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Pine Species Influence Suppression of *Fusarium* Root Rot by the Ectomycorrhizal Fungus *Paxillus involutus*

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Abstract

Seedlings of *Pinus banksiana* Lamb., *Pinus resinosa* Ait., *Pinus strobus* L. and *Pinus sylvestris* L. grown in test tubes were inoculated with the ectomycorrhizal fungus *Paxillus involutus* Fr. and the root pathogenic fungus *Fusarium oxysporum* Schlecth. emend Snyd. & Hans. f. sp. *pini* to investigate the effect of tree species on disease suppression by *P. involutus*. Disease suppression was assessed using seedling survival, sporulation of the pathogen in the tubes and fungitoxic activity of the ethanol-soluble rhizosphere extractives on *F. oxysporum* microconidia germination. Controls consisted of seedlings of all four pine species inoculated with discs of sterile modified Melin-Norkrans medium and *F. oxysporum*. Disease suppression by *P. involutus* was most effective on *P. resinosa* while lower levels of protection were observed on the other three pine species. The protective effect of the isolate of *P. involutus* used is therefore specific to *P. resinosa*.

Keywords: *Pinus, Paxillus, Fusarium*, biological control, ectomycorrhizae

1. Introduction

Ectomycorrhizal fungi have been reported to control root diseases of tree seedlings *in vitro* (Chakravarty and Unestam, 1985, 1987a, 1987b; Ross and Marx, 1972; Stack and Sinclair, 1975) and *in situ* (MacFall, 1976; Sampangi

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et al., 1985). However, the protective influence of biological control agents including ectomycorrhizal fungi is variable (Perrin, 1985a; 1985b; Perrin and Nouveau, 1985; Sinclair et al., 1975; Whipps, 1986) and it can be difficult to predict whether a given application of an ectomycorrhizal fungus will result in disease control. Research on the mechanisms of disease protection by ectomycorrhizal fungi may help solve this problem. Successful disease protection by ectomycorrhizal fungi is dependent on the host, the ectomycorrhizal fungus, the pathogen(s), and environmental conditions (Perrin, 1985a, 1985b; Perrin and Nouveau, 1985). The extent of the variation caused by each of these factors has yet to be determined.

Seedlings of *Pinus resinosa* Ait. can be protected against *Fusarium* root rot *in vitro* by inoculation with the ectomycorrhizal fungus *Paxillus involutus* Fr. (Duchesne et al., 1988a). This phenomenon is associated with the presence of antifungal compounds in the rhizosphere of the seedlings but not in their tissues. *Paxillus involutus* is responsible for the production of the fungitoxic components in the seedling rhizosphere before the occurrence of *Fusarium* root rot (Duchesne et al., 1989). Moreover, the synthesis of fungitoxic substances by *P. involutus* is stimulated by *P. resinosa* root exudate (Duchesne et al., 1985b). Although these results are encouraging with regard to field application of *P. involutus*, it is also important to determine whether *P. involutus* is effective at controlling *Fusarium* root rot of other tree species, particularly pine species which are grown in Canadian forest nurseries. The objective of this paper was to compare suppression of *Fusarium* root rot *in vitro* by *P. involutus* on seedlings of *Pinus banksiana* lamb, *Pinus resinosa* L., *Pinus strobus* L. and *Pinus sylvestris* L.

2. Materials and Methods

Fungal cultures

P. involutus isolate No. 0262 was obtained from Dr. J.A. Fortin, Université Laval, Sainte-Foy, Québec, Canada. Cultures of *F. oxysporum*, which were originally isolated from a dead *P. strobus* seedling, were obtained from Dr. G. Hofstra and P. Williams, University of Guelph. *Paxillus involutus* was maintained on modified Melin-Norkrans (MMN) medium in petri dishes (Marx, 1969), and *F. oxysporum* was maintained on potato dextrose agar (Difco) in pitri dishes at 25°C in the dark. The pathogenicity of *F. oxysporum* was confirmed every three weeks by reisolation from diseased *P. resinosa* and *P. sylvestris* seedlings.

Seedling cultivation

With a few exceptions, all aspects of seedling preparation and incubation have been described previously (Duchesne et al., 1988a). Seeds of *P. banksiana*, *P. resinosa*, *P. strobus*, and *P. sylvestris* (Canadian Forestry Service seed lots No. 6830070.0, 7030150.1, 8181280.0, 7230350.0, respectively) were surface disinfected (45 min, 30% H_2O_2), rinsed in autoclaved distilled water, and germinated for 12 to 15 d in petri dishes prior to transfer to sterile test tubes containing nutrient solutions. The test tube-growth system consisted of a modification of the paper wick method of Sylvia and Sinclair (1983). Before seedling transfer, the test tubes (Pyrex, 1.5 cm×18 cm) were lined with a strip (3 cm×10 cm) of Whatman 3MM chromatography paper, stoppered with a foam plug (2 cm×3.5 cm) and the foam plug covered with a plastic cap (Kim-cap). The tubes were autoclaved for 20 min at 120°C, 103 $kN.m^{-2}$. After autoclaving, 5 ml of autoclaved nutrient solution (Sylvia and Sinclair, 1983) were dispensed into each test tube. One seedling (12– to 15– d old) was placed between the chromatography paper and the wall of each test tube under aseptic conditions. The test tubes were placed in a growth room under a 16 hr photoperiod (100 μmol $m^{-2}s^{-1}$) and temperatures of 27°C and 20°C (night).

Disease suppression by P. involutus

Eighty seedlings of each of the four pine species were tested. Forty seedlings of each species were inoculated one day after transfer to the tubes, with four discs (5-mm diam) from the periphery of 2– to 3–w-old mycelial mats of *P. involutus*. Inoculation was performed by placing the discs between the test tube wall and the chromatography paper with the mycelial face against the paper wick. Two inoculum discs were placed 1 cm from the top of the paper strip and two others were placed 1 cm lower on the paper strip. All inoculum discs were placed ca. 5 mm from the root of the seedling. The remaining 40 seedlings of each species were inoculated with four agar plugs of sterile MMN medium. The seedlings were then incubated in a growth room for 1 day before being challenged with *F. oxysporum*. Inoculation with *F. oxysporum* was carried out by dispensing 1 ml of a *F. oxysporum* spore suspension (10^5 spores/ml in autoclaved distilled water) to each test tube. After inoculation, the seedlings were kept in a growth room for 2 weeks as described above. Disease severity, sporulation of *F. oxysporum* and fungitoxic activity of the rhizosphere were analyzed 2 weeks after inoculation with *F. oxysporum*. This experiment was conducted three times.

Disease severity

Disease severity was determined as described in (Duchesne et al., 1988a) from all of the 80 inoculated seedlings of each experiment. Seedlings were scored dead if both the primary root and the hypocotyl were macerated on more than 75% of their length as determined by observation under dissecting microscope. Seedlings were also examined for the presence of mycorrhizae. The percent survival of the seedlings was transformed using the arcsine method of Sokal and Rohlf (1981) to allow analysis of variance using the t-test at $P < 0.01$ and $P < 0.05$ (Sokal and Rohlf, 1981). The values reported are the average of the three experiments without arcsine transformation.

Sporulation of F. oxysporum

At each experiment, quantitation of *F. oxysporum* sporulation from 20 tubes of each treatment was carried out as a measure of its growth in the rhizosphere of the seedlings (Duchesne et al., 1988a). For this, the paper strips and nutrient solution were transferred to sterile petri dishes (1.5 cm×10 cm) and 5 ml of autoclaved distilled water were added. The paper strips were agitated gently with a sterile spatula to suspend the spores in the water. Spore counts were carried out in triplicate using a haemocytometer (Canlab, Canada). The results were expressed as the total number of spores per test tube and statistical analysis performed using the t-test (Sokal and Rohlf, 1981) at $P < 0.01$. The values reported are the average of the three experiments.

Bioassay of rhizosphere extracts

Extraction of the ethanol-soluble, non-volatile, components of the seedling rhizosphere was performed by transferring the contents of test tubes from all 80 seedlings (including the paper wick but excluding the seedlings) into 95% ethanol (5 ml ethanol/test tube). These were stored at 4°C in the dark for at least 24 hr, evaporated to dryness *in vacuo* at 50°C, taken up in 10% aqueous HPLC-grade acetonitrile (100 μl/test tube) and stored in the dark at −10°C. In order to allow comparison of the fungitoxic activity of each treatment a standard unit was adopted (Duchesne et al., 1988a). The extracts from one test tube were designated as one seedling extractive equivalent (1 SEE). A total of 8 different crude extracts was obtained from this experiment: 4 from the rhizosphere of seedlings of each of the four pine species inoculated with *P. involutus* and 4 from the rhizosphere of seedlings inoculated with sterile MMN plugs.

Table 1. Suppression of *Fusarium* root by *Paxillus involutus* on four pine species

Pine species	% Survival		
	P. involutus (A)	Control (B)	(A–B)
P. resinosa	62.2 a+	0.9 b 2*	60.3 1
P. bansiana	39.4 a	36.9 a 1	2.5 2
P. strobus	44.8 a	37.0 a 1	7.8 2
P. sylvestris	15.1 a	0 b 2	15.1 2

+Within lines, values followed by a different letter are significantly different at $P < 0.01$.

* Within columns (B) and (A–B), values followed by a different number are significantly different at $P < 0.01$.

The presence of antifungal compounds in the crude extracts was determined by bioassay on *F. oxysporum* microconidia germination (Duchesne et al., 1988a). The ED_{50} value of each crude extract, defined by the concentration of crude extract that prevented germination of 50% of *F. oxysporum* microconidia within 14 hr as compared to the germination rate of spores incubated without extractives, was determined. Duplicate aliquots of the crude extracts (10–200 μl; 0.1–2.0 SEE) were evaporated to dryness under an air stream at 50°C. Each dry residue was dissolved in 100 μl of 4% ethanol containing 10^5 microconidia of *F. oxysporum* and incubated for 14 hr on microscope glass slides in petri dishes lined with wet filter paper at ca. 20°C in the dark. The spores were fixed with lactophenol blue (Peacock, 1966) and microconidial germination determined by light microscopy at 40×. The ED_{50} values were determined by graphical interpolation. This test was conducted 3 times for each of the experiments. At least 150 microconidia were scored on each glass slide. Statistical analysis was carried using the t-test (Sokal and Rohlf, 1981) at $P < 0.01$. The results reported are the average of the 3 experiments.

3. Results

Disease severity

Inoculation with the ectomycorrhizal fungus *P. involutus* increased the survival of *P. resinosa* seedlings, but did not improve ($P < 0.01$) the survival of seedlings of *P. bansiana*, *P. strobus* and *P. sylvestris* (Table 1). At

Table 2. Sporulation of *F. oxysporum* in the rhizosphere of four pine species inoculated with *Pazillus involutus*

Pine species	spores/tube (10^6)	
	P. involutus	Control
P. resinosa	2.81 a+	7.92 b
P. bansiana	4.97 a	5.22 a
P. sylvestris	8.88 a	11.49 a
P. strobus	6.34 a	9.86 a

+Within lines, values followed by a different letter are significantly different at $P < 0.01$.

$P < 0.05$, however, the inoculation of *P. sylvestris* with *P. involutus* also increased the survival to infection by *F. oxysporum*. None of the pine seedlings formed mycorrhizae during the course of this experiment. The controls of the 4 pine species had different ($P < 0.01$) levels of endogenous resistance to *F. oxysporum* (Table 1).

Sporulation of F. oxysporum

Inoculation of pine seedlings with *P. involutus* depressed ($P < 0.01$) the sporulation of *F. oxysporum* only in the case of *P. resinosa* seedlings. There was no difference between sporulation in the rhizosphere of the other pine seedlings inoculated with *P. involutus* and their respective controls (Table 2). Nevertheless, there is a tendency toward suppression of sporulation of the pathogen by *P. involutus* in the seedling rhizosphere of *P. sylvestris* and *P. strobus*.

Bioassay of rhizosphere extracts

Bioassay of the crude extracts from the rhizosphere of the seedlings showed that inoculation with *P. involutus* increased the fungitoxic activity of the rhizosphere for *P. resinosa* only (Table 3).

4. Discussion

Only a few reports have compared disease suppression on different tree species using the same ectomycorrhizal fungus. Marx and Davey (1969) observed suppression of *Phytophthora cinnamomi* Rands. root rot of *Pinus taeda* L. and *Pinus echinata* Mill. using the ectomycorrhizal fungi *Leucopaxillus*

Table 3. Fungitoxic activity of rhizosphere extracts of four pine species inoculated with *Pazillus involutus*

	ED_{50} Value	
	P. involutus	Control
P. resinosa	0.37 a[+]	1.56 b
P. bansiana	1.17 a	1.78 a
P. sylvestris	1.17 a	1.40 a
P. strobus	1.59 a	1.50 a

[+]Within lines, values followed by a different letter are significantly different at $P < 0.01$

cerealis var. *piceina* Peck, *Pisolithus tinctorius* (Pers.) Coker & Couch and *Suillus luteus* (L. ex Fr.). There was little difference, presumably not significant, between the pine species in the level of protection which was provided by the ectomycorrhizal fungi. Sampangi et al. (1985) inoculated seedlings of *Picea abies* Karst. and *Pseudotsuga menziesii* (Mirb.) Franco with the ectomycorrhizal fungi *Laccaria bicolor* (Maire) Orton (one isolate) and *Laccaria laccata* (Scop.: Fr.) Berk. & Br. (3 isolates). Although these isolates showed different levels of protection against *Fusarium* root rot, there was little or no difference between the protection levels provided to *Picea abies* and *Pseudotsuga menziesii*. These results suggest that the influence of the tree species in disease suppression by ectomycorrhizal fungi is minimal.

Contrary results were presented by Perrin and Garbaye (1983), who reported a reduction in the growth of *Pythium ultimum* Trow in the rhizosphere of *Fagus* sp. seedlings, but not on *Quercus* sp. seedlings inoculated with the ectomycorrhizal fungus *Hebeloma crustuliniforme* (Bull. ex St-Am.) Quel. Unfortunately, disease symptoms on the seedlings were not reported (Perrin and Garbaye, 1983). Other results suggesting an important influence of the host on disease suppression have been published by Ross and Marx (1972). These authors inoculated two races of *Pinus clausa* (Chap.) Vasey with *Pisolithus tinctorius* and *Phytophthora cinnamomi*. Protection against the pathogen was observed on mycorrhizal seedlings of one of the two races of *Pinus clausa* (Ross and Marx, 1972). In the present study, disease suppression by the ectomycorrhizal fungus *P. involutus* was observed on *P. resinosa*, and to a lesser extent on *P. sylvestris*, but not on *P. bansiana* or *P. strobus*. Evaluation of the pathogen sporulation in the rhizosphere of the seedlings shows a positive correlation with the seedling survival rate.

The present data suggests that, over the time period of this experiment, the

root exudates of the 3 other pine species are, for either qualitative or quantitative reasons, less effective at stimulation of antibiosis by *P. involutus* than those of *P. resinosa*. The protective effect of *P. involutus* on *P. resinosa* may vary, however, because disease suppression is controlled by the interaction of a number of factors. Disease suppression may be dependant on fungal isolates (Laiho, 1970; Lapeyrie and Bruchet, 1986), on pine species and provenances (Cline and Reid, 1982; Marx and Bryan, 1971; Walker et al., 1986), of environmental conditions (Theodorou and Bowen, 1987), and on seedlings developmental stage. Nevertheless, an important conclusion of this study is that field application of ectomycorrhizal fungi as a means of biological control requires careful screening of ectomycorrhizal fungi to ascertain potential for disease protection. Furthermore, the use of ectomycorrhizal fungi in tree nurseries will require field screening to ensure effectiveness (Duchesne et al., 1988c).

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