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SHORT COMMUNICATION

Pathogenicity of Spanish isolates of *Heterobasidion annosum s. s.* in *Pinus pinaster* seedlings

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Summary

The aim of this study was to test the pathogenicity of two Spanish isolates of *Heterobasidion annosum sensu stricto* in 2-year-old *Pinus pinaster* seedlings. Two types of inocula (woodchips and sawdust) were used to infect the seedlings by two different routes (stem inoculation and soil infestation). The mortality rates of the stem-inoculated seedlings differed significantly from controls, but those of the seedlings infected via soil infestation did not differ. For both types of inoculation, the lesions were longer, and wilting symptoms were more severe in the seedlings inoculated with *H. annosum* than in control seedlings. For stem inoculation, biomass allocation did not differ significantly between the infected and control seedlings. However, the percentage of fine roots was lower in seedlings infected via soil infestation than in the control seedlings. To our knowledge, this is the first pathogenicity test with *H. annosum* isolates and *P. pinaster*.

1 Introduction

Heterobasidion annosum sensu lato (Fr.) Bref. is a species complex that severely affects coniferous forests in Europe, Asia and North America. This hymenomycete fungus, which causes root and butt rot, is responsible for high economic losses in the forestry sector. In Europe alone, *Heterobasidion* infection of forest stands is estimated to cause economic losses of up to 800 million euros annually. *Pinus pinaster* Ait. is the most widespread conifer in Spain, covering over 700 000 ha in pure stands and 600 000 ha in mixed stands. Mediterranean maritime pine is planted for soil conservation purposes and for timber and resin production. In recent years, *P. pinaster* decline, characterized by unusual transparency at the crown, small needles, foliage discoloration and early tree death, has been observed in association with a high mortality rate in several forests in the centre of the Iberian Peninsula. Although *H. annosum* has occasionally been recorded on *Pinus sylvestris* and *Pinus nigra* in Spain, the presence of the fungus on *P. pinaster* in association with forest decline is a very recent finding (Prieto-Recio et al. 2012). This pathogen is very aggressive on Pinaceae; however, some pine species such as *P. pinea* and *P. halepensis* have shown a lesser susceptibility (Scire et al. 2011). To our knowledge, pathogenicity tests have not previously been carried out with *H. annosum* isolates and *P. pinaster*. Therefore, the aim of this study was to test the virulence of Spanish isolates of *H. annosum* in *P. pinaster* seedlings.

2 Material and methods

A total of 120 two-year-old *P. pinaster* seedlings (Meseta Castellana provenance, Spain) were obtained from a Government tree nursery in Castile and Leon. The Spanish isolates of *Heterobasidion* used in the study (H1 and H4) were previously isolated and identified as *H. annosum s. s.* (Prieto-Recio et al. 2012). The sequences were deposited in the EMBL/GenBank database (GenBank Accession No. FR850494 and FR850495, respectively).

Two types of inocula, woodchips and sawdust, were used to infect the seedlings via each of two routes, stem inoculation and soil infestation. Thus, 40 seedlings were inoculated with each *H. annosum* isolate (10 for each type of inoculum and route of inoculation). Control seedlings (n = 40) were prepared in the same way, but using sterile woodchips and sawdust. The inocula were prepared by growing the isolates of *H. annosum* on autoclaved woodchips (4 mm diameter, 4 mm depth) and sawdust, which were maintained on potato dextrose agar (PDA) for 3 weeks. For stem inoculations, the inocula were placed inside an oblique incision made in the stem at 6 cm above the soil line, and the inoculation site was then wrapped with Parafilm® (BEMIS, Neenah, WI, USA). For soil infestation, four infected woodchips and four pieces of PDA with infected sawdust were placed in the soil near the stem at a depth of 1.5 cm. The seedlings were incubated in a growth chamber at 22.5°C with a 14-h photoperiod and watered twice a week. In months 1, 2, 4, 6, 10 and 18, the survival and the visual severity of symptoms (percentage of wilting or chlorosis) were assessed in each plant according to the following scale (where 0 = healthy and 4 = dead): 0 = 0–10%, 1 = 11–33%, 2 = 34–66%, 3 = 67–99%, 4 = 100%. Disease progress curves for each plant were constructed by plotting the scores over time. The area under the disease progress curve (AUDPC) was calculated as the sum of the area of the corresponding trapezoids.

Eighteen months after inoculation, seedlings were cut into two pieces at the root collar. The height, diameter and length from the inoculation point to the last withered needle on the leader were measured. The stems were also split lengthways, with a scalpel, to enable measurement of the length of the lesions. The aerial part was subdivided into stem and needles and was dried in an oven at 80°C for 38 h for investigation of biomass allocation. Roots were washed to remove adhering

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soil, the main root length measured and the total root mass dried to estimate the biomass of primary, secondary and tertiary roots.

Log-linear analysis of frequency tables was used to test the effects of the isolates and the type of inoculum on the mortality rates. Analysis of variance (ANOVA) and *post hoc* Tukey's HSD tests were used to test the effects of the isolates and the type of inoculum on the biomass allocations, wilting symptoms and lesion lengths. However, because heteroscedasticity was observed in the AUDPC, linear mixed models (PRO MIXED) with six variance parameters and no random effects were used to test for the effects of the isolates and the type of inoculum on this variable.

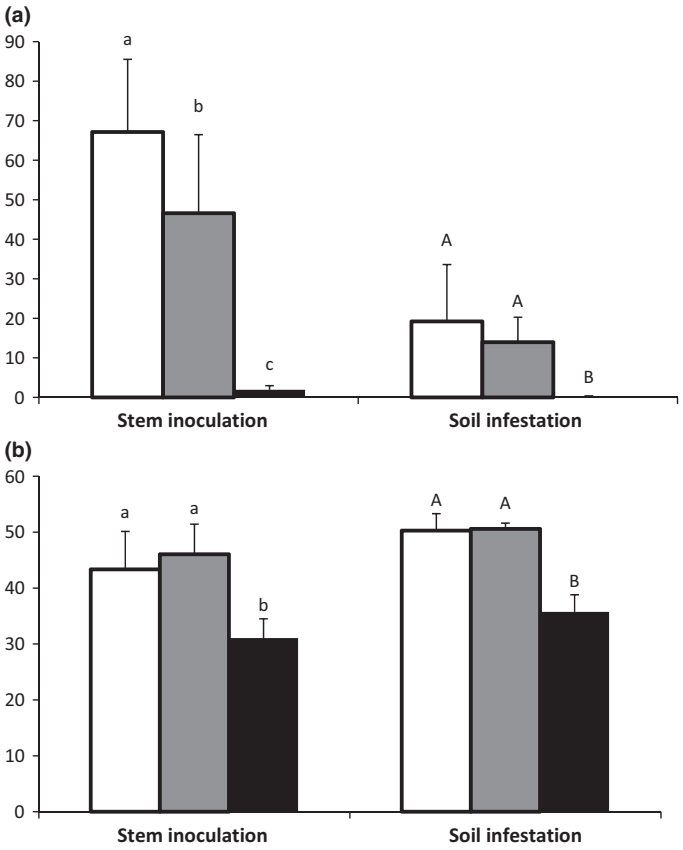

Fig. 1. (a) Length of lesions (mm) and (b) area under the disease progress curve (AUDPC) (±95% confidence intervals) for seedlings artificially infected with *Heterobasidion annosum* isolate H1 (white bars) and *H. annosum* isolate H4 (grey bars) and for the respective control seedlings (black bars). For each route of infection, different letters above the bars indicate significantly different means (two-tailed t-test, $\alpha = 0.05$).

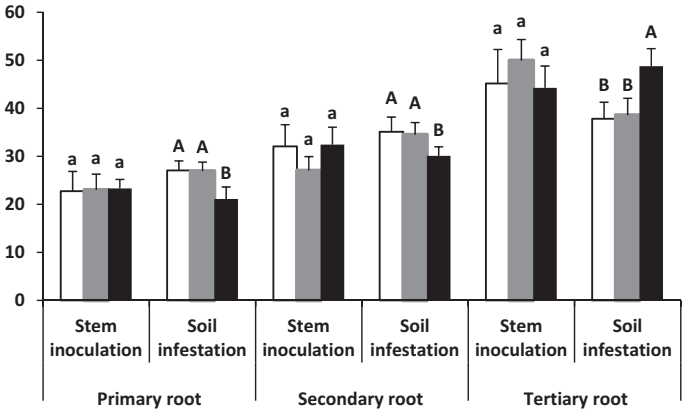

Fig. 2. Rate of biomass of primary, secondary and tertiary roots (%) (±95% confidence intervals) in seedlings infected with *Heterobasidion annosum* isolate H1 (white bars) and *H. annosum* isolate H4 (grey bars) and in the respective control seedlings (black bars). For each type of inoculation, different letters above the bars indicate significantly different means (*post hoc* Tukey's test, $\alpha = 0.05$).

3 Results and discussion

The mortality rate of seedlings infected with *H. annosum* via stem inoculation differed significantly from that of the respective control seedlings ($\chi^2 = 3.96$, $p = 0.04$). However, there was no difference in the mortality rate of the infected and control seedlings treated by soil infestation. Moreover, seedling mortality did not occur after 3 months from the inoculations, which is consistent with previous findings of maximal mortality rates in young seedlings at between 5 and 14 weeks after inoculation, which was attributed to occlusion of the pathogen from the plant material after this length of time (Swedjemark et al. 2001).

The mortality rates (approximately 35 and 10% for stem inoculation and soil infestation, respectively) were lower than reported for some North American pine species, such as *P. elliottii*, *P. echinata*, *P. palustris*, *P. strobus* and *P. taeda* (Delatour et al. 1998), and, to a lesser extent, for *P. sylvestris* (Swedjemark and Stenlid 1995), although inoculation methods were different from those used in this study. Nevertheless, rates were similar to or even higher than observed by other authors (Lehtijarvi et al. 2011; Scire et al. 2011), who reported low mortality rates in other pine species, such as *P. pinea*, *P. halepensis*, *P. nigra, P. sylvestris* and *P. brutia* seedlings. The fact that resin production was higher in the seedlings infected via stem inoculation than in the respective control seedlings ($\chi^2 = 5.64$, $p = 0.01$) may indicate that the relatively low mortality rate was due to physical and chemical host defences. Indeed, *P. pinaster* (also called resin pine) produces a large amount of oleoresin (62.8% of resin acids), which acts as mechanical barrier, and monoterpenes (28.7%), which may be fungitoxic (Asiegbu et al. 1998; Scire et al. 2011).

In addition to the differences in mortality rates, the lesions in the infected seedlings were also longer than in the control seedlings for both types of inoculation (Fig. 1a). The type of inoculum also affected lesion length: stem inoculation with woodchips caused more damage than stem inoculation with sawdust ($N = 60$, $F = 10.14$, $p < 0.01$). The lesion lengths in stem inoculations were consistent with other findings in *Picea abies* (Swedjemark et al. 2001), where an average lesion length of approximately 50 mm 182 days was found after inoculation. However, the lesion lengths were substantially higher than those observed in other pine species in Turkey, such as *P. brutia* (9.4 mm), *P. nigra* (15.7 mm) and *P. sylvestris* (15 mm) (Lehtijarvi et al. 2011). These differences may be due to (i) host defences and/or (ii) the maximum incubation temperature which in previous study reached approximately 31°C and may have halted the development of the disease. Furthermore, in the present study, the lesion lengths were measured 18 months after inoculation, whereas Lehtijarvi et al. (2011) harvested the seedlings 12 weeks after inoculation. Therefore, although the daily increase in lesion length was lower when plants were incubated for a long period of time (Swedjemark et al. 2001), an increase in the overall lesion length would be expected for longer incubation periods, depending on host species and defence mechanisms.

A similar finding was observed for AUDPC, which indicated that disease progress was greatest in the stem-inoculated seedlings (Fig. 1b). Furthermore, the curve stabilized after 4–6 months, which seems to confirm that occlusion of the pathogen from the plant material occurs after that time. Likewise, wilting symptoms were more severe in inoculated seedlings than in control seedlings, for both stem inoculation ($N = 60$, $F = 4.97$, $p = 0.01$) and soil infestation ($N = 60$, $F = 8.84$, $p < 0.01$) treatments.

For stem inoculation, biomass allocation did not differ significantly between the infected and control seedlings. However, the percentage of fine roots (tertiary) was lower in the seedlings infected via soil infestation than in the respective control seedlings (Fig. 2). This difference may be the result of the ability of *H. annosum* to parasitize fine roots in pine (Asiegbu et al. 1998) and also the inability of weakened seedlings (as a result of soil inoculation) to develop the same quantity of fine roots as control seedlings.

This paper reports the first pathogenicity test with *H. annosum* isolates and *P. pinaster,* and the results demonstrate the susceptibility of Mediterranean maritime pine to *H. annosum.* Further studies and surveys are essential to determine the role that *H. annosum* is playing in maritime pine decline in Spain.

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