

## Different Polypeptide Profiles from Tomato Roots following Interactions with Arbuscular Mycorrhizal (*Glomus mosseae*) or pathogenic (*Phytophthora parasitica*) Fungi

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### Abstract

In order to investigate the synthesis of arbuscular mycorrhiza-related polypeptides, protein extracts from tomato roots colonized with the arbuscular mycorrhizal fungus *Glomus mosseae* were analysed by two-dimensional gel electrophoresis. Polypeptide patterns of mycorrhizal tomato roots were compared not only to those of non-infected ones, but also to those of tomato roots infected with the pathogenic fungus *Phytophthora parasitica*. Comparisons of the various polypeptide profiles showed that additional polypeptides were induced specifically in response to arbuscular mycorrhizal symbiosis versus pathogenic interaction. Moreover, comparison of polypeptide patterns of *G. mosseae*-colonized and *P. parasitica*-infected tomato roots with those of either germinated spores of the arbuscular mycorrhizal fungus or of axenically grown pathogenic fungus, suggested that some of the additional polypeptides were of plant origin.

Keywords: Arbuscular mycorrhizal symbiosis, pathogen infection, *Lycopersicon esculentum*, roots, specific polypeptides, 2D-electrophoresis

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## 1. Introduction

Arbuscular mycorrhizas are ubiquitous symbioses formed between roots of a large majority of higher plants and soil fungi belonging to the order of Glomales (Harley and Smith, 1983). The benefits of such symbioses have received increased attention not only in terms of improved nutrition, but also because of the higher resistance they may give the plants to root pathogen attacks, and various abiotic stresses (Harley and Smith, 1983; Barea and Jeffries, 1995; Azcón-Aguilar and Barea, 1996; Caron, 1989; Linderman, 1994). The establishment and functioning of arbuscular mycorrhizal (AM) symbiosis is a complex process involving several developmental stages with concomitant biochemical, physiological and molecular changes. Although root colonization by AM fungi has received much attention focused on morpho-physiological and biochemical changes (Bonfante and Perotto, 1995; Gianinazzi-Pearson, 1995; Smith and Gianinazzi-Pearson, 1988; Smith et al., 1994), knowledge of the specific molecules and genes involved in such symbiosis still needs to be increased. Investigation at the protein expression level is in progress in various plant-microbe interaction models, and especially in beneficial root associations such as *Rhizobium*-leguminous and ectomycorrhizal symbioses (reviewed by Pueppke, 1996; Martin et al., 1995). In AM symbiosis however, information is more limited (Dumas-Gaudot et al., 1994; Garcia-Garrido et al., 1993; Wyss et al., 1990; Samra et al., 1997; Simoneau et al., 1994). There are several reasons for such a delay in characterising plant proteins specific to AM symbiosis. The fact that the AM fungi are obligate symbionts, which cannot be grown in pure culture without their host, has hampered the discrimination between plant or fungal origin among the additional polypeptides detected in mycorrhizal roots. Additionally, axenic cultures, which certainly represent an interesting tool to study the symbiosis in a very controlled and reproducible environment, are still limited to a small number of both plant and AM-fungal species (Benhamou et al., 1994; Simoneau et al., 1994). Moreover, it should be pointed out that they provide very limited amount of root material for biochemical analysis, and it has to be proven that they are representative of the events occurring in natural growth conditions. However, several working groups have tried to overcome these various problems by using different approaches such as targeted research on polypeptides which are common to different symbioses (Wyss et al., 1990), analysis of transformed roots axenically inoculated with AM fungi (Simoneau et al., 1994), comparative analysis of arbuscular mycorrhizas formed with AM fungi differing in their root colonization ability (Dumas-Gaudot et al., 1994) and, very recently, analysis of mycorrhiza-resistant plant mutants in comparison to wildtype during the early stages of the AM infection process (Samra et al., 1997). Another way to highlight AM symbiosis-related proteins consists of comparisons between plants inoculated with either AM or

pathogenic fungi. This approach was successfully used to reveal differential elicitation of specific molecular forms of some hydrolytic enzymes in various plant species inoculated with different AM and pathogenic fungi (Dassi et al., 1996; Dumas-Gaudot et al., 1992; Pozo et al., 1996). However, to our knowledge, it has not been used yet in combination with 2D-PAGE analysis. This technique is sensitive and allows direct comparison between complex extracts.

The aim of the present work was to compare the differential induction of polypeptides in fully established tomato root interactions with either arbuscular mycorrhizal or pathogenic fungi. Therefore, tomato plants were inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae* for five weeks, while other sets of three week-old plants were infected with zoospores of the pathogenic fungus *Phytophthora parasitica* for two weeks. To further characterize the AM symbiosis-related and the pathogenic-related polypeptides, 2D-PAGE profiles of tomato roots inoculated with either *G. mosseae* or *P. parasitica* were compared to those of either germinated spores of *G. mosseae* or of mycelium of *P. parasitica*, respectively.

## 2. Materials and Methods

### *Plant material, culture conditions and fungal inoculations*

Surface sterilized seeds of tomato (*Lycopersicon esculentum* cv. Earlymech) were pregerminated on sterile vermiculite for 10 days. Seedlings were then transplanted in plastic bottles containing sterile quartz sand layered (2:1) with either  $\gamma$ -irradiated clay loam soil (26 ppm Olsen P) for control and *Phytophthora parasitica*-infected plants, or *Glomus mosseae* (BEG 12) leek root inoculum for AM plants. After 3 weeks, *P. parasitica* (isolate 201, kindly provided by P. Bonnet, INRA Antibes, France) inoculation was performed on half of the control plants by watering the plant rhizosphere with 50,000 zoospores per plant. All sets of plants were grown under constant conditions (26/23°C; photoperiod 16h, light 330  $\mu\text{mol s}^{-1} \text{m}^{-2}$ ; relative humidity 60%) and watered 3 times per week with the Long Ashton nutrient solution, containing complete phosphorus for non-mycorrhizal and *P. parasitica*-infected plants and half phosphorus for mycorrhizal ones. Complete sets of plants for control, mycorrhizal- and pathogen-infected roots were repeated twice. Root samples were harvested after 5 weeks of *G. mosseae* inoculation and 2 weeks of *P. parasitica* infection, and were stored at -80°C until protein extraction.

### *Determination of mycorrhizal colonization and pathogenic infection*

Mycorrhizal colonization was evaluated microscopically following G.

*mosseae*-inoculated root clearing in 10% KOH and trypan blue staining (Phillips and Hayman, 1970) using the method of Trouvelot et al. (1986).

Evaluation of the amount of *P. parasitica* in tomato roots was achieved by ELISA, using the *Phytophthora* pathogen detection kit Agri-Screen (Sigma) according to the protocol provided by the supplier, except that incubation times were increased to 2 hours. As emphasised by the supplier, the commercial kit for *Phytophthora* detection does not recognize proteins from *in vitro* grown pathogen mycelium. Thus, the amount of pathogen has been expressed as absorbance values.

#### *Protein extraction and two-dimensional electrophoresis*

Phenol-extracted proteins from tomato roots were solubilized and analysed by 2D-polyacrylamide gel electrophoresis according to the protocol of Samra et al. (1997). Germinated spores of *G. mosseae* were obtained as described previously (Samra et al., 1996). For analysis of polypeptides from *P. parasitica*, the pathogen was grown in Petri dishes on malt agar. To handle *P. parasitica* mycelium more easily, the fungus was separated from the medium with sterile cellophane. After 7 days of growth at 23°C in darkness, the mycelium was carefully removed and stored at -80°C until further analysis. Fungal polypeptides from *G. mosseae* and *P. parasitica* were extracted according to Samra et al. (1996). One hundred µg of protein (both root and fungal extracts) were loaded on the IEF gels. Co-electrophoresis were carried out by loading 90 µg of proteins from control roots and 10 µg proteins from *G. mosseae*. Gel electrophoretic separations for each treatment were repeated at least twice.

#### *Staining and scoring procedure*

The gels were silver-stained following Blum et al. (1987). The molecular weight (MW) and isoelectric point (pI) of polypeptides were estimated using standard proteins (2D-standards, Bio-Rad, F.). Following silver staining, gels were thoroughly rinsed in MilliQ water (Millipore, F.) and photographed. Spot detection and gel to gel spot matching were visually performed.

### **3. Results**

#### *Mycorrhizal colonization and Phytophthora parasitica infection*

The purpose of our investigation was to study polypeptide modifications related to fully established root interactions. For a well established

mycorrhizal colonization, five weeks of inoculation with the AM fungus were necessary, while infection with the pathogen is quicker. Consequently, in order to compare plants of the same age, non-mycorrhizal tomato plants were inoculated with the pathogenic fungus after three weeks of growth and we obtained high level of infection for both fungi. In the two experiments, the frequency of mycorrhization reached respectively 87% and 93% after 5 weeks of *G. mosseae* inoculation. ELISA was used for detection of *P. parasitica* in tomato roots. For the two experiments, extracts from both uninoculated control and *G. mosseae*-inoculated roots gave absorbance values similar to the background level of the *Phytophthora* detection kit. In contrast, absorbance values of the roots infected with *P. parasitica* for two weeks reached 3.4, which is the maximum level for the kit detection.

#### *Comparison of two-dimensional polypeptide patterns*

Root soluble proteins from the various plant treatments were extracted and analysed by 2D-PAGE. On Fig. 1 are illustrated the 3 general patterns of proteins extracted from tomato roots uninoculated (Fig. 1A), *G. mosseae*-inoculated for 5 weeks (Fig. 1B) and *P. parasitica*-inoculated with zoospores for 2 weeks (Fig. 1C). At least 600 polypeptides were separated by the 2D-PAGE system. The polypeptides were mainly distributed from 25–80 kDa and had pIs ranging from 5–7, a lower density of polypeptides was detected in a range of 14 to 25 kDa. Tomato root inoculation with either *G. mosseae* or *P. parasitica* resulted in several modifications of polypeptide patterns. In general, *G. mosseae* inoculation induced more changes in acidic and neutral polypeptides, while, on the contrary, *P. parasitica* infection resulted mainly in modifications of neutral and basic polypeptides (Figs. 1B and C). Detailed analysis of 2D patterns for the acidic part of the gels and for molecular weights ranging from 100 to 40 kDa revealed that several polypeptides accumulated in mycorrhizal roots (Fig. 2B) compared to control uninoculated ones (Fig. 2A). These additional polypeptides were named according to their apparent molecular weights M 88, M 81, M 69, M 62 and M 45. M 81 corresponded in fact to several polypeptides with same MW but with different pIs. None of them were detected in tomato roots inoculated with *P. parasitica*, but four additional polypeptides, named P 90, P 44, P 43 and P' 43, were induced in response to pathogenic infection in the equivalent zone of the gels (Fig. 2C). For the part of the gels with MW between 25 and 15 kDa, additional polypeptides named M 24, M 21.5, M' 21.5 and M 16.5, were revealed in tomato roots colonized with the AM fungus (Fig. 2E), while only two different additional polypeptides P 19.5 and P 24 were detected in *P. parasitica*-inoculated roots (Fig. 2F). According to its MW and pI, the polypeptide P 24 could correspond to the polypeptide

named M 24 detected in *G. mosseae*-inoculated roots. In the neutral part of the gels and for molecular weights ranging from 32 to 21 kDa, two additional polypeptides M 28 and M' 28 were revealed in *G. mosseae*-inoculated roots, while in the pathogen-infected ones we observed the appearance of a different polypeptide P 27 (Fig. 2I). Details of the most basic part of gels are not shown.

All the additional polypeptides detected in tomato roots inoculated with either *G. mosseae* or *P. parasitica* were characterized by their respective molecular weight and isoelectric point (Table 1).

2D-PAGE analyses were then carried out on proteins extracted from germinated spores of *G. mosseae* and from mycelium of *P. parasitica* grown axenically. The polypeptide patterns of the two fungi appeared completely

Table 1. List of all the additional polypeptides induced in tomato roots by either mycorrhizal or pathogenic inoculation

Protein	Induced by	MW	pI
M 16.5	<i>G. mosseae</i>	16.5	5.5
M 21.5		21.5	5.7
M' 21.5		21.5	5.8
M 23.5		23.5	6.4
M 24		24	4.7
M 28		26	6.7
M' 28		26	6.8
M 32		32	6.1
M 41		41	6.4
M 45		45	5.8
M 62		62	5.4
M 69		69	5.3
M 81		81	5.4-5.7
M 88		88	5.7
P 19.5	<i>P. parasitica</i>	19.5	5.8
P' 19.5		19.5	7.5
P 24		24	4.7
P 27		27	6.8
P 32		32	6.7
P' 32		32	6.75
P 33		33	6.4
P 43		43	5.0
P' 43		43	5.2
P 44		44	5.0
P 90	90	5.1	

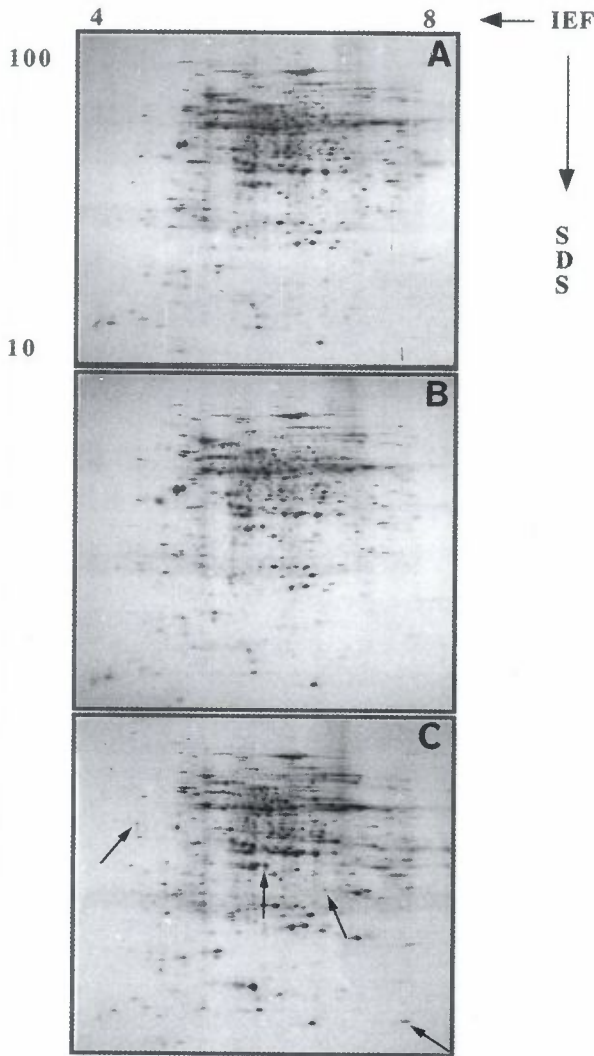


Figure 1. Protein profiles from (A) non-infected tomato roots, (B) tomato roots inoculated with *Glomus mosseae* for 5 weeks, or (C) with *Phytophthora parasitica* for 2 weeks (C).

different (Figs. 3A and B). As previously reported by Samra et al. (1996), more than 300 polypeptides could be detected in germinated spores of *G. mosseae* (Fig. 3A). However, when this 2D-PAGE profile was compared to the polypeptide pattern of tomato roots inoculated with the arbuscular mycorrhizal fungus (Fig. 1B), none of the additional polypeptides corresponded

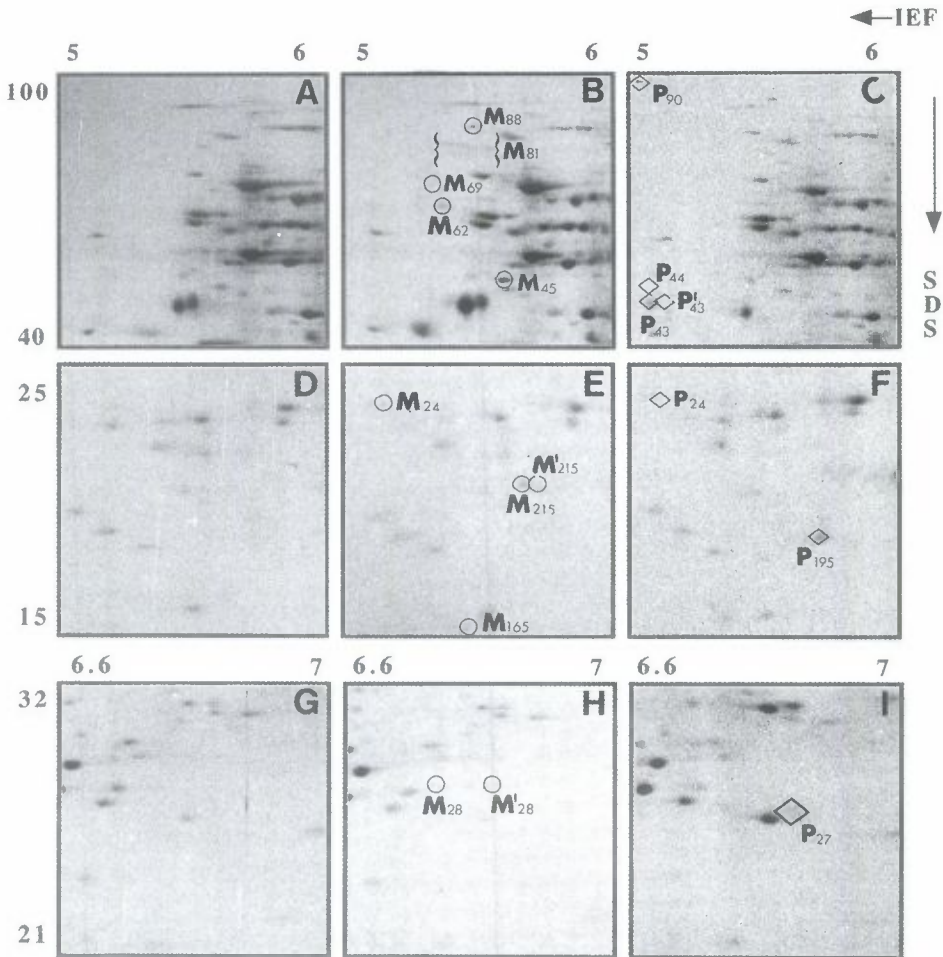


Figure 2. Detailed portions of two-dimensional gels of Fig. 1. Polypeptides were from non-infected tomato roots (A, D, G), and from tomato roots inoculated with *Glomus mosseae* (B, E, H) and with *Phytophthora parasitica* (C, F, I). A, B and C cover a range of molecular weights from 40 to 100 kDa and isoelectric gradients from 5 to 6. In D, E, F, for the same isoelectric gradient, polypeptides are separated between 15 to 25 kDa. In G, H, I the polypeptides are resolved between 21 and 32 kDa and the isoelectric gradient from 6.6 to 7.

to the fungal ones. Moreover, when co-electrophoresis with control root and extract from germinated spores of *G. mosseae* were carried out, we did not find polypeptides corresponding to the additional polypeptides detected in mycorrhizal tomato roots (data not shown). Although the polypeptide pattern from mycelium of *P. parasitica* was quite different from the one corresponding to



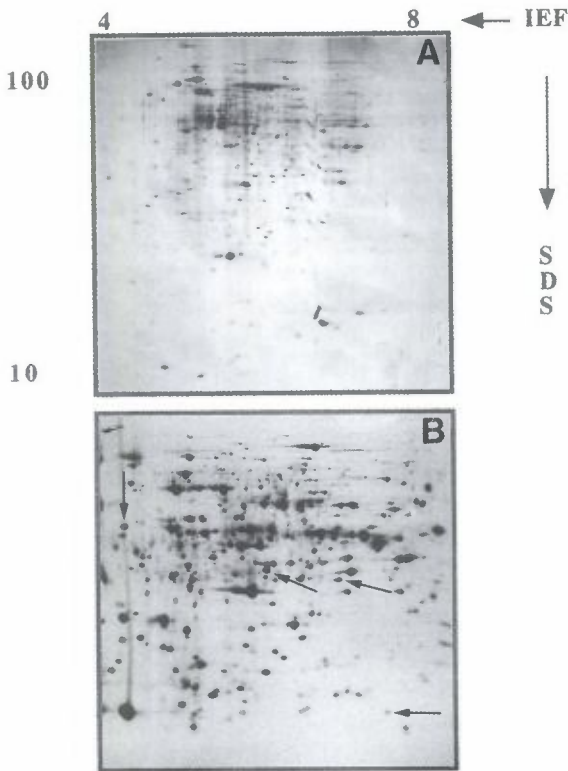


Figure 3. Protein profiles from (A) germinated spores of *Glomus mosseae* or (B) *Phytophthora parasitica* mycelium.

*P. parasitica*-infected roots, at least four polypeptides were detected in both mycelium (arrows on Fig. 3B) and root extracts from infected plants (arrows on Fig. 1C).

#### 4. Discussion

In this study we compared the polypeptide patterns of extracts from uninoculated, *G. mosseae*-colonized and *P. parasitica*-infected tomato roots in order to demonstrate the altered plant polypeptide synthesis in relation to symbiotic versus pathogenic interactions. We detected 14 additional polypeptides, identified by their molecular weights and pIs, present in *G. mosseae*-colonized tomato roots. Additional polypeptides in AM symbiosis have been previously reported (Dumas-Gaudot et al., 1994; Simoneau et al., 1994; Samra et al., 1997). In our experimental system, the major additional

polypeptides found in response to AM symbiosis possess acidic pIs and MWs ranging from 16.5 to 88 kDa. To further determine the origin of the additional polypeptides we compared the 2D-PAGE profiles of mycorrhizal tomato roots to those from germinated spores of *G. mosseae*. None of the additional polypeptides were observed in extracts from germinated spores of *G. mosseae*. They could therefore be plant AM symbiosis-related polypeptides. However, they could also be fungal polypeptides which are expressed only during the AM symbiosis. Indeed, a differential expression of fungal proteins has already been reported for AM fungi cultivated under symbiotic or non-symbiotic conditions (Saito, 1995; Thingstrup et al., 1995).

In *P. parasitica*-inoculated tomato roots, 11 additional polypeptides were detected. Interestingly, only one of these polypeptides (M24/P24) was present in both tomato root interactions and could therefore represent either a plant response common to infection by one or the other fungal species or a fungal polypeptide present in both fungi. However, since this polypeptide was neither detected in extracts from germinated spores of *G. mosseae* nor in mycelium of *P. parasitica*, its plant origin seems more likely. In mycorrhizal Ri T-DNA transformed roots of tomato, Simoneau et al. (1994) have revealed two major acidic polypeptides of 24 and 39 kDa, which were considered by these authors as polypeptides possibly related to early post-infection stages of the AM symbiosis. Because of differences in our experimental conditions and plant/fungus material, the identity of the 24 kDa polypeptide detected in our analysis and in those from Simoneau et al. (1994) cannot be ascertained. However, it is interesting that a polypeptide with very similar electrophoretic characteristics has been reported in two different works. Nevertheless, if this polypeptide is the same, then our results indicate that it is more likely to be a general plant response to fungi than a specific response to AM symbiosis.

To our knowledge, this is the first analysis by 2D-PAGE of protein from *P. parasitica*. Four polypeptides from *P. parasitica* mycelium and from *P. parasitica*-infected tomato roots showed similar electrophoretic characteristics. They could therefore be of fungal origin.

The 2D-PAGE analyses have given rise to more accurate determination of additional polypeptides in AM root symbiosis than previous 1D-PAGE analyses (Dumas et al., 1989; Pacovsky, 1989; Schellenbaum et al., 1992; Arines et al., 1993). Moreover, our present investigation brings clear evidence of a differential response of tomato plants to AM symbiosis compared to pathogen infection. Molecular approaches to study AM symbiosis-related genes have recently been carried out in our group (Martin-Laurent et al., 1997). Further studies at the protein level will now be focused on microsequencing of identified symbiosis-related polypeptides. This will allow us to identify their biological function(s).

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