

Chitinase and peroxidase activities are induced in eucalyptus roots according to aggressiveness of Australian ectomycorrhizal strains of *Pisolithus* sp.

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(Received 5 October 1993; accepted 3 February 1994)

SUMMARY

Peroxidase and chitinase activities were measured in roots of *Eucalyptus globulus* spp. *bicostata* (Maid. *et al.*) Kirkp. during colonization by *Pisolithus* sp. isolated from under *Eucalyptus*. Ten fungal isolates, ranging from poor to good root colonizers, were selected to represent a range of ectomycorrhizal aggressivity. The induction of chitinases and peroxidases was strongly related to the aggressiveness of the fungal strain. Only good colonizers, that is strains which rapidly form differentiated ectomycorrhizas, induced a strong response in the plant. Therefore, it can be concluded that these enzymes are not responsible for poor root colonization by the less aggressive strains. The chitinase response of *Eucalyptus* roots to contact with fungal extracts differed only slightly between weakly and strongly aggressive strains. This suggests that a major component of differential induction observed *in vivo* is the consequence of root colonization, tissue penetration and the ability to deliver elicitors to the plant prior to and during root colonization.

Key words: *Eucalyptus*, *Pisolithus*, chitinase, peroxidase, ectomycorrhizas.

INTRODUCTION

Ectomycorrhizas are common symbiotic associations between roots and fungi. As they are characterized by differentiation of specialized tissues, the mantle and the Hartig net (Dexheimer & Pargney, 1991), the mechanisms leading to development of such structures should be highly regulated and under the control of recognition factors.

Numerous authors have reported interspecific and intraspecific differences among fungi with regard to the development of symbiotic structures, such as thickness of the mantle or the Hartig net (Debaud *et al.*, 1988; Malajczuk, Lapeyrie & Garbaye, 1990; Lei *et al.*, 1990; Wong, Piché & Fortin, 1990). Differential gene expression, which depends on a complex set of signals exchanged between the two partners, should determine some of these developmental differences. Certain genes, for example, could control fungal growth while others could determine resistance mechanisms within the host. It is postulated that expression of such genes will

determine the ectomycorrhizal aggressivity of a fungus. Aggressivity may be assessed by measuring the rate at which an ectomycorrhizal fungus colonizes a root apex and the extent of formation of symbiotic tissues such as the Hartig net (Lei *et al.*, 1990). It may also be assessed by the number of ectomycorrhizas which form on inoculated root systems (Wong *et al.*, 1990).

In a previous study (Burgess, Dell & Malajczuk, 1994), *Pisolithus* isolates from carpophores collected under *Eucalyptus* spp. in Australia displayed a range of ectomycorrhizal aggressivity when inoculated onto *E. grandis* seedlings *in vitro*. Based on morphological criteria, fungal sheath structure and Hartig net development, ten strains of contrasting aggressiveness were selected from among approximately one hundred isolates. We postulated that these ten strains would induce different responses in the plant, leading either to resistance of the host with the least aggressive isolate, or to differentiation of ectomycorrhizas with most aggressive.

Root peroxidase and chitinase activities in *Eucalyptus globulus* spp. *bicostata* (Maid. *et al.*) Kirkp. were measured during colonization by the ten strains

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of *Pisolithus*. These enzymes are known to contribute to resistance mechanisms (Fehrmann & Dimond, 1967; Roby, Toppan & Esquerré-Tugayé, 1987, 1988; Vögeli, Meins & Boller, 1988; Trudel, Audy & Asselin, 1989; Voisey & Slusarenko, 1989; Broglie *et al.*, 1991; Reimers, Guo & Leach, 1992). They are developmentally regulated, induced by abiotic or biotic stresses and involved in local and/or systemic acquired resistance. They have numerous isoforms with different cellular localization, suggesting a specific role for each isoform (Flach, Pilet & Jollés, 1992).

MATERIALS AND METHODS

Biological material

Seeds of the host, *Eucalyptus globulus* ssp. *bicostata* (Maid. *et al.*) Kirkp. were collected at Tumbarumba NSW, Australia and purchased from Kyliisa seeds, ACT (Seedlot: 91/24). The ectomycorrhizal fungi were Australian *Pisolithus* sp. isolates, MH56 (Murdoch University, Western Australia); and H4461, H2144, H445, H556, H506, H8101 H98, H4320 (CSIRO Herbarium, Division of Forestry, Western Australia). These isolates were chosen to cover a range of ectomycorrhizal aggressivity based on the morphology of 7 d-old root tips of *E. grandis* (Burgess *et al.*, 1994). In addition a Brazilian isolate, 441, collected under *Eucalyptus citriodora* was included for comparison with earlier studies (Albrecht *et al.*, 1993).

Root inoculation

Before inoculation, fungi were grown for three weeks at 25 °C on cellophane laid over modified low sugar Pachlewski's medium (7.3 mM KH_2PO_4 ; 5 mM glucose; 2.7 mM diammonium tartrate; 7.3 mM MgSO_4 ; 2.9 μM thiamine-HCl; 1 ml of trace element stock solution (Kanieltra 6 Fe from Hydro Azote Co, France) and 20 g of agar per litre). One-week old plants were grown under aseptic conditions and gently laid over the edge of the fungal colony (Malajczuk *et al.*, 1990). Plates were incubated in a controlled environment chamber (light/dark period, 16/8 h; temperature, 22 °C; photosynthetic photon flux density, 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Strictly identical treatments were applied to uninoculated control seedlings, except that fungal hyphae were absent. Seedlings were sampled 1, 2, 4 and 7 d after inoculation. Entire roots excised from 20 replicate seedlings, and free living hyphae from five control colonies of corresponding age, were pooled separately and stored in liquid nitrogen before protein extraction.

Protein extraction

Inoculated roots, free living mycelia and control uninoculated roots were powdered in liquid nitrogen

using a mortar and pestle. The powder was extracted using potassium phosphate buffer (100 mM, pH 7; 1:10, w/v). Extracts were centrifuged at 4 °C (10000 *g* for 20 min). The supernatants were used for chitinase and peroxidase assays, and the pellets were extracted to estimate the fungal mass by the ergosterol assay.

Ergosterol assay

Pellets were resuspended in 0.5 ml absolute methanol, shaken and centrifuged for 5 min at 14000 *g* at 4 °C. The supernatant was filtered through a 0.45 μm acetate filter (Sartorius, Paris) and analysed for free ergosterol by HPLC (Martin, Delaruelle & Hilbert, 1990). The fungal fresh weight in ectomycorrhizas was calculated by relating their ergosterol content to the ergosterol concentration of pure cultures sampled under similar conditions.

Preparation of fungal extracts and application to Eucalyptus roots

Three week-old fungal colonies, grown over cellophane, were collected, freeze-dried and powdered in liquid nitrogen using a mortar and pestle. The powder (20 mg) was resuspended in 200 μl of phosphate buffer (100 mM, pH 7). After centrifugation at 4 °C (10000 *g* for 20 min), the supernatant was filtered (Sartorius filter, 0.2 μm). Twenty seedlings, 7-d old, were placed in 300 μl of this medium in a watch glass and incubated in a controlled environment chamber as previously. Roots were collected for chitinase analysis 24 h later.

Native polyacrylamide gels and chitinolytic activity

Sample preparation and staining for chitinase activity, after polyacrylamide gel electrophoresis (PAGE) under native conditions for the separation of acidic or neutral proteins (Davis, 1964), were performed as described by Trudel & Asselin (1989). Extracts were loaded on gels by total fresh weight, no correction was made to compensate for mycelium present on the root surface. Chitinase activity was detected as dark (non-fluorescent) bands against a UV fluorescent background of 0.01% (v/v) glycolchitin embedded in the gel and stained with Calcofluor White M2R. Computerised densitometric analysis of the gel photographs was used to quantify and compare lysis zones from the same gel. The density of the lytic zones on gels was found to be linear for volumes of root extracts from 2.5 to 25 μl .

Peroxidase activity

Peroxidase activity was measured as described by Bashan, Okon & Henis (1987). The reaction mixture

contained: 0.5 ml of 0.1 M phosphate buffer pH 7; 0.4 ml of a solution prepared from 0.2 ml of guaiacol (1%) dissolved in absolute ethanol; 0.2 ml of 10 mM H₂O₂ in distilled water; and 25 μ l protein extract. Absorbance at 470 nm was recorded for 3 min. One enzyme unit was defined as the increase in 0.01 absorbance units min⁻¹. Each value is the mean of two separate replicate experiments.

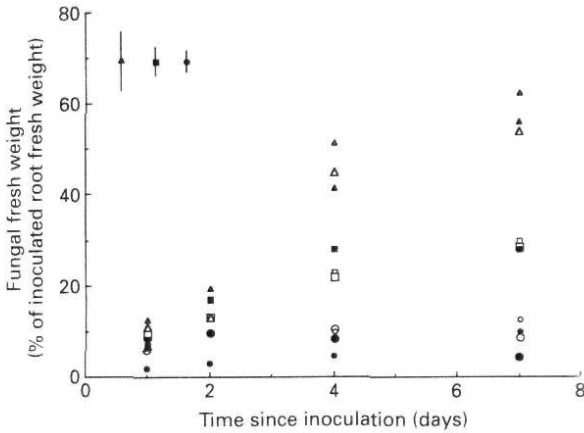


Figure 1. Time-course of colonization of roots of *Eucalyptus globulus* ssp. *bicostata* by ten strains of *Pisolithus* sp. *Pisolithus* strains: Δ , MH56; \blacktriangle , H4461; \triangle , H2144; \square , H445; \square , 441; \blacksquare , H556; \circ , H506; \bullet , H8101; \circ , H98; \bullet , H4329. Each value, estimated from the ergosterol content of infected roots, is the mean of two replicate experiments. Error bars show the mean standard deviation for strains belonging to the same aggressiveness group (triangle; very aggressive strains, square: aggressive strains, circle: poorly and non-aggressive strains).

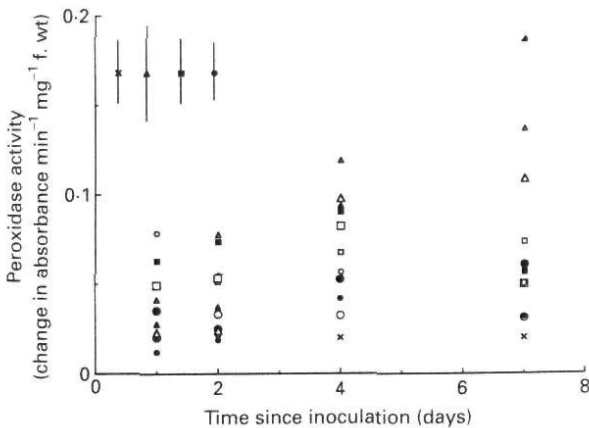


Figure 2. Time-course of peroxidase activity in roots of *Eucalyptus globulus* ssp. *bicostata* following colonization by ten strains of *Pisolithus* sp. Symbols for fungal strains as in Figure 1, X: non-inoculated roots. Each value is the mean of two replicate experiments. Error bars shows the mean standard deviation for strains belonging to the same aggressiveness group (triangle: very aggressive strains, square: aggressive strains, circle: poorly and non-aggressive strains) or non-inoculated roots.

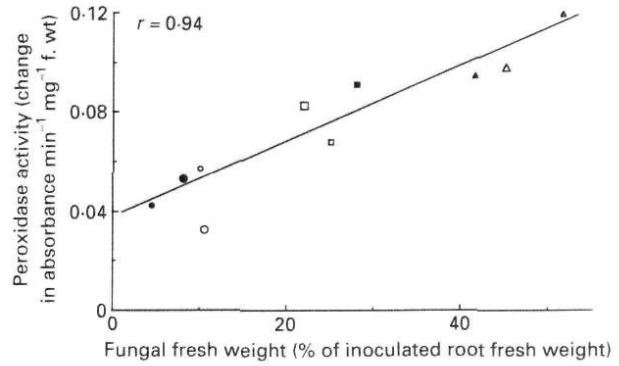


Figure 3. Relationship between intensity of colonization of roots of *Eucalyptus globulus* ssp. *bicostata* by ten strains of *Pisolithus* sp. (symbols for fungal strains as in Figure 1) and soluble peroxidase activity in roots ($r = 0.94$).

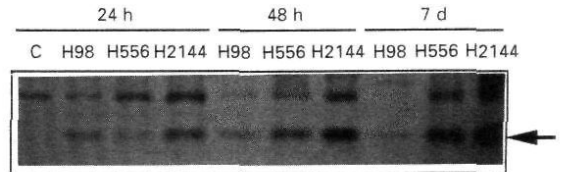


Figure 4. Chitinase activities of roots of *Eucalyptus globulus* ssp. *bicostata* after native 15% polyacrylamide gel electrophoresis (PAGE) for acidic proteins (Davis system). The second isoform activity (arrow head) from root extracts was compared 24 h, 48 h and 7 d after inoculation with three strains of *Pisolithus* sp. previously characterized as very aggressive (H2144), aggressive (H556) and poorly aggressive (H98). Control plants (C) are non-inoculated. Samples each contained 0.5 mg fresh weight material.

RESULTS

Root colonization

All the fungal isolates were previously shown to be able to infect eucalypt roots (Burgess *et al.*, 1994). However, in that study, great variability in aggressiveness toward *Eucalyptus grandis* was described *in vitro* on the basis of a morphological examination of 7 d-old tips. Here, fungal aggressiveness towards *E. globulus bicostata* was assessed by the kinetics of ergosterol accumulation in roots sampled over 7 d (Fig. 1). Fungal isolates were classified into three groups.

(i) Poorly aggressive and non-aggressive strains: H506, H8101, H98, H4320. The fresh weight of mycelium colonizing the root by day 7 was less than 15% of root fresh weight. This sparse hyphal envelope may simply have resulted from hyphal proliferation on root exudates and may not have required typical fungal-root attachment characterized by a glycoproteic fibrillar network (Lei *et al.*, 1990).

(ii) Aggressive strains: H445, 441, H556. Hyphal mass attached to the root increased linearly to reach between 25% and 35% of infected root fresh weight. In *E. grandis*, roots showed typical ectomycorrhizal

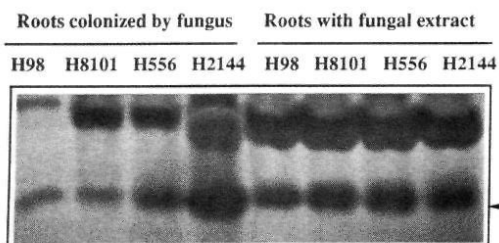


Figure 5. Chitinase activities of roots of *Eucalyptus globulus* ssp. *bicostata* after native 15% polyacrylamide gel electrophoresis (PAGE) for acidic proteins (Davis system). The second isoform activity (arrow head) from root extracts was compared 4 d after inoculation with four strains of *Pisolithus* sp. previously characterized as very aggressive (H2144), aggressive (H556) and poorly aggressive (H98, H8101) or 24 h after treatment with extracts from these strains. Samples each contained 0.8 mg fresh weight material.

features, namely a differentiated mantle and a Hartig net partially or uniformly developed in the outer cortical layer (Burgess *et al.*, 1994).

(iii) Very aggressive strains: MH56, H4461, H2144. Fungal colonization increased rapidly from day 4 to reach more than 50% of infected root fresh weight after 7 d. Ectomycorrhizas formed on *Eucalyptus grandis* by these strains were characterized by a thick mantle sometimes divided into two layers and a Hartig net uniformly developed in the outer cortex (Burgess *et al.*, 1994).

Peroxidase activity during fungal colonization

No peroxidase activity was detected in protein extracts prepared from mycelia in pure culture.

Uninoculated control plants had low peroxidase activity throughout the experiment (Fig. 2). Twenty four hours after inoculation, increases in peroxidase activity were detected in plants in contact with most fungal isolates. Significant differences between plants inoculated with poorly aggressive and very aggressive strains were detected 4 d after inoculation. The peroxidase activity of roots colonised by poorly aggressive strains remained low over the 7 d period. The peroxidase activity of plants inoculated with very aggressive strains increased with time of contact.

Four days after inoculation, root peroxidase activity in the three aggressiveness groups was highly correlated ($r = 0.94$) with the amount of fungal mass, estimated from the ergosterol content, attached to the roots (Fig. 3).

Chitinase activity following fungal inoculation

The fungal chitinase did not migrate into the gel. The electrophoretic pattern of chitinase activity in root extracts showed two isoforms. Twenty four

hours after contact between symbionts, the second isoform was strongly stimulated (Fig. 4). The activity of the second isoform increased in roots inoculated with all fungal strains. However, differential root chitinase induction by strains belonging to the three classes of aggressiveness was observed:

(i) with poorly aggressive strains: chitinase were fully induced after 24 h of contact and their activity remained low over the 7 d period,

(ii) with aggressive and very aggressive strains: chitinase activity increased up to the final harvest (7 d of contact). Enzyme activity was higher with the very aggressive strains.

There was a positive correlation between the activity of the second root chitinase isoform and root colonization from 2 to 7 d after contact ($r = 0.76$ after 2 d, 0.74 after 4 d, 0.76 after 7 d). Each r value is the mean from 2 separate replicate experiments. There was no correlation at 24 h after contact ($r = 0.49$).

Chitinase activity following contact with crude fungal extracts

Root chitinase activity was compared following induction for 4 d by the ectomycorrhizal fungus and contact for 24 h with fungal extracts. Results from four strains, representative of the three groups defined earlier (two from group 1), are presented (Fig. 5). The root chitinase activity can be compared only among fungi within the same treatment group. Chitinase activity of the second isoform, as previously observed, was much higher in roots colonized by a highly aggressive strain (H2144) than in roots colonized by poorly aggressive strains (H98 and H8101). Activity of the second isoform in roots colonized by H98 was 30% of that in roots colonized by the most aggressive strain (H2144).

The same trend was observed when roots were placed in contact with fungal extracts prepared from these strains. However, there was less response by the plant to strains of variable aggressiveness; extracts from the less aggressive strain induced only 60% of the chitinase activity induced by extracts from the most aggressive strain.

DISCUSSION

Extra chitinase and peroxidase activities were induced in *Eucalyptus* roots following ectomycorrhizal inoculation and root colonization. Stimulation of chitinase activity has previously been reported during ectomycorrhizal infection of *Eucalyptus* roots (Albrecht *et al.*, 1993) as well as in mature ectomycorrhizas (Dumas-Gaudot *et al.*, 1992a, b). Extracts from ectomycorrhizal fungi repeatedly proved able to induce chitinase accumulation in roots (Albrecht *et al.*, 1993) and in plant cells in culture

(Sauter & Hager, 1989). Here, comparing a large number of well characterized fungal strains, we have been able to show that the plant response was strongly related to the aggressiveness of the fungal strain. Up to now, stimulation of peroxidase activity by symbiotic fungi has been observed only during endomycorrhizal infection, where cell wall peroxidase was assessed (Spanu & Bonfante-Fasolo, 1988). No significant differences were observed in peroxidase activity between ectomycorrhizal and nonmycorrhizal short roots of *Pinus sylvestris* (Ronald & Söderhäll, 1985).

These enzymes are often considered to have a role in plant defence. This could be questioned considering their presence and stimulation in symbiotic relationships. The induction of both groups of enzymes, chitinase and peroxidases, appears to be strongly related to fungal strain aggressiveness. Only good colonizers, that is fungal strains which rapidly form mature ectomycorrhizas, induce a strong response from the plant. Therefore, it can be assumed that these enzymes are not responsible for poor root colonization by the less aggressive strains. However, the sensitivity of ectomycorrhizal fungi to those chitinases induced during infection still has to be assessed. Indeed, fungal penetration through root tissue might be regulated by very localized production of minor isoforms.

It is feasible that these plant enzymes may not be directed toward the fungus but the plant organs themselves. Indeed, apart from controlling fungal growth (Boller *et al.*, 1983; Schlumbaum *et al.*, 1986; Mauch, Mauch-Mani & Boller, 1988), chitinases may participate in the generation of plant signal molecules which regulate development and organogenesis (Neale *et al.*, 1990; De Jong *et al.*, 1992, 1993; Staehelin *et al.*, 1992). Further, while peroxidases can contribute to the deposition of lignin and other phenolic polymers which serve as physical barriers preventing fungal spread, they could also participate actively in differentiation processes (Coppens & Dewitte, 1990; De Jong *et al.*, 1992; Sherf & Kolattukudy, 1993). In ectomycorrhizas, for example, they could act by triggering, and contributing to, developmental processes leading to specialised structures such as the Hartig net.

When the roots were in contact with an equal amount of fungal extract from each strain, a measurement of chitinase induction did not allow the aggressive and less-aggressive strains to be easily discriminated. Indeed, the response of *Eucalyptus* roots did not differ strongly between extracts. This suggests that among these strains of *Pisolithus tinctorius* isolated in Australia (except strain 441) from under eucalypts, a minor component of differential induction observed with live mycelium is the consequence of elicitor concentration or specificity, while a major component is the consequence of root colonization and tissue penetration.

ACKNOWLEDGEMENT

The authors wish to thank Dr N. Malajczuk (CSIRO, Perth) for providing most of the *Pisolithus* cultures and Dr F. Martin (INRA, Nancy) for valuable discussions.

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