

## Induction of catalase and ascorbate peroxidase activities in tobacco roots inoculated with the arbuscular mycorrhizal *Glomus mosseae*

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Catalase and ascorbate peroxidase enzymatic activities were examined during the interaction between *Nicotiana tabacum* and the arbuscular mycorrhizal *Glomus mosseae*. Transient enhancements of both enzymatic activities were detected in the inoculated plant roots coinciding in time with the stage of appressoria formation in the root surface. The analysis of free salicylic acid content in roots revealed that the increases in enzymatic activities were coincident in time with the accumulation of SA in inoculated roots. These data indicate that the first reaction of the root cells to the invasion of arbuscular mycorrhizal fungi is a defence response.

### INTRODUCTION

Arbuscular mycorrhizas (AM) are symbiotic associations between zygomycetes and the roots of most terrestrial flowering plants. The establishment of this mutualistic association is a successful strategy to improve the nutritional status of both partners. The plant supplies the biotrophic fungus with carbon, and the fungus assists the plant with the acquisition of phosphate and other mineral nutrients from the soil. In addition to this nutritional effect other agronomic and ecological benefits of the association, particularly in plant resistance to invading pathogens (García-Garrido & Ocampo 1988, 1989) and soil conservation (Jeffries 1987) have been reported.

The establishment of compatible AM symbiosis requires recognition by the plant host. The plant clearly exerts a degree of control over AM fungi development since it restricts its growth to the cortical parenchyma. Some plant resistance marker molecules and genes have been investigated previously in compatible AM fungus-root interactions. During the early stage of colonisation a transient and weak plant defence response is induced and then subsequently suppressed (Kapulnik *et al.* 1996). A weak transient accumulation of phytoalexins has been reported in roots system of soybean (Morandi, Bailey & Gianinazzi-Pearson 1984); and *Medicago* species (Harrison & Dixon 1993, Volpin *et al.* 1995). In addition, the expression of genes encoding the early phenylpropanoid biosynthetic enzymes phenyl ammonia lyase (PAL), chalcone synthase (CHS) and chalcone isomerase (CHI) are enhanced concomitant with isoflavonoid accumulation (Harrison & Dixon 1993, Volpin *et al.* 1994, 1995). Enhanced peroxidase, chitinase and  $\beta$ -1,3 glucanase activities coincided with early plant-fungal interactions in AM and strongly diminish as roots colonisation proceeds (Spanu & Bonfante-

Fasolo 1988, Spanu *et al.* 1989, Lambais & Mehdy 1993, Vierheilig *et al.* 1994, Volpin *et al.* 1994).

Hypersensitive-like reactions were observed in Ri T-DNA transformed roots of *Medicago sativa* during colonisation by *Gigaspora margarita* (Douds *et al.* 1998). Moreover, Salzer, Corbière & Boller (1999) have shown that a locally restricted oxidative burst is involved in control of the colonisation of *Medicago truncatula* cells by *Glomus intraradices*. This oxidative burst was characterised by the accumulation of H<sub>2</sub>O<sub>2</sub> around hyphal tips and cells in which arbuscules were clumped and little branched.

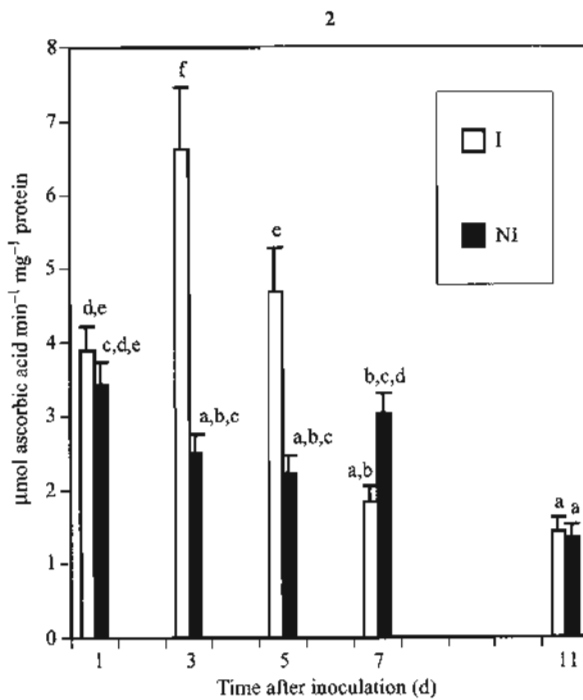
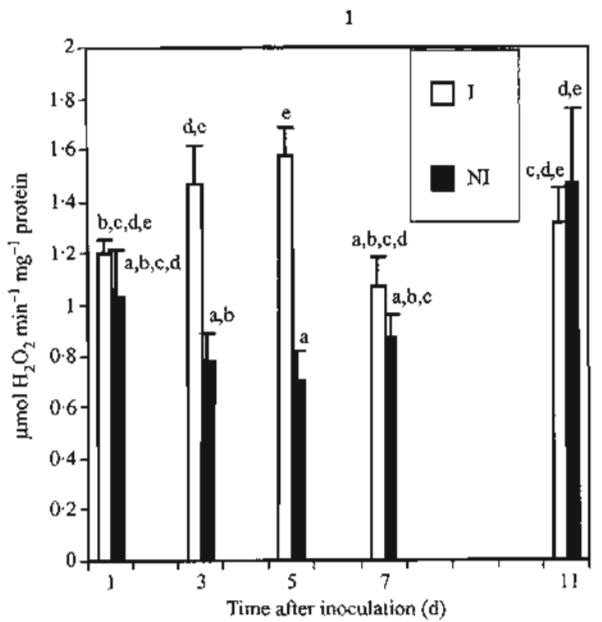
Peroxidases and catalases are involved in the defence mechanisms of plants in response to pathogens either by their direct participation in cell wall reinforcement, or by their antioxidant role in the oxidative stress generated during plant pathogen interaction (Mehdy 1994). Catalase and ascorbate peroxidase are induced by oxidative stress, since they convert H<sub>2</sub>O<sub>2</sub> to H<sub>2</sub>O. It has been demonstrated that H<sub>2</sub>O<sub>2</sub> activates the acid-benzoic 2-hydroxylase (Leon, Lawton & Raskin 1995), an enzyme required for salicylic acid (SA) biosynthesis.

Here we report the analysis of catalase and ascorbate peroxidase enzyme activities in tobacco roots inoculated with *Glomus mosseae* at the early stages of their interaction. We also analysed the content of free salicylic acid (SA) in the root in order to correlate the increases in enzyme activity with a marker of plant defence response.

### MATERIAL AND METHODS

#### *Plant material, methods of inoculation and growth*

Tobacco (*Nicotiana tabacum* cv. Tennessee) seeds were surface sterilised with 10% of sodium hypochlorite for 5 min and



Figs 1, 2. Catalase and ascorbate peroxidase activity in tobacco roots non-inoculated (NI) and inoculated (I) with *Glomus mosseae*. s.e. are shown ( $n = 3$ ). Values sharing the same letter are not significantly different (Duncan's multiple range test:  $P = 0.05$ ). Fig. 1. Catalase. Fig. 2. Ascorbate peroxidase.

germinated at 28 °C in darkness. Seedlings were grown in a sand vermiculite 50:50 (v:v) seed bed for 4 wk after which they were transplanted in 80 ml pots containing a sterile mixture of sand: vermiculite: peat, 50:50:1 (v:v). The inoculation was performed at the moment of transplanting using 1 g of soil inoculum of *Glomus mosseae* (BEG 12) per pot. The soil inoculum was spread around the root of the transplanted plant to assure a rapid colonisation. Control pots were inoculated with 10 ml of water filtrate corresponding to 1 g of inoculum (McAllister *et al.* 1997).

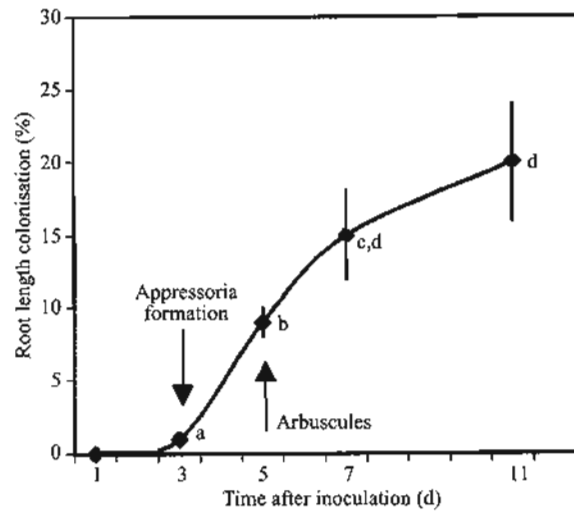


Fig. 3. AM root length colonisation (%) in tobacco inoculated with *Glomus mosseae*. The appearance of typical AM structures is indicated by arrows. s.e. are shown ( $n = 3$ ). Values sharing the same letter are not significantly different (Duncan's multiple range test:  $P = 0.05$ ).

Plants were grown under greenhouse conditions and harvested at 1, 3, 5, 7 and 11 d after inoculation. There were three replicate control and inoculated pots sampled at each harvest.

The root system of each replicate pot was divided into three portions, one for the determination of the mycorrhizal colonization as described by Ocampo, Martin & Hayman (1980), the other for protein and SA assays.

#### Protein extraction

Root tissue (0.5 g) was ground in liquid nitrogen and homogenised in 2 ml of extraction buffer (6% of polyvinyl-pyrrolidone; 0.1% of Triton X-100; 1 mM of phenyl-methylsulphonyl fluoride; 1 mM of dithiothreitol and 50 mM of dipotassium hydrogen phosphate, pH 7). After centrifugation at 10000 g for 10 min, the supernatant was filtrated and precipitated with 2 vol. of cold acetone for 4 h at 4 °C, the pellet was recovered by centrifugation at the same conditions as described previously and dissolved in 50 mM potassium phosphate at pH 7.

Protein concentration was determined as described by Lowry *et al.* (1951). Catalase and ascorbate peroxidase activities were measured as described by Aebi (1984) and Amako, Chen & Asada (1994) respectively.

#### Extraction and quantification of free salicylic acid

For SA extraction 0.5 g of roots tissues were used as described by Rasmussen, Hammerschmidt & Zook (1991). After ethanol extraction and centrifugation, the pellets were dried and dissolved in 100 µl 5% trichloroacetic acid and extracted with 2 vol. of cyclopentane:ethyl acetate:isopropanol, 50:50:1 (v:v) according to Malamy, Henning & Klessing (1992). The organic extract was dried under nitrogen, resuspended in 10 µl of ethanol and analysed by tlc. The samples were spotted onto silica gel 60 A chromatography plates (Merck) and developed in toluene:dioxane:acetic acid, 90:25:4 (v:v)

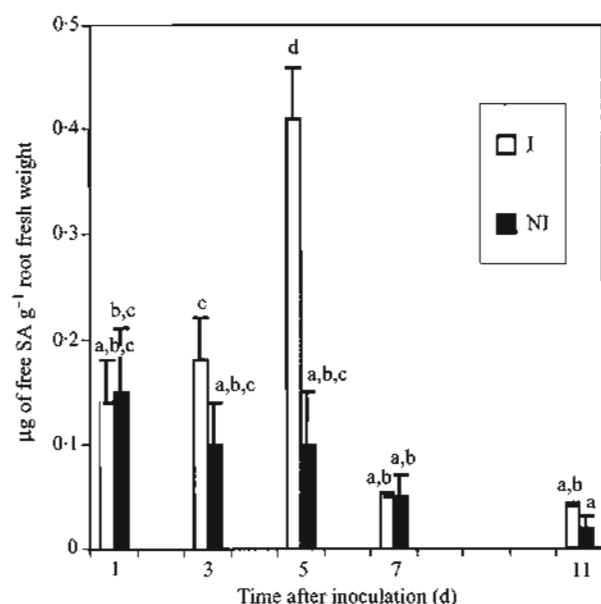


Fig. 4. Time-course of free salicylic acid accumulation in tobacco roots non-inoculated (NI) and inoculated (I) with *Glomus mosseae*. s.e. are shown ( $n = 3$ ). Values sharing the same letter are not significantly different (Duncan's multiple range test:  $P = 0.05$ ).

(Rasmussen *et al.* 1991). The fluorescent band corresponding to salicylic acid was visualised and identified in the plate by viewing under uv light (302 nm). The band was eluted from the silica gel with 1 ml of 95% ethanol and used for fluorimetric quantification (excitation wavelength = 310, emission wavelength = 400 nm). Different ethanol aliquots of salicylic acid (Sigma) solution were run in parallel, recovered from the silica gel, quantified and used as standard. The value of each replicate is the average of 10 fluorescent readings taken over 10 s. The limit of detection for salicylic acid in a final volume of 1 ml was 1 nmol.

#### Data analysis

The results were evaluated statistically using Duncan's multiple range test ( $P = 0.05$ ). The data presented correspond to a representative experiment.

## RESULTS AND DISCUSSION

Catalase activity in control roots increased at the final harvest (Fig. 1) and ascorbate peroxidase activity in control plants tended to decrease throughout the experiment (Fig. 2).

In *G. mosseae* inoculated plants a transient increase of enzymatic activities was observed when compared with non-inoculated plants. Catalase activity in inoculated roots showed a weak, but significant increase after 3 and 5 d of inoculation in comparison to the non-inoculated roots harvested at the same age (Fig. 1). After reaching the maximum, catalase activity in inoculated roots decreased to that found in noninoculated roots. The increase in catalase activity in the control plants at the end of the experiment

suggests that at this stage the enzyme was independent of *G. mosseae* inoculation.

Similar results were obtained for ascorbate peroxidase in inoculated roots. Ascorbate peroxidase activity was higher in mycorrhizal roots than in control roots at 3 and 5 d after inoculation (Fig. 2). The peak of enhanced activity was transient, and within days the activity decreased to that found in non-inoculated roots (Fig. 2).

The observed changes in enzymatic activities occurred at the initial interaction between mycorrhizal fungus and roots. The first entry points and appressoria were observed after 3 d of inoculation, and arbuscules were evident after 5 d (Fig. 3). Once the fungus had infected it rapidly proliferated within the root, reaching  $15 \pm 3\%$  and  $20 \pm 4\%$  of root length colonized after 7 and 11 d respectively (Fig. 3). At these latter stages of development both enzymatic activities reached similar values to those seen in non-inoculated controls.

The transient increases of catalase and ascorbate peroxidase coincided in time with appressoria formation in the root surface at the onset of the interaction of tobacco roots with the arbuscular mycorrhizal *G. mosseae*. This result could indicate that the first reaction of the root cells to the AM fungi invasion is a defence response. Previous studies have demonstrated the increase of plant defence response at the early stage of colonisation (reviewed by Gianinazzi-Pearson *et al.* 1996). Interestingly, in some plant-AM fungus interactions the initial response of colonised cells was a hypersensitive-like reaction, with accumulation of phenolics compounds (Douds *et al.* 1998) or  $H_2O_2$  around hyphal tips (Salzer *et al.* 1999). These findings suggest that AM fungi can elicit defence responses. It has been reported that appearance of stunt disease in tobacco plants may be correlated with inoculation with AM fungi (Modjo & Hendrix 1986).

The increases in catalase and ascorbate peroxidase observed in our experiments may be related with their antioxidant role against any active oxygen molecules generated during the initial stage of fungus penetration. Further studies are required to correlate enzyme activity with biochemical alterations in the oxidative metabolism in colonised cells.

To relate the increase of catalase and ascorbate peroxidase activities with defence responses occurring during the early stage of colonisation, we measured free SA levels in tobacco roots throughout the colonisation process. As shown in Fig. 4, a transient increase of free SA was found in colonised roots. This increase was a maximum significantly higher at 5 d of colonisation, after which SA concentration decreased to meet that found in non-inoculated roots. This suggests that an initial defence-like response does take place in tobacco cells colonised by *G. mosseae*. Nevertheless, the response is transient and does not prevent colonisation, since the fungus colonised the root normally. Increases in SA also have been shown in the incompatible interaction between *Myc<sup>-</sup>, Nod<sup>-</sup>* pea plants and *G. mosseae* or *Rhizobium leguminosarum* and a role for the SA in the resistance mechanism has been proposed (Blilou 1998).

On the other hand, it is possible that the transient increase in catalase and peroxidase activities observed in our experiments could be due to oxidative stress in colonised cells. As a consequence of this oxidative alteration, the intracellular

level of SA could be increased in these cells. There may be a synergetic relation between SA and  $H_2O_2$  in some plant defence responses (Van Camp, Van Montagu & Inzè 1998).

The results presented here indicate that enzymes such as catalase and ascorbate peroxidase, which are normally induced by oxidative stress, are induced at the commencement of mycorrhizal infection, but are subsequently suppressed. The induction was coincident in time with the transient enhancement of free SA level, a molecular marker of defence response in plants. The defence responses in AM interactions are typically restricted to the infected tissues, and have been reported to be significantly lower than those observed in roots challenged with pathogens (Gianinazzi-Pearson *et al.* 1996). The role and significance of the mechanisms of defence activated during AM interaction remains unclear. Investigation of the regulatory mechanisms underlying this activation event may contribute to our understanding of the mechanisms underlying the establishment of an effective symbiosis.

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