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Peroxidase Activity in Poplar Inoculated with Compatible and Incompetent Isolates of *Paxillus involutus*

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Peroxidase activity of the hybrid poplar *Populus x canescens* (Ait.) Sm. (= *P. tremula* L. x *P. alba* L.) inoculated with compatible and incompetent isolates of *Paxillus involutus* (Batsch) Fr. was investigated. Screening of the ectomycorrhizal fungal isolates was initiated with exploration of mycelial growth characteristics and mycorrhizal ability *in vitro* with poplar. Both traits varied within the fungus although they did not seem to be genetically correlated. While isolates SCO1, NAU, and 031 grew faster than others, only isolates MAJ, SCO1, and 031 were able to form ectomycorrhiza with poplar. Isolates MAJ (compatible) and NAU (incompetent) were subsequently selected for further experiments. Activity of peroxidase, one of the defense-related enzymes, was examined in pure culture and short root components of compatible and incompetent interactions between poplar and *P. involutus*. Peroxidase activities increased significantly in poplar inoculated with incompetent isolate of the fungus compared to control, while induction of the same enzyme was not detected in compatible associations.

Key words: ectomycorrhiza, *Paxillus involutus*, peroxidase, plant defense, poplar

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INTRODUCTION

Despite widespread recognition of important roles of ectomycorrhizal fungi, a little is known about their physiological and molecular interactions with plants. The nature of these mutually symbiotic associations between fungi and roots is still in its infancy and remains poorly understood. There have been indications, however, that compared with much tighter plant-pathogen interactions, mycorrhizal symbiosis lacks specificity between the fungi and the plants. It is clear that we need information in a number of areas including the genes and proteins present in symbiotic tissues and how they are regulated. The question of whether plants recognize the presence of symbiont at all, or rather that the defense responses are repressed by the symbiotic fungus in order to allow a compatible interaction is yet to be elucidated.

In plant-mycorrhizal associations, a number of different approaches to investigate mechanisms occurs during the symbiosis have been under taken. Most of the strategies, however, have involved comparisons with other plant-microbe interactions about which we have more extensive information (Smith 1999). This includes production of hydrogen peroxide (H_2O_2) and peroxidases (Morandi *et al*. 1984; Gianinazzi-Pearson *et al*. 1992; Olson & Varner 1993; Lamb & Dixon 1997; Karpinski *et al*. 1999; Pellinen *et al*. 1999; Orozco-Cárdenas *et al*. 2001; Salzer & Boller 2001; Schützendübel *et al*. 2001; Tagu *et al*. 2001; Gafur *et al*. 2004) and the expression of genes coding for pathogenesis-related (PR) protein such as chitinases (Gianinazzi 1991; Dumas-Gaudot *et al*. 1992; Gianinazzi-Pearson *et al*. 1992). Information on similarities and differences between the interactions will subsequently lead to a good understanding of the molecular nature of mycorrhizal development which can be linked to the physiological and biochemical function of mycorrhizal symbioses of different types (Smith 1999).

The present study was aimed at investigating whether active defense mechanisms, such as activation of peroxidases, are induced in hybrid poplar *Populus x canescens* (Ait.) Sm. (= *P. tremula* L. x *P. alba* L.) in response to inoculation with *Paxillus involutus* (Batsch) Fr. (Basidiomycetes, Boletales, Paxillaceae). The involvement of peroxidases in induction of plant resistance has been confirmed. Generally, peroxidases enhance their activity after pathogen attack (Lebeda *et al*. 2001). To facilitate this exploration, *in vitro*, rapid synthesis of ectomycorrhiza under aseptic conditions and in systems which produce uniform infection needed to be established (Gafur *et al*. 2005). Isolates of *P. involutus* with different mycorrhizal abilities were also required for perfect comparisons of the interactions. As the fungus represents broad-range host fungal species (Langenfeld-Heyser *et al*. 2007), the poplar-*P. involutus* system described in this study may also be employed in exploration of other ectomycorrhizal interactions.

MATERIALS AND METHODS

Plant Culture, Fungal Culture, and Growth Media. After cutting, plantlets of hybrid poplar were grown for three to four weeks on MS medium (Murashige & Skoog 1962) to allow sufficient rooting under sterile conditions before use for ectomycorrhization. To ensure optimal growing conditions for the plant, special attention was focused to the humidity of the growing system, as young poplars are sensitive to low humidity. Eleven isolates of *P. involutus* used in this study (Table 1) were stock cultures available at the Institute for Forest Botany, University of Göttingen, Göttingen, Germany.

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They were collected from various hosts and environmental conditions in different places of Germany and other European countries. Prior to use as inoculum for the aseptic synthesis of ectomycorrhiza, five small plugs (10 mm diameter) of actively growing mycelia of *P. involutus* were transferred onto Modified Melin Norkrans (MMN) agar medium with further modifications and covered with cellophane. The medium contains (l^{-1}) 0.5 g KH_2PO_4, 0.25 g $(NH_4)_2SO_4$, 0.15 g $MgSO_4 \cdot 7H_20$, 1 ml of 1% $FeCl_3$, 0.05 g $CaCl_2$, 0.025 g NaCl, 5 g glucose, 3 g malt extract, 100 µg thiamine HCl. After addition of 2.5 ml trace elements, the pH was adjusted to 4.5. In order for the agar not to dry out, 30-35 ml of media was added to each of the 9 cm Petri dish.

Ectomycorrhizal Ability and Growth Characteristics of Fungal Isolates. For physiological investigations, both compatible and incompetent isolates that express optimal growing ability in pure culture were required. All isolates of *P. involutus* used in the present study were tested both for their capability to form ectomycorrhizal associations with poplar and for growth characteristics. Ectomycorrhization was carried out according to the technique developed by Hampp *et al.* (1996) with modifications. Rooted plantlets from sterile culture (about three weeks old) were carefully freed from agar particles and, for better handling, the lowermost leaves were removed. The root system of the plantlets was transferred directly to Petri dishes (9 cm diameter) containing 30-35 ml of modified MMN agar covered with a sheet of cellophane on which five plugs of fungal inoculum had been pregrown for one week. The root was carefully expanded to allow optimal contact with the agar surface. The lower end of the shoot was positioned in an opening of the sidewall of the dish punctured with a hot needle serving as an exit gate of the plants from the Petri dish. The Petri dish was closed, sealed with a strip of parafilm and then wrapped with aluminum foil to keep the roots in darkness. Symbiont culture was maintained in a special planting chamber, the bottom of which was covered with wet filter papers. As additional measures to prevent evaporation, the planting chamber was covered with a plastic cover. Quick transfer and high humidity were essential for keeping the plantlets alive. The whole system was maintained at 20 °C under continuous illumination. Roots were examined for mycorrhizal formation after one, four, and seven weeks of inoculation. Control plants were grown under the absence of other organisms. In the meantime, fungal growth characteristics were expressed as mycelial radial growth measured three weeks after incubation. Six plates of each isolate were examined. The cultures were maintained in Petri dishes by successive transfer on modified MMN agar medium.

Histochemical Staining for Mantle and Hartig Net Localization. Root tips of control, non-mycorrhizal and mycorrhizal tissues were vacuum infiltrated with freshly prepared 5 mM $CeCl_3$ in 50 mM 3-(*N*-morpholino)-propanesulfonic acid at pH 7.2 for 30 min. Tissues were then fixed in 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer (CAB), pH 7.2, for 1 hr at room temperature and kept overnight at 4 °C. After fixation, tissues were washed twice for 10 min in CAB and postfixed for 45 min in 1% (v/v) osmium tetroxide in CAB and dehydrated in a graded acetone series (30, 50, 70, 80, 90, and 100% [v/v]). The tissues were then progressively embedded in rising concentrations of acetone-resin mixtures, and finally incubated in two 24-hr changes of pure epoxy resin before polymerization at 60 °C for 48 hr. Sections of control, compatible, and incompetent root tissues were examined under microscope and subsequently photographed with a digital camera (Coolpix 990, Nikon, Tokyo) for observation of anatomical characteristics of the interactions with the emphasis on the formation of mantle and Hartig net.

Peroxidase Activity. Peroxidase accumulation induced as plant defense responses was investigated in the present poplar-*Paxillus* interactions. The enzymatic activities of the guaiacol peroxidase were determined according to the method of Pütter (1970), modified after Polle *et al*. (1990). Briefly, the assay reaction contained 500 µl of 100 mM potassium phosphate buffer pH 5.25, 400 µl of 100 mM guaiacol, 50 µl extract supernatant, and 50 µl of 200 mM H_2O_2. Assays were initiated by addition of H_2O_2 and the change of optical density at 436 nm was measured for 5 min at 25 °C (Beckman DU 640, Beckman Instruments Inc., CA, USA). Peroxidase activity was expressed as the change in optical density on the basis of protein contents. Short roots of control, compatible, and incompetent associations were harvested after one, four, and seven weeks, respectively, of inoculation for root sample preparations. Five plants were examined for each host-fungal isolate interaction and incubation period combination. Collected roots were powdered in liquid nitrogen using mortar and pestle. The powder was extracted 15 min at 4 °C in 1 ml of cold extraction buffer (10 mM Tris-HCl, 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.1 mM Na-EDTA, 10 mM Mercaptoethanol, 1 mM PMSF, 2% polyvinylpolypyrrolidone (PVPP), 0.1% [v/v] Triton X-100). The extract was centrifuged (30 min, 24,000 x *g*, 4 °C) and the supernatant was passed through a Sephadex G-25 column (NAP-5 column, Amersham Pharmacia Biotech AB, Uppsala, Sweden) and equilibrated with 100 mM potassium phosphate, pH 7.8. For fungal pure culture extraction, mycelia were grown for three weeks in Petri dishes on modified MMN agar. Suspension cultures were derived by homogenization of fungal inoculum from these cultures in liquid modified MMN medium and grown for two weeks on a shaker. After another homogenization, the suspension was transferred to new liquid medium for physiological studies. After incubation for five and ten days, respectively, fungal mycelia were homogenized in a frozen state. Five flasks were examined for each treatment. Extraction procedure was as explained above for plant roots with only minor modifications.

RESULTS

Selection of Fungal Isolates. Three (MAJ, SCO1, and 031) of the 11 isolates of *P. involutus* tested were found to consistently form ectomycorrhiza with poplar, whereas the others failed (Table 1). Failure to associate successfully may indicate that the isolates were incompetent with poplar. The table also showed variations in the growth ability within the species. All fungal isolates grew on modified MMN agar, except isolate B12. However, the growth form of mycelia varied

Table 1. Origin, host, mycelial radial growth, and ectomycorrhizal ability with hybrid poplar of isolates of *P. involutus* (Batsch) Fr. used in this study

Isolate	Origin	Host*	Radial growth (mm)**	Ectomycorrhiza***
B05	Belgium	ND	43.00 ± 4.16	-
B08	Belgium	ND	46.83 ± 5.58	-
B12	Belgium	ND	00.00 ± 0.00	-
B13	Belgium	ND	27.17 ± 1.57	-
MAJ	France	Poplar	49.50 ± 2.22	+
NAU	France	Oak	78.83 ± 2.48	-
P19	Poland	Birch/pine	31.00 ± 2.38	-
SCO1	Scotland	ND	85.00 ± 0.00	+
031	Germany	Spruce	65.17 ± 3.08	+
032	Germany	Spruce	27.83 ± 0.69	-
533	Germany	Spruce	26.17 ± 1.57	-

*ND: not determined; **Radial growth was measured three weeks after the fungi were grown on MMN. Values are expressed as means of six replicates; ***Ectomycorrhiza was formed (+) or not (-)

among isolates, with particular isolates (SCO1, NAU, and 031) producing more rapid and dense mycelia during growth on agar-based media than others. Two isolates with reasonable growth characteristics, compatible MAJ, and incompetent NAU (Figure 1), were selected for further experiments. MAJ was selected for the compatible interaction because the isolate produced the most consistent ectomycorrhizal association with poplar.

Morphological and Anatomical Characteristics. Root tips morphological changes of compatible association characteristics of mycorrhizal formation were visible one week after inoculation. The mycorrhiza continued to develop and they were mature by five to seven weeks. Development of poplar both grown in the absence and presence of *P. involutus* under currently described Petri dish system occurred normally.

Figure 1. Root systems of poplar in control plant (left) and plants inoculated with compatible MAJ (middle) and incompetent NAU (right) *P. involutus* isolates.

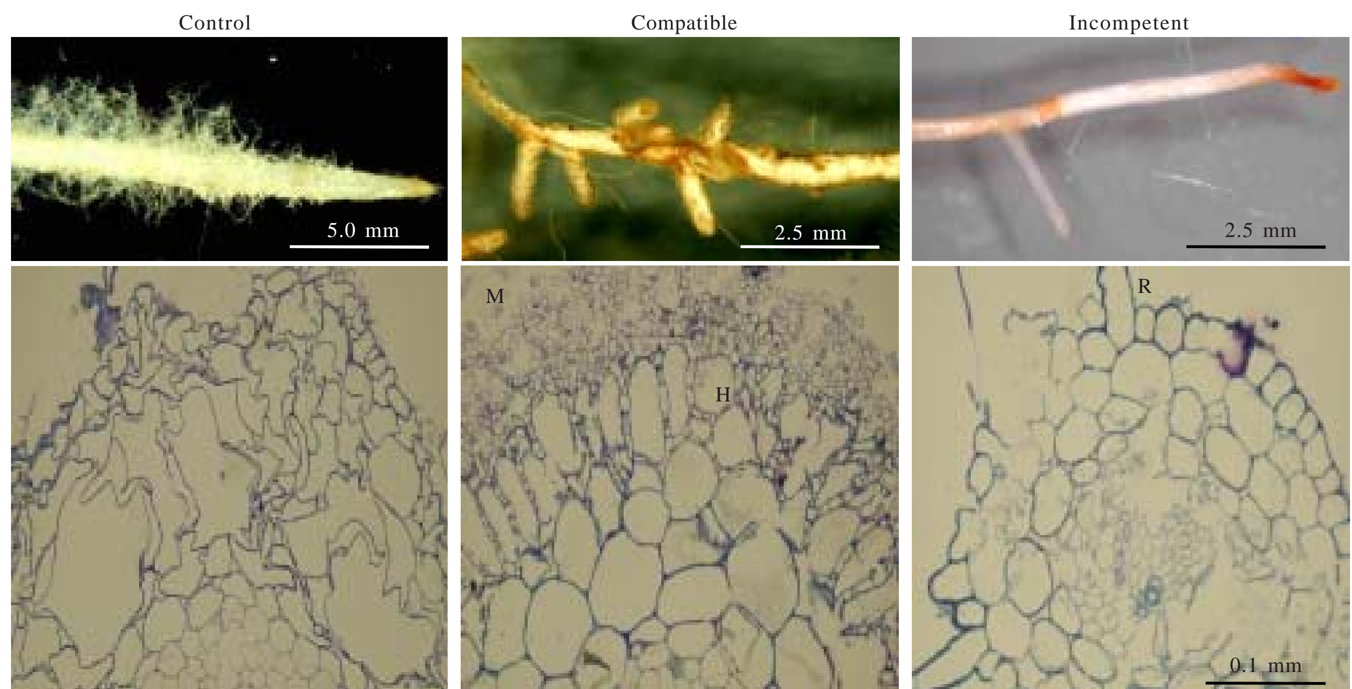

Figure 2. Morphology of poplar root tips (top) and anatomical characteristics of the cross sections of the root tips (bottom) in control, compatible, and incompeten isolates of *P. involutus* four weeks after inoculation. H: Hartig net, M: mantle, R: root hair. Control plants were grown in the absence of the fungus.

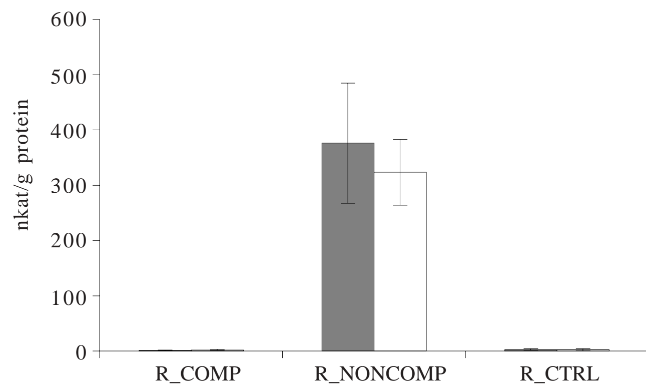

Figure 3. Peroxidase activities in interaction between poplar and *P. involutus*. Data were taken four (left bar) and seven (right bar) weeks after inoculation. Values are expressed as means of five replicates. Peroxidase activities were induced only in incompetent interactions (R_NONCOMP), not in compatible associations (R_COMP) or in control plants (R_CTRL).

All the plants exhibited a well-developed root system (Figure 1). Mycorrhiza formed by association between poplar and compatible isolate of *P. involutus* were initially pale white to yellowish and then became brown with age. Mycorrhized plants in this study were characterized among others by dramatic changes in root morphology such as stimulation of lateral root production and suppression of root hair formation. As shown in Figure 2 (top), root hair formation was restricted in compatible association. Anatomical characteristics of the roots of control, compatible, and incompetent associations were shown in Figure 2 (bottom). The mantle of compatible isolate surface consisted of loose web-like prosenchymatous elements. Mantle thickness varied and was irregularly layered. Hartig net developed showed intercellular penetration by hyphae, mostly in the form of a single hyphal row, to the first cortical layer. Upon maturity, the Hartig net fully enveloped the cells of epidermal layer forming a periepidermal net.

Peroxidase Accumulation. Peroxidase activity increased significantly in roots of poplar in response to incompetent isolate of *P. involutus* (Figure 3). Elevated levels of the enzymatic activity were maintained seven weeks after inoculation. In contrast, no significant changes in peroxidase activity were observed in compatible tissues after inoculation with mycorrhizal isolates MAJ or in control roots.

DISCUSSION

The results of the present study support the view that *P. involutus* has a broad variation of host range at the isolate level, as this has been previously reported by Heslin and Douglas (1986) and Molina and Trappe (1982). A number of studies also attest to significant variation in the ability of isolates of other fungal species to form ectomycorrhiza with particular host taxa (Bonfante *et al*. 1998; Cairney 1999). Similar growth characteristics of different ectomycorrhizal fungal species have also been earlier reported by Kieliszewska-Rokicka (1992) and Littke *et al*. (1984).

Despite the fact that both growth characteristics and mycorrhizal ability of *P. involutus* vary among the tested isolates, the two traits do not seem to be correlated in the fungus. For example, isolates NAU and 031 whose growth rate is at a similar level, differ in their ability to form ectomycorrhiza. While isolate 031 was able to form ectomycorrhiza with poplar, isolate NAU failed. In contrast, the growth rate of compatible isolates MAJ and 031 was different. Nevertheless, this lack of correlation between the two traits of mycelial growth and ectomycorrhizal ability enabled us to select isolates perfectly suitable for further experiments, i.e. compatible and incompetent isolates with optimal growth ability.

Increased peroxidase activity has been reported in many pathogenic interactions between plants and fungi (Svalheim & Robertsen 1990; Asiegbu *et al*. 1994; Fossdal *et al.* 2001). The fact that overall peroxidase activity was not significantly increased in the mycorrhizal roots of poplar supports earlier observations that mycorrhizal colonization does not activate full-scale plant-defense responses (Nylund 1981; Nylund 1987; Münzenberger *et al.* 1997) or elicitation of defense responses is avoided (Mohr *et al*. 1998). In the meantime, failure to detect peroxidase activities to an appreciable amount in short roots of control plants may well explain that defense-related peroxidases, in the absence of root microbial pathogens or other organisms, are not expressed.

As a defense mechanism, plants are able to respond to invading organisms in a variety of ways including production of components which modify cell walls including peroxidases, making them more difficult to penetrate (Hahlbrock *et al*. 1986). In symbiotic associations, on the other hand, both cell wall bound peroxidase and chitinase increased above control levels during the first days of mycorrhization, only to return to or fall below those of non-mycorrhizal roots at later stages of established symbiosis (Salzer & Boller 2001). However, Bonfante-Fasolo and Spanu (1992) pointed out that most of the experiments had not always been of a comparative nature, and a more systematic approach considering both compatible and incompetent mycorrhizal and pathogenic associations was needed. The present study, therefore, sheds some light on and contributes to better understanding of the mechanism of recognition in plant-microbe interactions.

Accumulation of peroxidase was not induced by all isolates of *P. involutus* tested in pure culture (data not shown). All isolates, including poplar-incompetent, did not seem to induce peroxidase accumulation to an appreciable amount. Similar results that no peroxidase activities are detected in extracts of extramatrical mycelium or pure culture mycelium have also been reported in different isolates of *Suillus bovinus* and *P. involutus* by Timonen and Sen (1998). Cairney and Burke (1998) also noted that there is currently no convincing evidence to support production of peroxidase activity by ectomycorrhizal fungi *in vitro*.

Isolates of MAJ and NAU served as perfect models to investigate physiological interactions between poplar and *P. involutus*. At the genomic level we have shown that the two isolates are very similar with DNA sequence identity of 98.9% although the difference is more significant in terms of expressed genes after contact with birch roots (Le Quéré *et al*. 2004). Thus, the currently described novel system should

also enable investigations on ectomycorrhizal interactions in different systems.

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