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Short communications

The pathogenicity of the blue stain fungus *Ophiostoma clavatum* in Scots pine seedlings

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INTRODUCTION

The aim of this research was to determine the pathogenicity of the bark beetle *Ips acuminatus* (Gyllenhal) associated fungus *Ophiostoma clavatum* Math.-Käärik (Kirisits 2004; Linnakoski et al., 2012, 2016) on Scots pine seedlings (*Pinus sylvestris* L.). This question arose as *I. acuminatus* has become more aggressive in Finland in the last two decades (Siitonen 2014), as well as in the alpine regions of Central Europe (Wermelinger et al. 2008), following the shift towards hotter and drier summers in these areas, which in turn has weakened the defence mechanisms of the pines (Chinellato et al. 2014, Wermelinger et al. 2008). The combination of increased bark beetle population size and availability of susceptible host trees (Allen et al. 2010, Siitonen 2014, Wermelinger et al. 2008), is believed to be the driving factor for increased tree mortality (Siitonen 2014, Wermelinger et al. 2008). It was, therefore, interesting to determine the pathogenicity of the fungus compared to mock-inoculated Scots pine seedlings, to see if it contributes to tree mortality.

MATERIALS AND METHOD

The research was carried out at the Forest Pathology Laboratory at the University of Helsinki, Finland. The fungal strain of *O. clavatum* originated from an adult beetle infesting Scots pine in Finland. The fungal cultures were grown in 70 mm petri dishes containing 2% malt extract agar (MEA) in an incubator in stable conditions at 25°C.

Frozen one-year old Scots pine seedlings were procured and placed in a fridge at 3-4°C with a diurnal light cycle. Two weeks later, the seedlings were planted individually in pots sized 10x10x10 cm and placed in an incubator room to adjust to potted life. In total, 90 seedlings were used. The potted seedlings were placed on a tray with 15 pots in each tray, and six trays in total. The trays were then placed on shelves within the temperature and light controlled incubator room. The incubator room had diurnal light cycles and the temperature was a steady 20°C. A fan kept the air moving, and the trays and the pots within the trays were rotated on weekly basis to ensure uniform conditions for every seedling.

After another two-week adjustment period, the inoculation of the seedlings was performed. The seedlings were randomly divided into three groups according to the treatment they would receive. One group was inoculated with *O. clavatum* and the other two groups were control groups, one mock-inoculated with 2% MEA and one left untouched with no inoculation

or wounding on the bark. Before starting the inoculation, the height of the seedlings was measured and each seedling was examined to determine its quality, leaving 88 healthy seedlings, of which 66 were inoculated (*I*), ten mock-inoculated (*CM*), and 12 were control seedlings (*C*).

The inoculation was performed by stripping the woody part of the stems of needles on the first-year growth, on an area approximately 2 cm in length. A 3x4 mm lesion was cut into the bark to expose the sapwood on *I* and *CM* seedlings. To prevent contamination, the *CM* group was inoculated first with 2% MEA and then the test group was inoculated with the fungus by placing a piece of the malt agar or fungal cultures on the wound. Parafilm® was used to seal in the MEA and fungus.

Following the treatment, the seedlings were watered twice per week. Notes on the health of each seedling were taken once per week. Discolouration of needles, resin production around the lesions, and any other signs of stress were documented.

After eight weeks of observations, the seedlings' height was measured and each seedling was removed from their individual pots, the soil cleaned off, and the wounds examined. Both the *CM* and *I* seedlings' bark was first polished off with a fine-grained sandpaper (P400), and then pealed with a scalpel around the lesions to examine if any infection had manifested around the inoculation site. Any infection detected was measured using a digital Vernier calliper. Depending on the depth and size of the infected area, seedlings were assigned to one of four classes: 0 = no infection detected; 1 = minimal infection (barely visible < 0.5 mm); 2 = modest infection (infection surrounding the wound by 0.5-2 mm); and, 3 = considerable infection (infection growing > 2 mm along the stem from the wound). Additionally, random *I* seedlings were chosen, using the randomising command in Excel, and samples from the infected areas were taken to be regrown in agar plates and later reisolated to confirm infection of *O. clavatum*.

The seedlings were placed in a drying oven at 40°C for 48 hours after which they were weighed to determine their dry weight. Roots, stems, and needles were taken from the dry samples and weighed separately to establish if there was any difference in biomass allocation of infected and non-infected seedlings.

SAS Basic 9.4® software was used for statistical analysis. The variables analysed were: height at inoculation (H1), height at the end of the observation period (H2), height differences in cm (Hdiff), height differences percentage (Hdiff_rel), infection class (Infection), total dry weight (DW), dry needles (Needle), dry roots (Root), and dry stems and branches (Stem). Finally, the needle-mass ratio (NMR), root-mass ratio (RMR), and stem and branch mass ratio (SMR) were compared against each other and between treatments, as well as the root-shoot ratio (RSR). A Spearman´s ranking correlation was used and an ANCOVA test, for the statistical analysis.

RESULTS

During the eight-week observation period the seedlings showed no visual signs of stress, such as discolouration of the top needles. There was no visible discolouration in the mechanical wounding of *I* and *CM* seedlings, though dark and dried-up hyphae leftovers created a film at the top of many of the wounds; resin was visible in and around the wounds.

There was evidence of infection in 45%, or 30 of a total 66, of the *I* seedlings (data not shown). This left 55% with no infection (class 0). The distribution between the infection classes was 23%, 19%, and 3% (2 seedlings) for classes 1 to 3, respectively. Most of the infections were superficial with little or no depth within the sapwood of the seedlings. None of the mock-inoculated seedlings or the control group showed evidence of infection.

To determine if there was any relation to be found between the visually estimated infection class and other variables, the Spearman's rank correlation was used across all treatments (Table 1). The Spearman's test showed no significant correlation to be found between the infection class and other measured components.

Even though no significant relationship

Table 1. Spearman's rank correlation coefficients, N = 88, showing no relationship between the infection class of Scots pine seedlings infected by *Ophiostoma clavatum* and other categories. See text for definitions of variables.

	r	p
H1	-0,0508	0,6386
H2	-0,0753	0,4857
Hdiff	-0,0717	0,5069
Hdiff_rel	-0,0842	0,4353
DW	-0,1353	0,209
Needle	-0,0701	0,5163
Root	-0,117	0,2777
Stem	-0,0753	0,4854
NMR	0,04239	0,695
RMR	-0,0138	0,8983
SMR	-0,026	0,8101
RSR	-0,022	0,8386

was found between the infection class and the different variables, additional statistical analysis was applied, where initial size differences between the individual treatment plants were included in an ANCOVA model as a covariate. This test showed a significant difference in the total DW between the *I* and the *C* seedlings

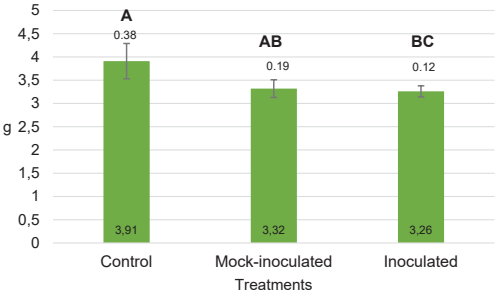

Figure 1: Results of ANCOVA test showing means and standard error of total dry weight (g) of Scots pine seedlings that had been inoculated by *Ophiostoma clavatum*, mock-inoculated and control with no treatment. Different letters indicate significant difference of $P < 0.05$.

Table 2. Results of Analysis of covariance (ANCOVA) where initial height (H1) is the covariate value on the difference Scots pine seedlings that had been inoculated by Ophiostoma clavatum, mock-inoculated and control group with no treatment. Dependent variables are: height at the end of the observation period (H2), the difference between the two height measurements in cm (Hdiff) and in percentage (Hdiff_rel), weight of the dry needles (Needle), dry roots (Root), and dry stem and branches (Stem), needle-mass ratio (NMR), root-mass ratio (RMR), stem and branches ratio (SMR), and root-shoot ratio (RSR). RMR, SMR, and RSR, were not determined because not all the treatment results had a normal distribution.

	Control		Mock-inoculated		Inoculated		Significance
Variable	**Mean**	**SE**	**Mean**	**SE**	**Mean**	**SE**	**Pr > F**
H1	18,35	1,23	18,42	0,80	18,65	0,36	n/a
H2	20,33	1,37	20,26	0,87	20,71	0,41	0,7589
Hdiff	1,98	0,26	1,84	0,21	2,07	0,11	0,7589
Hdiff_rel	0,11	0,01	0,10	0,01	0,11	0,01	0,8227
Needle	2,20	0,21	2,01	0,08	2,04	0,06	0,3870
Root	0,81	0,05	0,79	0,04	0,78	0,02	0,7722
Stem	1,00	0,11	0,95	0,08	0,99	0,03	0,7561
NMR	0,55	0,02	0,54	0,02	0,53	0,00	0,6663
RMR	0,21	0,01	0,21	0,01	0,21	0,00	nd.
SMR	0,25	0,01	0,25	0,01	0,26	0,00	nd.
RSR	0,27	0,02	0,27	0,01	0,26	0,01	nd.

(Figure 1). There was, however, no significant difference between the *CM* seedlings and the other two groups. No other measured variables were significantly different between treatments in the ANCOVA analysis (Table 2).

DISCUSSION

Ophiostoma clavatum was shown to be of low virulence. Seedling mortality was not observed during the experiment. No measured growth components were significantly affected by the inoculation, apart from the difference between the total dry weight of the untreated *C* group and the *I* group. It was only the combined effect of the wounding (simulating bark-beetle effect) and the inoculation that led to significant growth reductions, while the *CM* was not significantly different from the *I* group or the *C* group. This suggests that the mechanical wounding and the fungus had only a small physiological effect on the growth of Scots pine seedlings, with no additional disturbances or stressors other than wounding and fungal infection.

Similar results have been found by Guérard et al. (2007) when inoculating 3-year old Sctos pine saplings with *O. bruenneo-ciliatum* (an *O. clavatum complex* species); the inoculation alone, without environmental stressors, did not seem to overly disturb the saplings. In another study, Krokene et al. (2000) showed that low intensity exposure to *O. canum* infection increased the resilience of Scots pine to more aggressive inoculation few weeks later. This result might suggest that the previous infection induced defence responses which then benefitted the tree in later infection. Largely, though, it has been found that the virulence of many *Ophiostoma* species has proven weak, contributing to tree mortality in only a limited way (Harrington 1993). Indeed the pathogenicity of most blue stain fungi is linked with the aggressiveness of the insect vector (Kirisits 2013), which is in turn then associated with population size and favourable environmental conditions (Siitonen 2014; Wermelinger et al. 2008). It can, therefore, be surmised that the single mechanical wounding of the seedlings in this experiment was not sufficient to severely affect the seedling.

In conclusion, the *O. clavatum* strain used in this study does not seem to seriously stress Scots pine seedlings, when kept in favourable conditions, in cases where a single infection is introduced through mechanical wounding under the bark. Further studies on the role environmental stressors play in the aggression of the fungus and how it manifests in its host are, however, warranted.

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