was inserted and the entire area was covered with duct tape. The average number of holes per tree was 71 and 283, respectively, for trees inoculated at 200 and 800 cork borer holes m^{-2}. The distance between evenly spaced inoculation points was 3.1 and 7.1 cm at high and low density, respectively. On 7 July 2004, 280 days after inoculation, trees were felled and bolts of approximately 1.4 m were transported back to the laboratory for further analysis.

On 5 July 2004, five lodgepole pines were inoculated with *G. clavigera* and four with sterile agar using the bark flap inoculation method. To insert fungal inoculum, a three-sided flap (2×10 cm) was cut into the bark (Figure 1) and peeled back using a chisel. Flaps were evenly spaced in a ring around the tree, with 4–5 cm between flaps. A second ring was cut 20 cm below the first ring. Rings were spaced to avoid vertical lesion overlap between upper and lower rings. Under each flap, a square of MEA (2×2 cm) with fungal inoculum was smeared from the top to the bottom of the inner bark. The flap was flattened back over the inoculated area. The area inoculated with fungi, comprising both rings of bark flap inoculations and the intervening space, was covered with duct tape to prevent infection or

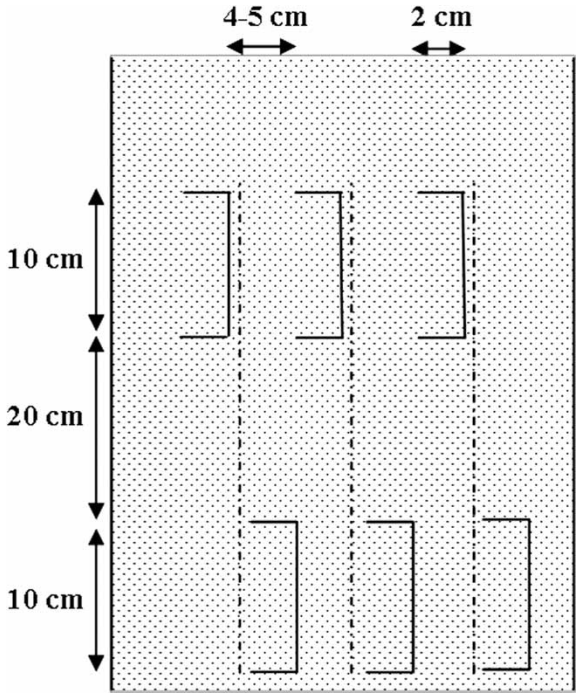

Figure 1. Bark flap inoculation pattern used for inoculating *Grosmannia clavigera* into *Pinus contorta* trees. Each bark flap measured 2 × 10 cm with 4–5 cm between flaps. Two rings of flaps separated by 20 cm were made around the tree. Since the majority of lesion development occurs in a vertical manner, the upper and lower flaps were spaced vertically to avoid overlapping fungal growth.

invasion by secondary beetles. Each tree received, on average, six to seven bark flap inoculations per ring for a total of 12–14 total bark flap inoculations per tree, resulting in approximately 260 cm^2 of area inoculated with fungi in a 40 cm band completely circling the tree at dbh. This is equivalent to 48 flaps m^{-2} or 960 cm^2 of fungal inoculum m^{-2} of tree surface. Control trees received the same peeling as treated trees but were inoculated with fungus-free media. Trees inoculated in 2004 had an average dbh of 18 (13–22) cm and an average age of 145 (121–161) years. All trees were felled on 3 July 2005, 363 days after inoculation. The weather conditions during these incubation periods are shown in Table I.

Measuring pathogenic symptoms

Occlusion area and moisture content were determined using the same procedures as Lee et al. (2006*a*). In brief, tree bolts of 1.4 m were cut and transported back to the laboratory. The occluded sapwood area on stem disks cut near inoculation points or flaps was determined after tracing the area on Mars Vellum paper (Staedtler, Mississauga, ON, Canada), cutting the drawings and weighing them. To determine the living sapwood area, stem disks were immersed overnight in 1% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC; Sigma-Aldrich, Oakville, ON, Canada) solution in the dark. Regions showing red staining were considered viable, as TTC stains living plant cells red while non-viable cells remain whitish in appearance (Towill & Mazur, 1975).

Moisture content was determined by removing pieces of sapwood near fungal inoculation areas and weighing them immediately. Pieces of wood were then dried overnight at 95°C and reweighed the following day. Moisture content was determined as a percentage by subtracting the dry weight from the wet weight and then dividing by the dry weight. Normal sapwood moisture content in lodgepole pines ranges from 85 to 150% (Reid, 1961).

In November 2003 and April 2004, the foliage colour was assessed for trees inoculated using the cork borer. Trees were left standing to allow symptoms to develop further. The foliage colour was assessed in October 2004 and April 2005 and on 20 May 2005 for trees inoculated using the bark flap method. Trees with green crowns were considered still viable, while trees with yellow or red needles were considered to be dead. Yellow needles are a precursor to red needles, which is usually an indicator of successful beetle attack and tree mortality (Amman et al., 1990).

Fungal reisolation

Reisolation points near fungal inoculum were selected from bolts and transported back to the laboratory. Three points were selected from each

Table I. Average monthly temperature and total monthly precipitation during the two field seasons.

	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	June
Average monthly temperature (°C)												
Season 1				11.1	−1.6	−0.7	−4.3	0.1	7.2	12.1	14.8	20.8
Season 2	23.4	22.2	14.4	8.7	3.3	0.1	−5.6	1.1	6.8	10.9	16.5	17.2
Total monthly precipitation (mm)												
Season 1				15.1	26	3.9	32.4	13.4	11.2	11.5	52.1	23.4
Season 2	39.6	55.5	38.9	30.8	39.1	31	24.1	17	16.6	0.2	19.7	86.2

Note: season 1 started in October 2003 and ended in June 2004. Season 2 started in July 2004 and ended in July 2005. Temperatures were similar between the two field seasons, but season 2 was wetter than season 1.

tree. The wood was placed on 2% malt extract agar and incubated at 22°C for 1 week before being re-examined for the presence of fungi.

Statistical analysis

The normality of data sets was verified using the Shapiro–Wilk p-value and visually inspecting normality plots of residuals for outliers. Comparisons between all treatment and control means were performed using an anova (GLM procedures) using SAS software followed by a Bonferroni correction for multiple pairwise comparisons.

Treatments were categorized by inoculum type (control or *G. clavigera*), and method and density (200 holes m^{-2}, 800 holes m^{-2} or bark flap inoculation).

A significance threshold of 0.05 was used. Pairwise comparisons were limited to (1) treatment versus control for the same inoculation level (three comparisons), (2) within treatment (three comparisons) and (3) within controls (three comparisons), for a total of nine comparisons. As such, a Bonferroni significance level of 0.0055 (number of total comparisons divided by 0.05 acceptance level of error) was used when comparing pairwise significance levels.

Results

The average occlusion area for trees inoculated at 200 cork borer holes m^{-2} was much lower than for trees inoculated at 800 cork borer holes m^{-2} or using flap inoculations (Figure 2). Occlusion areas between trees inoculated at 800 holes m^{-2} and using bark flap inoculations were not significantly different ($p = 0.595$), whereas the occlusion areas of trees inoculated at 200 holes m^{-2} were significantly different ($p < 0.0001$ in both cases) from the other two treatments. Control trees inoculated at 200 cork borer holes m^{-2} had an average occlusion of 1.2%, an average occlusion area of 1.5% for trees inoculated at 800 holes m^{-2} and an average occlusion area of 1.6% for trees inoculated using flap inoculations. The occlusion areas for controls of all levels were not significantly different from each other (p ranged from 0.847 to 0.947). These values are grouped together in Figure 2. The occlusion area in treated trees was always significantly higher than it was in control trees ($p < 0.0001$ in all cases).

The average moisture content of trees receiving 200 cork borer holes m^{-2} of fungal inoculum was higher than in trees receiving 800 cork borer holes m^{-2} of fungal inoculum or in trees receiving bark flap inoculations (Figure 3). The sapwood moisture content of disks in trees that received bark flap

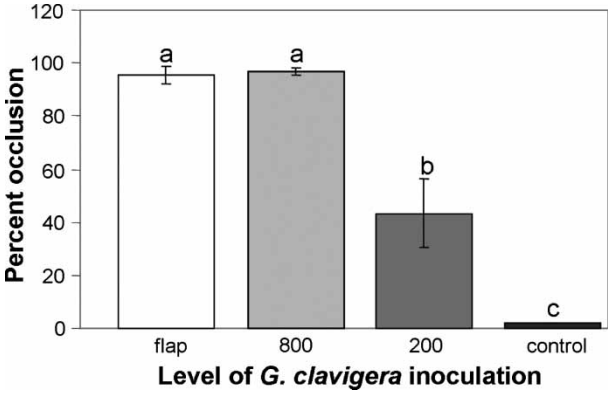

Figure 2. Percentage occlusion in stem disks of trees inoculated with *Grosmannia clavigera* at three inoculation levels: 200 and 800 cork borer inoculations m^{-2} and bark flap inoculation. Error bars represent means ± SD. Bars with the same letter above them are not significantly different at the 0.05 level. Flap = bark flap inoculation; 800 = 800 cork borer inoculations m^{-2}; 200 = 200 cork borer inoculations m^{-2}; Control = sterile agar control.

inoculations was not different from trees that received cork borer inoculations at 800 holes m^{-2} ($p = 0.922$). Using a significance level of 0.0055 based on Bonferroni corrections, comparisons between sapwood moisture content for inoculation at 200 and at 800 cork borer holes m^{-2} ($p = 0.006$) or bark flap inoculations ($p = 0.007$) were borderline significant. No differences were found between levels of moisture content in control trees receiving either cork borer density or flap inoculations (p ranged from 0.799 to 0.984). The moisture content in control trees was always significantly higher than in treatment trees (200 cork borer holes m^{-2} $p = 0.0003$; 800 cork borer holes m^{-2} $p < 0.0001$; bark flap inoculation $p = 0.0002$). The sapwood moisture content of control trees was within the 85–150% moisture content range that is considered normal for lodgepole pine trees (Reid, 1961), while

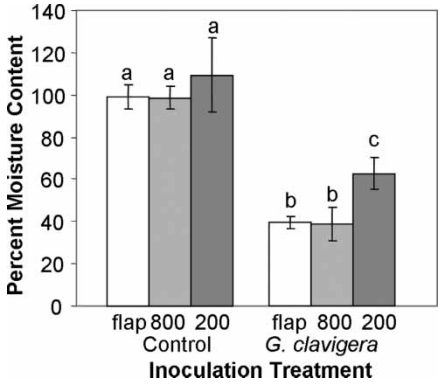

Figure 3. Percentage sapwood moisture content in pieces of sapwood taken from trees after inoculation treatment. Error bars represent means ± SD. Bars with the same letter above them are not significantly different at the 0.05 level. Flap = bark flap inoculation; 800 = 800 cork borer inoculations m^{-2}; 200 = 200 cork borer inoculations m^{-2}; Control = sterile agar control.

trees inoculated with *G. clavigera* at either density or with either technique had sapwood moisture contents well below 85%. *Grosmannia clavigera* was successfully recovered from all trees in at least one of the three inoculation points sampled from all three treatments, but was not reisolated from agar control trees.

In November 2003 and April 2004, the foliage colour remained unchanged for trees inoculated at 200 and 800 cork borer holes m^{-2}. Trees were left standing to allow symptoms to further develop. By 7 July 2004, 280 days after inoculation, for trees inoculated with *G. clavigera* at 800 holes m^{-2}, four out of six trees had yellow needles, one tree had yellow and green needles, and one tree had only green needles. All trees inoculated at 200 cork borer holes m^{-2} and all control trees had green needles.

In October 2004 and April 2005, the foliage colour remained unchanged for trees inoculated using the bark flap method. By 20 May 2005, foliage discolouration had started to occur. By 3 July 2005, 363 days after inoculation, four out of five trees inoculated using the bark flap method had yellow needles and one tree had green needles. All control trees had green needles.

It took six people 15 h (i.e. 90 person-hours) to inoculate nine trees (six with *G. clavigera* and three controls) at a density of 800 holes m^{-2}. The same six people took 6 h (i.e. 36 person-hours) to inoculate nine trees at a density of 200 holes m^{-2}. It took five people a total of 5 h (i.e. 25 person-hours) to inoculate nine trees using flap inoculations.

Discussion

Inoculating the fungus *G. clavigera* at a density of 800 cork borer holes m^{-2} produced pathogenic symptoms (sapwood occlusion, moisture content decrease and foliage discolouration) that were comparable to inoculations using the bark flap method. Pathogenic symptoms were less intense in trees inoculated at 200 cork borer holes m^{-2}. However, results between inoculation methods must be interpreted with caution because of differences in the length of time for fungal colonization and in yearly weather conditions. Fungi inoculated using the bark flap technique had almost three additional months of summer weather to colonize trees, while fungi inoculated using the cork borer method were inoculated in October, when the average mean monthly temperature was only 11°C. *Grosmannia clavigera* has an optimal growth rate between 22.0 and 25.0°C and showed reduced growth at lower temperatures (Solheim & Krokene, 1998). This could result in virulence symptoms being stronger in the second season when the bark flap method was used. The amount of precipitation, especially during the spring and summer months, was much higher during the second field season (July 2004 to July 2005) than it was for the first field season (October 2003 to June 2004). More precipitation is likely to reduce the stress on trees, which could lead to trees responding more vigorously to the presence of fungi in the second field season when the bark flap method was used. Horntvedt (1988) has shown that the resistance of *Picea abies* to *Ips typographus* varied significantly during different months of the summer, and Krokene and Solheim (1998) noticed considerable variation between two consecutive years of treatments. The present results might have been affected by similar variations.

Declines in tree health and foliage discolouration were observed in the bark flap technique used by Yamaoka et al. (1995). They found that one of two trees receiving *G. clavigera* inoculations had turned brown after 84 days, while the crown of the second tree turned brown within 1 year. Control trees and trees inoculated with *O. montium* had green crowns after 1 year. The dimensions of their bark flaps differed from those used in this study. They were shorter and wider. Despite the small sample size used, the authors indicated that the inoculation method used seemed reliable to assess the ability of a fungus to kill mature lodgepole pine trees within 1 year. The present results also suggested that the majority of trees receiving bark flap inoculations would have died because of the small percentage of viable sapwood and foliage discolouration, although the foliage discolouration (yellow) was not as severe as the brown discolouration observed by Yamaoka et al. (1995). Strobel and Sugawara (1986) also used a bark flap technique to inoculate 20-year-old lodgepole pines with *O. montium*. Their flaps were shorter and wider than those used in this study. Trees appeared healthy after one growing season, but after two growing seasons, three out of eight trees were dead and another four trees showed signs of decline. While this work is not directly comparable to the present study because of the age of the trees and the fungal species used, the results showed that the bark flap inoculation can be used to determine fungal virulence and tree mortality.

Although both inoculation techniques are capable of inducing tree mortality, each technique has its limitations. A low inoculation density of *G. clavigera* (200 cork borer holes m^{-2}) was insufficient to kill mature lodgepole pines after 1 year, while at a higher density (800 holes m^{-2}), *G. clavigera* was lethal to trees (Lee et al., 2006). However, Waring and Pitman (1983) found that successful tree attacks were characterized by beetle densities of 40–160 per m^2, depending on the health of the tree, which they

estimated by wood production/unit leaf area. Thus, inoculating fungi into small holes may not give a good indication of the beetle density needed to overcome host defense mechanisms. Krokene and Solheim (1997) noted that one beetle gallery of *I. typographus* was probably a more severe challenge to the tree's defenses than one cork borer inoculation. It is likely that the effects of an MPB boring a vertical gallery are more detrimental to the tree than an individual point inoculation. Neither inoculation technique mimics the full area that beetles naturally attack. Much larger sections of a host tree compared to the 0.6 or 0.4 m bands of inoculations used in this study are normally attacked but, regardless of pattern, it is likely that the total number of inoculations is more important than the inoculation pattern (Christiansen, 1985*a*; Krokene & Solheim, 1997). The cork borer method also does not simulate how beetles disseminate fungal spores while constructing galleries. The bark flap method more accurately reflects this, but does not simulate how larvae spread fungi in constructing galleries perpendicular to the main gallery.

Bark flap inoculations required 30.6% less time than using a density of 200 cork borer holes m^{-2} and 72.2% less time than using a density of 800 cork borer holes m^{-2}. Given the efficiency with which this method delivers fungal inoculum to a tree, it makes it practical for a small team to characterize fungal virulence on a relatively large number of hosts, which may be important for understanding genetic variability in fungal virulence or host resistance. In contrast, cork borer inoculations produce small wounds, seem less damaging to a tree and may be more appropriate at low densities for two applications: monitoring individual symptom development in hosts (Krokene & Solheim, 2001; Rice et al., 2007*a*, *b*) and inducing tree resistance to subsequent beetle or pathogen invasion (Christiansen et al., 1999; Krokene et al., 1999, 2000).

To assess the new inoculation approach further in the context of characterizing large-scale genetic variability, follow-up studies should examine the repeatability of these results between field seasons, and address whether the approach could be used with other beetle–fungal–tree complexes.

Acknowledgements

This work was supported by Natural Resources Canada through the Mountain Pine Beetle Initiative funds. We thank Dr L. Maclauchlan (BC Ministry of Forests, Kamloops) for her help with fieldwork.

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