



Xyloglucanases in the interaction between saprobe fungi and the arbuscular mycorrhizal fungus *Glomus mosseae*

Elisabet Aranda, Inmaculada Sampedro, Rosario Díaz, Mercedes García, Juan Antonio Ocampo, Inmaculada García-Romera*

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, Prof. Albareda 1 Apdo. 419, E-18008 Granada, Spain

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KEYWORDS

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Summary

We studied the production of xyloglucanase enzymes of pea and lettuce roots in the presence of saprobe and arbuscular mycorrhizal (AM) fungi. The AM fungus *Glomus mosseae* and the saprobe fungi *Fusarium graminearum*, *Fusarium oxysporum*-126, *Trichoderma harzianum*, *Penicillium chrysogenum*, *Pleurotus ostreatus* and *Aspergillus niger* were used. *G. mosseae* increased the shoot and root dry weight of pea but not of lettuce. Most of the saprobe fungi increased the level of mycorrhization of pea and lettuce, but only *P. chrysogenum* and *T. harzianum* inoculated together with *G. mosseae* increased the dry weight of pea and lettuce respectively. The AM and saprobe fungi increased the production of xyloglucanases by plant roots. The level of xyloglucanase activities and the number of xyloglucanolytic isozymes in plants inoculated with *G. mosseae* and most of the saprobe fungi tested were higher than when both microorganisms were inoculated separately. The possible relationship between xyloglucanase activities and the ability of AM and saprobe fungi to improve the dry weight and AM root colonization of plants was discussed.

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Introduction

Arbuscular mycorrhizal (AM) fungi form symbiotic associations with higher plants in which the fungi receive fixed carbon and, in turn, transport phosphate to the host, enhancing phosphorus uptake and growth of the plant (Cooper and Tinker,

Abbreviations: AM, arbuscular mycorrhizal; CFUs, saprobe colony-forming units

*Corresponding author. Tel.: +34 958 181600x274; fax: +34 958 129600.

E-mail address: igarcia@eez.csic.es (I. García-Romera).

1978). On the other hand, saprobe fungi are important and common components of the soil rhizosphere because of the large amount of microbial biomass they supply to the soil, and because of their role in plant residue decomposition (Domsch et al., 1980; Finlay and Soderstrom, 1992). Several experimental results confirm the existence of antagonistic, neutral or synergistic interactions between AM and saprobe fungi in the rhizosphere and in the plant root (Gryndler, 2000). Some authors confirm the existence of synergistic effects of saprobe fungi on plant root colonization by AM fungi and on the effectiveness of AM fungi on plant resistance to heavy metals in soils (Arriagada et al., 2004, 2005; Vogel-Mikus et al., 2005). The mechanisms implicated in the effects of saprophytic fungi on AM fungi are unknown. However, it is known that exudates produced by saprophytic fungi influence the level of germination of AM fungal spores and the development and function of the AM symbiosis (Fracchia et al., 2004). The effect of saprobe fungi on AM fungi takes place primarily when the arbuscular fungi are in the extraradical phase of the symbiosis (McAllister et al., 1994; García-Romera et al., 1998; Fracchia et al., 1998, 2004). Nevertheless, the results from pot experiments indicate that saprobe fungi can also affect AM symbiosis when the AM fungi are inside the root (McAllister et al., 1995; Martinez et al., 2004).

Plant cell-wall degradation may be important to fungi not only for penetration and ramification inside the plant tissue but also for obtaining nutrients (Radford et al., 1996). Plant pathogenic fungi synthesize and secrete large quantities of cell-wall-degrading enzymes to invade the plant tissue and their regulation has been extensively studied (Deising et al., 1995; Mendgen et al., 1996; Tonukari, 2003). In contrast, saprobe fungi, which produce lower and strictly regulated amounts of cell-wall-degrading enzymes, have been studied very little (Mendgen and Deising, 1993). On the other hand, there are cell-wall-degrading enzyme activities during the establishment of the AM symbiosis and various cell-wall protein-encoding genes appear to be differentially regulated in mycorrhiza (Balestrini and Bonfante, 2005). Cellulase, pectinase and xyloglucanase activities have been found in colonized root and in the external mycelium of AM fungi (García-Romera et al., 1991; García-Garrido et al., 1992; Rejón-Palomares et al., 1996). These hydrolytic enzymes seem to be involved in the penetration and development of AM fungi in plant roots (García-Garrido et al., 2002). Xyloglucan is the major structural hemicellulose in primary cell walls of plants (Fry, 2004). In addition to its structural role, xyloglucan can be hydrolyzed

by plant and fungal hydrolytic enzymes and the products used as a source of signalling molecules (Hayashi, 1989) and as a nutrient reserve (McDougall and Fry, 1989). Of all the different hydrolytic enzymes, xyloglucanases are the least well known; however, they play an important role in plant cell-wall degradation (Hoson et al., 1995). The products of cell-wall-degrading enzyme genes may be involved either in facilitating hyphae penetration by allowing localized cell-wall loosening or in modifying the structure of xyloglucans in the cell wall (Liu et al., 2004; Maldonado-Mendoza et al., 2005). There is evidence that hemicellulases, including xyloglucanases, are involved in the colonization of root by the AM fungi (Rejón-Palomares et al., 1996). Production of xyloglucanases by saprobe fungi has also been observed (Tribak et al., 2002).

The aim of this work was to study whether the plant xyloglucanolytic enzymes produced in presence of AM and saprobe fungi were implicated in the beneficial effect of saprobe fungi on AM root colonization.

Material and methods

Plant culture and inoculation procedures

The assays were carried out in 0.3L pots filled with a grey loam soil obtained from the grounds of the Estación Experimental del Zaidín (Granada, Spain). The soil had a pH of 8.1 in a 1:1 soil:water ratio. *P*, *N* and *K* were determined using the methods of Mingorance (2002); NaHCO_3 -extractable *P* was 6.2 mg kg^{-1} , *N* was 2.5 mg kg^{-1} and *K* was 132 mg kg^{-1} . The soil texture was 358 g kg^{-1} sand, 436 g kg^{-1} silt, 206 g kg^{-1} clay and 18 g kg^{-1} organic matter. The soil was steam-sterilized and mixed with sterilized sand to a proportion of 2:3 (V:V). Seeds of pea (*Pisum sativum* L. var. Lincoln) and lettuce (*Lactuca sativa* L. var. Romana invernata) were surface-sterilized with HgCl_2 for 10 min, thoroughly rinsed with sterilized water and sown in moistened sand. After germination, uniform seedlings were planted and grown in a greenhouse with supplementary light provided by Sylvania incandescent and cool-white lamps, $400 \text{ E m}^{-2} \text{ s}^{-1}$, 400–700 nm, with a 16/8 h day/night cycle at 25/19 °C and 50% relative humidity. Plants were watered from below and fed with 10 mL of Long Ashton nutrient solution containing 25% of the *P* concentration (Hewitt, 1966).

The AM fungus used was *Glomus mosseae* (Nicol. and Gerd.) Gerd. and Trappe (BEG 12). The *G.*

mosseae inoculum consisted of soil, spores, mycelium and infected root fragments from an open pot culture of sorghum plant (*Sorghum vulgare* L.). Five grams of inoculum which contained spores, mycelium and colonized root fragments (an average of 30 spores/g and 75% of root infected), were added to each pot at the time of transplanting. Soil filtrate (Whatman No.1 filter paper) from the rhizosphere of mycorrhizal plants was added to the AM non-inoculated treatment. The filtrate contained common soil microorganisms, but no propagules of AM fungi.

The saprobe fungi used were: *Fusarium graminearum* and *Fusarium oxysporum*-126 (Booth, 1977), *Trichoderma harzianum* (Rifai, 1969), *Penicillium chrysogenum* (Samsom, 1974), *Pleurotus ostreatus* (Domsch et al., 1980) and *Aspergillus niger* (Raper and Fennell, 1965). The fungi were isolated from soil by the particle washing method (Widden and Bisset, 1972) using a multichamber washing apparatus. Thirty washings were required to remove sclerotia, spores, and other fungal structures from soil particles. Twenty soil particles (2 mm) were dried on sterilized filter paper and plated on 2% malt extract agar containing antibiotics ($5 \mu\text{g L}^{-1}$ streptomycin and $10 \mu\text{g L}^{-1}$ tetracycline). The ligninolytic fungus *Pl. ostreatus* was directly isolated from infected black poplar wood. The fungal isolates were transferred to tubes of potato dextrose agar (DIFCO) and 2% malt extract at 4°C as stock cultures. An aqueous suspension in sterile distilled water containing approximately 4×10^8 spores mL^{-1} of each saprobe fungus was prepared from cultures grown in potato dextrose agar for 1 week at 27°C and 2.5 mL of this suspension were inoculated per pot.

Four treatments were used: (1) non-inoculated controls (2) soil inoculated with saprobe fungi (3) soil inoculated with *G. mosseae* and (4) soil inoculated with both saprobe fungi and *G. mosseae*. Plants were inoculated with *G. mosseae* at the time of transplanting (after 10 days of growth). The saprobe fungi were inoculated 1 week after *G. mosseae*.

To evaluate the population of saprobe fungi inoculated to soil, about 1.5 g of rhizosphere soil was taken from each of the experimental pots and 10 times aqueous dilution series (from 10^{-1} to 10^{-4}) were prepared for each sample. One millilitre of each solution was plated on potato dextrose agar. The number of saprobe colony-forming units (CFUs) in suitable dilutions of such samples, taken from the five replicate pots of each treatment, was counted. All the saprobe fungi were identified by using microscopic structures (conidiophores, conidia, chlamidospores) and by using colony charac-

teristics such as growth rate, aerial mycelium, pigmentation and sclerotial bodies (Raper and Fennell, 1965; Rifai, 1968; Samsom, 1974; Booth, 1977; Domsch, et al., 1980). Soil was dried at 105°C and weighed. The number of CFUs was expressed per gram of dry soil.

Plants were harvested after 8 weeks and dry mass was determined. After the harvest, the root system was washed and rinsed several times with sterilized distilled water and three samples of the same fresh weight were taken from the entire root system at random. One of the samples was used to estimate the dry weight, the second sample for enzymatic activity determinations, and the third was cleared and stained for microscope examination (Phillips and Hayman, 1970). The percentage of total root length colonized by AM fungi was measured (Giovannetti and Mosse, 1980).

Preparation of extracts for enzyme assays

Roots (10 g fresh weight) were pulverized in a mortar under liquid nitrogen. The resulting powder was homogenized in 30 mL of 100 mM Tris-HCl buffer (pH 7) plus 0.02 g polyvinyl-polyrrolidone, 10 mM MgCl_2 , 10 mM NaHCO_3 , 10 mM β -mercaptoethanol, 0.15 mM phenylmethyl sulfonyl fluoride and 0.3% (wt/vol) X-100 Triton. Sodium azide (0.03%) was added to all solutions. The liquid was filtered through several layers of cheesecloth and centrifuged at 20 000g for 20 min. The supernatant (20 mL) was dialyzed (Spectra/Por membrane, MWCO: 6–8000) against 5 L of the same diluted extracting solutions (1:9, v/v) for 16 h at 4°C . The samples were then frozen until used.

Total proteins were measured using a Bio-Rad kit with albumin serum bovine as standard (Bradford, 1976).

Enzyme assays

The extracts were assayed to determine the endoxyloglucanase activity by the viscosity method (Rejón-Palomares et al., 1996) using xyloglucan from nasturtium seed as substrates. The reduction in viscosity was determined at 0–30 min intervals. Approximately 0.8 mL of the reaction mixture was sucked from a 2 mL tube into a 1-mL syringe through its needle and the time taken for the meniscus to flow from the 0.70 to 0.20 mL (about 1–3 min) mark was recorded. The reaction mixture in the 2-mL tube contained 1 mL of 0.5% substrate in 50 mM citrate-phosphate buffer (pH 5) and 0.2 mL enzyme. Viscosity reduction was determined at 37°C . One unit of enzyme activity was expressed

as specific activity (U/mg protein; U is the reciprocal of time in hours for 50% viscosity loss $\times 10^3$) (Rejón-Palomares et al., 1996). Controls for all enzyme assays were autoclaved enzyme supernatants and autoclaved buffers.

Polyacrylamide gel electrophoresis

Xyloglucanase enzymes were separated by non-denaturing electrophoresis on 8% polyacrylamide slab minigels (MiniProtean II, Bio-Rad) amended with 0.1% xyloglucan in 50 mM Tris–0.1 M glycine buffer (pH 8.8) (García-Garrido et al., 1992). The electrode tank contained the same Tris–Glycine buffer (pH 8.8) as used in the gel. The wells were filled with 30 μ L of root or extract (175 μ g protein) and 3 μ L 0.05% bromophenol blue. Electrophoresis was done at 4 °C and a constant current of 20 mA per gel for 4 h.

The gels were incubated with 50 mM citrate–phosphate buffer (pH 5) at 37 °C for 16 h, after which they were stained with 0.1% Congo red for 15 min; washings in 1 M NaCl followed this until the bands became visible.

Statistical analysis

The data were subjected to one-way ANOVA. The mean values of five replicate pots were compared using Duncan's multiple-range test ($P = 0.05$). Percentage data were subjected to arcsine transformation before analysis.

Results

The shoot dry weight of pea inoculated with *G. mosseae* was higher than the non-inoculated control (Fig. 1a). The saprobe fungi did not increase the shoot dry weight of pea except when *P. chrysogenum* was inoculated together with *G. mosseae*. The inoculation of pea with *G. mosseae* increased the dry weight of the root but the saprobe fungi did not increase the root dry weight of mycorrhizal plants (Fig. 1b). All saprobe fungi tested, except *F. graminearum*, increased the percentage of root length colonization of pea by *G. mosseae* (Fig. 1c). Endoxyloglucanase activity in plant roots of pea inoculated with the saprobe fungi *F. oxysporum*, *T. harzianum* and *Pl. ostreatus* was higher than non-inoculated controls (Fig. 1d). Higher endoxyloglucanase activity of pea roots inoculated with *G. mosseae* relative to non-inoculated controls was found (Fig. 1d). These enzymatic activities increased when *G. mosseae* was inocu-

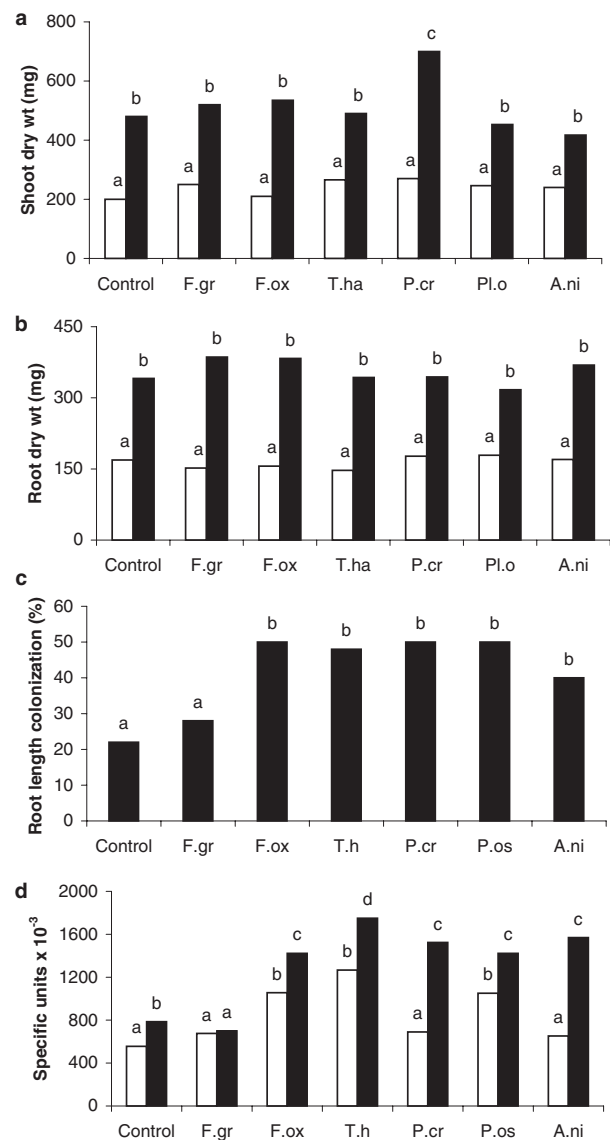


Figure 1. Shoot (a), root (b), percentage of AM root length colonization (c) and xyloglucanase activity (d) of pea (*Pisum sativum*) plants inoculated with AM fungus alone, in combination with saprobe fungi or non-inoculated control plants. C = Plants no inoculated with saprobe fungi; F.gr = *Fusarium graminearum*; F.ox = *F. oxysporum* 126; T.h = *Trichoderma harzianum*; P.cr = *Penicillium chrysogenum*; P.os = *Pleurotus ostreatus* and A.ni = *Aspergillus niger*. Values followed by the same letter are not significantly different as determined by Duncan's multiple-range test ($P = 0.05$). □ = Plant non-inoculated with *G. mosseae* and ■ = plants inoculated with *G. mosseae*.

lated with the saprobe fungi, except when it was inoculated together with *F. graminearum* (Fig. 1d). The number of CFUs of saprobe fungi was similar in all the saprobe-inoculated treatments (data not shown).

The inoculation of lettuce with the saprobe fungi, inoculated either alone or together with *G. mosseae*, did not increase the shoot dry weight of the plants except when *G. mosseae* and *T. harzianum* were inoculated together (Fig. 2a). The joint inoculation of the saprobe and arbuscular fungi did not increase the root dry weight of lettuce

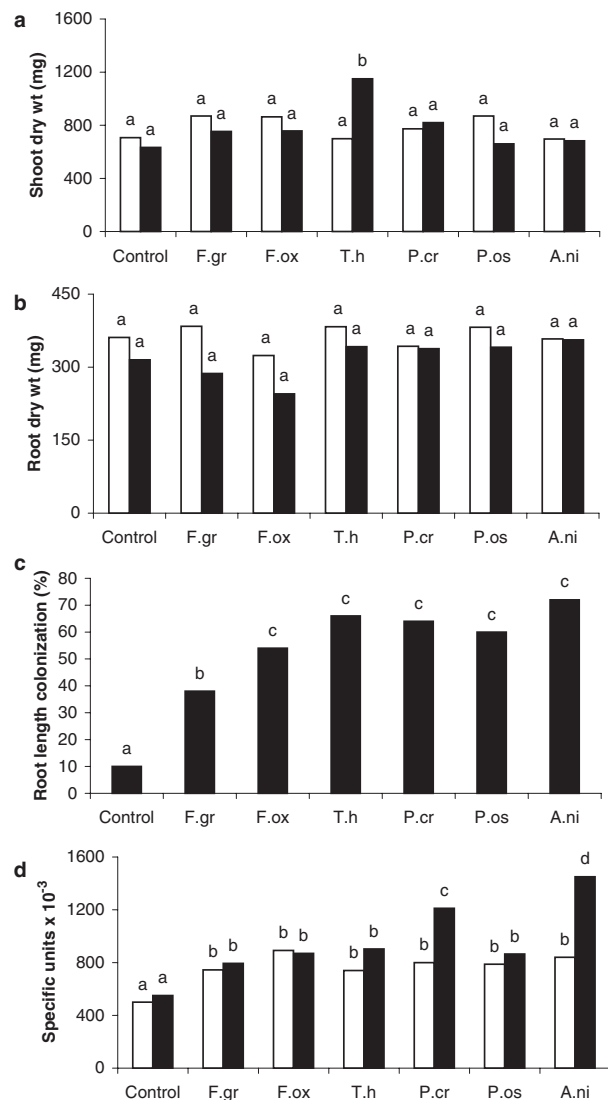


Figure 2. Shoot (a), root (b), percentage of AM root length colonization (c) and xyloglucanase activity (d) of lettuce (*Lactuca sativa*) plants inoculated with AM fungus alone, in combination with saprobe fungi or non-inoculated control plants. C = Plants no inoculated with saprobe fungi; F.gr = *Fusarium graminearum*; F.ox = *F. oxysporum* 126; T.h = *Trichoderma harzianum*; P.cr = *Penicillium chrysogenum*; P.os = *Pleurotus ostreatus* and A.ni = *Aspergillus niger*. Values followed by the same letter are not significantly different as determined by Duncan's multiple-range test ($P = 0.05$). □ = Plant non-inoculated with *G. mosseae* and ■ = plants inoculated with *G. mosseae*.

(Fig. 2b). The saprobe fungi increased the percentage of root length colonization of lettuce by *G. mosseae* (Fig. 2c). The inoculation of saprobe fungi alone or together with *G. mosseae* increased the levels of endoxyloglucanase activity of lettuce roots (Fig. 2d). When *G. mosseae* was inoculated together *P. chrysogenum* or *A. niger*, the highest level of endoxyloglucanase activity was observed (Fig. 2d).

Figure 3 shows that the electrophoretic Band 1 of xyloglucanase activity in root of pea and lettuce has the same mobility, but other bands with different electrophoretic mobility in pea (Band 2) and lettuce (Band 9) was also observed. The electrophoretic Band 4 of endoxyloglucanase activity in root of pea (Fig. 3a) and lettuce (Fig. 3b) colonized with *G. mosseae* was not detected in the non-inoculated controls. As can be observed in Fig. 3a, the inoculation of pea with *P. chrysogenum* and *A. niger* produced one band of enzymatic activity (Band 5) that were not detected in pea root not inoculated with these saprobe fungi. *F. graminearum* and *T. harzianum* did not change the pattern of bands of endoxyloglucanase activity of pea inoculated with *G. mosseae*. Nevertheless, the other saprobe fungi increased the number of bands of enzymatic activity in AM-inoculated pea roots (Bands 6, 7 and 8 for *P. chrysogenum*, Bands 8 and 9 for *Pleurotus ostreatus* and Band 8 for *A. niger*). Figure 3b shows that the pattern of bands of xyloglucanase activity was different between AM-colonized and non-colonized lettuce roots with the formation of new bands in the mycorrhizal treatment (Bands 4, 5, 10 and 11). New bands of xyloglucanase activity in lettuce inoculated with *F. oxysporum* 126 (Band 3), *Pl. ostreatus* (Band 10) and *A. niger* (Band 7) that did not appear in the non-inoculated control were observed. The joint inoculation of lettuce with *G. mosseae* and *F. graminearum* or *F. oxysporum* did not increase the number of bands of enzymatic activity. New bands of xyloglucanase activity in lettuce inoculated with *G. mosseae* and the saprobe fungi *T. harzianum* (Band 3), *P. chrysogenum* (Bands 3, 8 and 11) and *A. niger* (Bands 3 and 11) were observed. These bands were not detected when both microorganisms were inoculated separately.

Discussion

Although the effects of different AM fungal species on the same plant species have been observed (Bradbury et al., 1991), the type of plant appears to be more important in the beneficial effect of the AM fungi on the growth of the plants

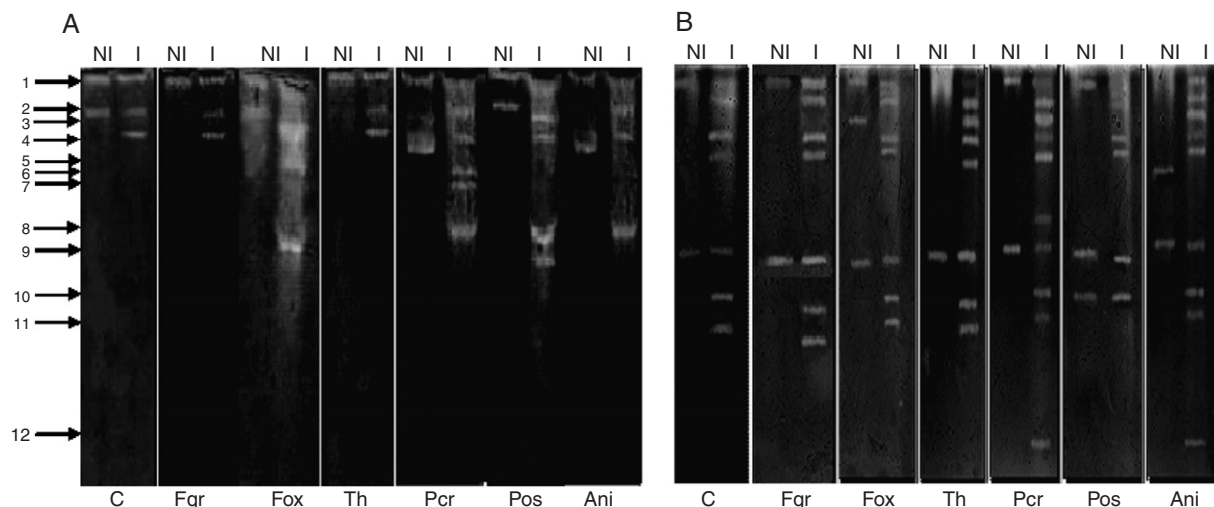


Figure 3. Non-denaturing polyacrylamide gel electrophoresis of xyloglucanase of pea (*Pisum sativum*) (A) and lettuce (*Lactuca sativa*) (B). Lane NI = extracts of non-mycorrhizal roots; Lane I = extracts of mycorrhizal roots. C = plants no inoculated with saprobe fungi; Fgr = *Fusarium graminearum*; Fox = *F. oxysporum* 126; Tha = *Trichoderma harzianum*; Pcr = *P. chrysogenum*; Pos = *Pleurotus ostreatus* and Ani = *Aspergillus niger*.

(Sturtz et al., 2000). Our assays showed that *G. mosseae* increased the dry weight of pea but not of lettuce, in spite of the fact that lettuce is considered to be a mycotrophic plant dependent on AM symbiosis for its optimal development and growth (Martín et al., 2002). However, the mycorrhizal effects of *G. mosseae* on plant growth differ not only between species, but also in different cultivars (Azcón and Ocampo, 1981; Vierheilig and Ocampo, 1991).

The saprobe fungi used in this work were selected for their capacity to increase the mycorrhization and growth of the plants when inoculated together with AM fungi (García-Romera et al., 1998; Godeas et al., 1999; Fracchia et al., 2000). Most of the saprobe fungi used increased the mycorrhization of pea and lettuce, but only *P. chrysogenum* and *T. harzianum* inoculated together with *G. mosseae* increased the shoot dry weight of pea and lettuce, respectively. It is known that saprobe fungi can increase the infectiveness of AM fungi though they do not always increase its efficiency on plant growth (McAllister et al., 1997; Godeas et al., 1999). Moreover, it is common to find no apparent relationship between the percentage of AM colonization and the effect of the AM fungi on the growth of the plants (Vierheilig and Ocampo, 1991; Camprubi et al., 1995; García-Garrido et al., 2000).

Production of xyloglucanases by saprobe fungi has been described (Tribak et al., 2002). Our results show that saprobe fungi increased the production of these enzymes by plant roots. The increase in the production of hydrolytic enzymes varied ac-

cording to the plant type on which the saprobe fungi were inoculated. All the saprobe fungi tested increased the level of xyloglucanase activity of lettuce but only some saprobe fungi increased the level of this enzymatic activity in pea. Different patterns of xyloglucanase activity in both plants inoculated with the same saprobe fungi was observed indicating that xyloglucanase activities were induced in plant by saprobe fungi. Moreover, some saprobe fungi such as *F. oxysporum* 126, *P. chrysogenum* and *A. niger* in pea and *F. oxysporum* 126, *Pleurotus ostreatus* and *A. niger* in lettuce increased the number of xyloglucanase isozymes suggesting that these saprobe fungi were able to induce specific metabolic changes in the root. These results suggest that saprobe fungi induced the xyloglucanase activity of root and some of them were able to induce specific plant root xyloglucanase enzymes.

On the other hand, the hydrolytic activity produced in plants inoculated with *G. mosseae* was qualitative and quantitatively different according to the plant species (García-Garrido et al., 1999). In fact, *G. mosseae* increased the level of the xyloglucanase activity of pea whereas it did not increase the enzymatic activity level of lettuce. It is known that the AM fungi increases the hydrolytic activity of the root but, due to the very low activity of hydrolytic enzymes in the AM symbiosis, it is difficult to detect quantitative increases of these enzymatic activities (García-Garrido et al., 2002). The developmental stage of the fungus inside the root and the level of AM colonization reached in the root can also be decisive for the detection of

these enzymatic activities (García-Romera et al., 1991; Schubert and Wyss, 1995; García-Garrido et al., 1999). The low level of mycorrhization reached in lettuce roots might have influenced the absence of increase of the xyloglucanolytic activity level observed in AM roots. Nevertheless, the new xyloglucanolytic isozyme with the same electrophoretic mobility observed in pea and lettuce roots colonized with *G. mosseae* indicate the involvement of specific xyloglucanolytic enzymes in the colonization of the root by the AM fungi (García-Romera et al., 1991; García-Garrido et al., 2000).

The level of xyloglucanases activities, the number of xyloglucanolytic isozymes and the percentage of AM colonization of plant roots co-inoculated with *G. mosseae* and most of the saprobe fungi tested were higher than when both microorganisms were inoculated separately. The increase of xyloglucanase activity cannot be due to the increase of metabolic activity of the root and the formation of new root cell walls as a consequence of root growth, because the joint inoculation of saprobe and AM fungi used in our experiments did not increase the root dry weight. The independence of hydrolytic activity from the metabolic status of the plant root was also observed with a xyloglucan endotranshydrolase gene induced by colonization of root with AM fungi, which was not related to the phosphate status of the root (Maldonado-Mendoza et al., 2005). On the other hand, saprobe fungi are able to produce xyloglucanase enzymes (Tribak et al., 2002) and some experiments revealed the AM fungi increased the population of saprobe fungi (Godeas et al., 1999). However, the increase of xyloglucanase activity observed in the joint inoculation of AM and saprobe fungi cannot be attributed to the increase in the number of CFUs of saprobe fungi because no increase of the number of CFUs in presence of AM fungi was found. This lack of effect has been observed for several saprobe fungi co-inoculated with AM fungi (García-Romera et al., 1998; Fracchia et al., 2000). No increase in the level of xyloglucanases activities, in the number of xyloglucanolytic isozymes and in the percentage of AM colonization of pea root inoculated with *F. graminearum* and *G. mosseae*, which were independent of their effect on the number of CFUs of saprobe fungi and on the root dry weight were observed. These results reinforce the idea that the increase in enzymatic activities observed in plants co-inoculated with *G. mosseae* and saprobe fungi were not related to their effect on the number of CFUs of saprobe fungi and on the root dry weight, being more related to the beneficial effect that these saprobe fungi have on the AM colonization of plant roots.

Our results suggest that plant xyloglucan hydrolases induced by saprobe fungi can be involved in modification of plant cell-wall structure that facilitate development and proliferation of the AM fungus into the root as has been suggested for other xyloglucan hydrolases such as xyloglucan endotranshydrolase (Maldonado-Mendoza et al., 2005).

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