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

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Abstract

We studied the production of xyloglucanase enzymes of leguminous roots in the presence of *Rhizobium* and arbuscular-mycorrhizal (AM) fungi. The AM fungus *Glomus mosseae* and *Sinorhizobium meliloti* GR4 for alfalfa, *Rhizobium leguminosarum* bv. *viceae* Rlv 13 for pea, *R. leguminosarum* bv. *trifolii* 2152 for clover, *R. tropici* CIAT 899 for bean and *Mesorrhizobium mediterraneum* USDA 3392 for chickpea. The inoculation of plants with *G. mosseae*, with *Rhizobium* or with both microorganisms together increased the shoot dry weights of almost all plants tested. The shoot dry weight of plants was higher when both symbionts were inoculated together than when they were inoculated separately and this benefit was related to the increase in the percentage of root length colonization and to the increase in the number of nodules observed in the joint inoculated plants. The AM fungus and *Rhizobium* increased the production of xyloglucanases in plant roots. The level of xyloglucanase activity and the number of xyloglucanolytic isozymes in plants roots inoculated with *G. mosseae* together with most of the *Rhizobium* tested was higher than when both microorganisms were inoculated separately. The possible relationship between xyloglucanase activities and the ability of AM and *Rhizobium* to improve the dry weight and AM root colonization of plants was investigated.

Keywords: Arbuscular mycorrhizal, *Glomus mosseae*, hydrolytic enzymes, *Rhizobium*, xyloglucanases

1. Introduction

Xyloglucan is the major structural hemicellulose in the primary cell walls of plants (Fry, 2004). Besides its structural role, xyloglucan can be hydrolyzed by plant and microbial 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 various hydrolytic enzymes, xyloglucanases are the least well known; however, they play an important role in plant cell-wall degradation (Hoson et al., 1995). The xyloglucan-specific endo-β-1,4-glucanases (EC 3.2.1.151) are characterized by being active against xyloglucan, having no or limited activity on cellulose or cellulose derivatives like carboxymethylcellulose (Adriano-Anaya et al., 2005; Aranda et al., 2005; Sianidis et al., 2006).

The arbuscular mycorrhizal (AM) fungi penetrate the plant cell wall at the site of contact during the establishment of the symbiosis indicating that hydrolytic enzymes may be involved in the penetration and development of AM fungi in plant roots (García-Garrido et al., 2002; García-Romera et al., 1991). Molecules, common in the plant's primary cell wall, such as xyloglucans have been found in the interface compartment between the plant cell and AM fungus. These molecules are not fully assembled into a structural wall. This may be the result of a weak lytic activity by the fungus, which impairs assembly mechanisms by producing hydrolytic enzymes (Balestrini and Bonfante, 2005). The occurrence of xyloglucanase activities in spores and the external mycelia of *G. mosseae*, the presence of xyloglucanase activity peaks during AM infection (Rejón-Palomares et al., 1996), and the detection of a fungal xyloglucanase isozyme in AM root extracts (García-Garrido et al., 1999) all provide further evidence for the possible involvement of xyloglucanolytic enzymes in the process of root colonization. The products of cell wall degrading

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enzyme genes may be involved either in facilitating hyphal penetration by promoting localized cell wall weakening or in modifying the structure of xyloglucans in the cell wall (Liu et al., 2004; Maldonado-Mendoza et al., 2005).

Rhizobia have the ability to produce hydrolytic enzymes (Akimova et al., 2000; Chen et al., 2004; Hussain et al., 1995; Jimenez-Zurdo et al., 1996; Mateos et al., 1992, 2001). Electron microscopic studies of the infection process have shown a localized degradation of the root hair wall, suggesting the involvement of cell wall hydrolyzing enzymes in the penetration process (Callaham and Torrey, 1981; Higashi and Abe, 1980; Mateos et al., 2001; Ridge and Rolfe, 1985; Turgeon and Bauer, 1985). However, the precise mechanism whereby *Rhizobium* successfully infects temperate legumes remains unknown. There remains the important question as to whether the wall-degrading enzymes involved in the process are produced by the bacteria or locally induced in the plant by components of the bacteria (Van Spronsen et al., 1994). The production of xyloglucanases by *Rhizobium* has been described but the role of these enzymes in plant penetration by this bacterium is also unknown (Aranda et al., 2005).

The contribution of *Rhizobium* and arbuscular mycorrhizal fungi (AM) to soil fertility, productivity and crop yield has been well documented (Bethlenfalvay and Schüepp, 1994; Biró et al., 1993, 2000; Boddey et al., 1991). The success co-inoculation depends not only on the infectivity of microbes but also on the compatibility of the interactions between the participants (Lindermann, 1983; Puppi et al., 1994). Compatible combinations of nitrogen-fixing *Rhizobium* bacteria and AM may result in enhanced effects on plant development in the various microsymbiont legume systems (Barea et al., 1988; Biró et al., 1993, 2000).

The effects of single and dual microsymbiont inoculation, *Rhizobium* and AM fungi, were studied to evaluate the xyloglucanase activity of roots colonized by *Rhizobium* and arbuscular mycorrhizal fungi as a factor influencing plant growth and the microbial ability of root colonization.

2. Material and Methods

Plant culture and inoculation procedures

The assays were carried out in 0.3 l 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. The 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 sterilised sand to a proportion of 2:3 (V:V). Seeds of

alfalfa (*Medicago sativa* L.), pea (*Pisum sativum* L.), clover (*Trifolium repens*), bean (*Phaseolus vulgaris*) and chickpea (*Cicer arietinum* L.) were surface-sterilised with 50% NaClO for 15 min, thoroughly rinsed with sterilised 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 E m^{-2} 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 colonised root fragments (an average of 30 spores/g and 75% of infected root) 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 pots which were not treated with the AM inoculum. The filtrate contained common soil microorganisms, but no propagules of AM fungi.

The rhizobia *Sinorhizobium meliloti* GR4, *Rhizobium leguminosarum* bv. *viceae* Rlv 13, *R. leguminosarum* bv. *trifolii* 2152, *R. tropici* CIAT 899 and *Mesorrhizobium mediterraneum* USDA 3392 all from the Estación Experimental del Zaidín (Granada), were used to inoculate alfalfa, pea, clover, bean and chickpea, respectively. The rhizobial strains were grown in BINOS medium (Mateos et al., 1992) and applied at a rate of 1 ml per seeding (10^8 cell ml^{-1}).

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

Plants were harvested after 8 weeks and the number of nodules and dry mass were 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-polypyrrolidone, 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 20 000 g for 20 min. The supernatant (20 ml) was dialyzed (Spectra/Por membrane, MWCO: 6-8,000) against 5 l of the same diluted extracting solutions (1:9, vol/vol) 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 (EC 3.2.1.151) 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 ml 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 of citrate-phosphate buffer (pH 5) and 0.2 ml of enzymes. 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 h for 50% viscosity loss × 10^3) (Rejón-Palomares et al., 1996). In controls for all enzyme assays, autoclaved enzyme supernatants and autoclaved buffers were used.

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 μl of root or extract (175 μg protein) and 3 μl 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 the

Table 1. Relationships between xyloglucanase activities of roots colonized by *G. mosseae*, *Rhizobium* or both symbionts and the percentage of arbuscular mycorrhizal root length colonization or the number of nodules of *Rhizobium*.

Xyloglucanase activity	AM root length colonization		Number of nodules	
	r	P	r	P
Alfalfa				
G. mosseae	0.83	0.07	–	–
S. meliloti	–	–	0.83	0.08
G. mosseae + S. meliloti	0.81	0.04	0.82	0.08
Pea				
G. mosseae	0.78	0.08	–	–
R. leguminosarum viceae	–	–	0.83	0.07
G. mosseae + R. leguminosarum viceae	0.71	0.08	0.16	0.70
Clover				
G. mosseae	0.89	0.03	–	–
R. leguminosarum bv. *trifolii*	–	–	0.80	0.09
G. mosseae + R. leguminosarum trifolii	0.70	0.08	0.72	0.08
Bean				
G. mosseae	0.83	0.07	–	–
R. tropici	–	–	0.74	0.08
G. mosseae + R. tropici	0.77	0.07	0.78	0.03
Chickpea				
G. mosseae	0.78	0.06	–	–
M. mediterraneum	–	–	0.79	0.09
G. mosseae + M. mediterraneum	0.79	0.09	0.86	0.05

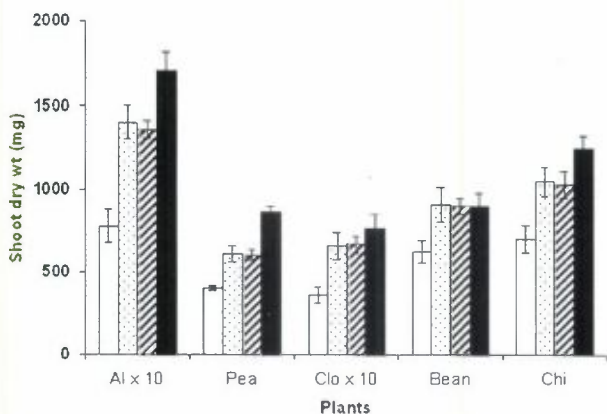Numbers are correlation coefficients (r) and probability values (P).

Figure 1. Shoot dry weights of alfalfa (Al), pea (Pea), clover (Clo), bean (Bean) and chickpea (Chi) plants inoculated with its specific *Rhizobium* alone (*S. meliloti* for alfalfa, *R. leguminosarum* bv. *viceae* for pea, *R. leguminosarum* bv. *trifolii* for clover, *R. tropici* for bean and *M. mediterraneum* for chickpea), *G. mosseae* alone, both symbionts together or non-inoculated control plants. Shoots dry weights of alfalfa and clover values were increased 10 times in the figure. Data are the means ± standard errors of mean of five replicate samples. Control plants (blank); plants inoculated with *G. mosseae* (dotted); plants inoculated with *Rhizobium* (striped); and plants inoculated with both symbionts (filled).
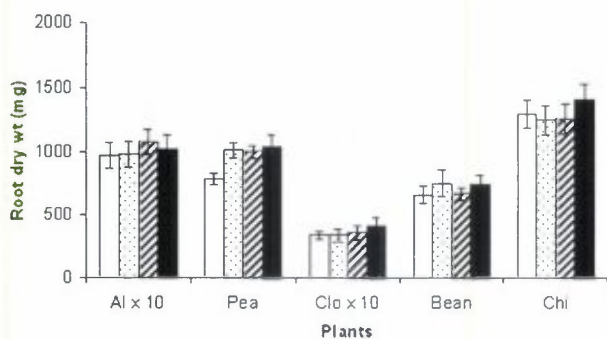

Figure 2. Root dry weights of alfalfa (Al), pea (Pea), clover (Clo), bean (Bean) and chickpea (Chi) plants inoculated with its specific *Rhizobium* alone (*S. meliloti* for alfalfa, *R. leguminosarum* bv. *viceae* for pea, *R. leguminosarum* bv. *trifolii* for clover, *R. tropici* for bean and *M. mediterraneum* for chickpea), *G. mosseae* alone, both symbionts together or non-inoculated control plants. Root dry weights of alfalfa and clover values were increased 10 times in the figure. Data are the means ± standard errors of mean of five replicate samples. Control plants (blank); plants inoculated with *G. mosseae* (dotted); plants inoculated with *Rhizobium* (striped); and plants inoculated with both symbionts (filled).

standard error of means test (P=0.05). Percentage data were subjected to arcsine transformation before analysis by linear regression.

3. Results

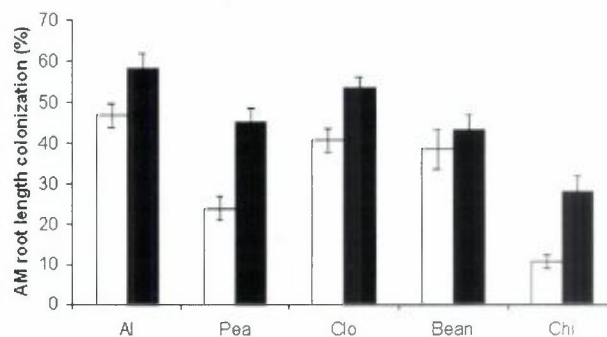The inoculation of plants with *G. mosseae*, with

Figure 3. Percentage of AM root length colonization of alfalfa (Al), pea (Pea), clover (Clo), bean (Bean) and chickpea (Chi) plants inoculated with *G. mosseae* alone or together with its specific *Rhizobium* (*S. meliloti* for alfalfa, *R. leguminosarum* bv. *viceae* for pea, *R. leguminosarum* bv. *trifolii* for clover, *R. tropici* for bean and *M. mediterraneum* for chickpea). Data are the means ± standard errors of mean of five replicate samples. Plants inoculated with *G. mosseae* (blank); plants inoculated with both symbionts (filled).
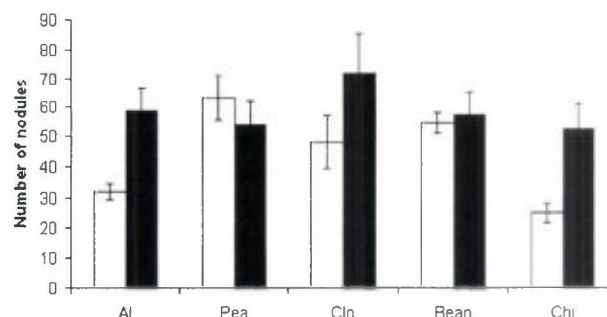

Figure 4. Number of nodules of alfalfa (Al), pea (Pea), clover (Clo), bean (Bean) and chickpea (Chi) plant root inoculated with its specific *Rhizobium* alone (*S. meliloti* for alfalfa, *R. leguminosarum* bv. *viceae* for pea, *R. leguminosarum* bv. *trifolii* for clover, *R. tropici* for bean and *M. mediterraneum* for chickpea) or together with *G. mosseae*. Data are the means ± standard errors of mean of five replicate samples. Plants inoculated with *Rhizobium* (blank); plants inoculated with both symbionts (filled).

Rhizobium or with both microorganisms increased the shoot dry weights of plants (Fig. 1). The shoot dry weights of alfalfa and pea inoculated with *S. meliloti* and *R. leguminosarum* together with *G. mosseae* were higher than when they were inoculated individually with each symbiont. *G. mosseae*, *Rhizobium* or both microorganisms inoculated together increased the root dry weights of plants but no significant differences among the various inoculation treatments were found (Fig. 2). The inoculation of plants with its specific *Rhizobium* increased the percentage of root length colonized by *G. mosseae*. However, a similar AM root colonization level in bean, whether inoculated with both symbionts together or separately, was observed (Fig. 3). The number of nodules was significantly higher when

plants were inoculated with *G. mosseae* together with each plant's specific *Rhizobium,* except in pea and bean which did not form more nodules than when each plant was inoculated with *R. leguminosarum* bv. *viciae* and *R. tropici* alone (Fig. 4). The level of xyloglucanase activity in plants inoculated with its specific *Rhizobium* was higher than in the non-inoculated controls, except in alfalfa inoculated with *S. meliloti,* which had a similar level of xyloglucanase activity to that of non-inoculated control (Fig. 5). The xyloglucanase activity of plants inoculated with *G. mosseae* alone, or together with its specific *Rhizobium,* was higher than that of the non-inoculated controls, this activity being higher in alfalfa, pea, clover and chickpea when both symbionts were inoculated together (Fig. 5). Correlations were found between endoxyloglucanase activity of alfalfa, clover, bean and chickpea roots colonized by *G. mosseae,* by its specific *Rhizobium* or by both symbionts and the percentage of AM root colonization or the number of nodules. However, no correlation between the number of nodules and the enzymatic activity of pea root inoculated with *R. leguminosarum* bv. *viciae* together with *G. mosseae* was found (Table 1).

Fig. 6 shows the electrophoretic bands of xyloglucanase activity in roots of the leguminous plants tested. Bands 3 appeared for alfalfa and pea, 4 and 5 for clover, 3, 5 and 6 for bean and 2 and 5 for chickpea inoculated with *G. mosseae* but not in the non-inoculated controls. Each leguminous species inoculated with its specific *Rhizobium* showed a different pattern of bands of enzymatic activity. Bands 1 appeared for alfalfa inoculated with *S. meliloti,* band 4 for pea inoculated with *R. leguminosarum* bv. *viciae,* band 3 for clover inoculated with *R. leguminosarum* bv. *trifolii,* bands 2 and 4 for bean inoculated with *R. tropici* and band 3 for chickpea inoculated with *M. mediterraneum.* None of these bands was found in the non-inoculated controls. Several bands of enzymatic activity show the same electrophoretic mobility for those plants inoculated with *G. mosseae* alone as for those also inoculated with their specific *Rhizobium,* such as band 3 for alfalfa and pea, 5 for clover, 5 and 6 for bean and 2 and 5 for chickpea. On the other hand, several plants inoculated with *Rhizobium* alone produced bands (Band 1 of alfalfa inoculated with *S. meliloti,* band 3 of clover inoculated with *R. leguminosarum* bv. *trifolii* and bands 2 and 3 of chickpea inoculated with *M. mediterraneum*) with the same mobility as for those also inoculated with *G. mosseae.* Different bands of activity appeared in plants inoculated with *Rhizobium* and *G. mosseae* together (Band 4 for alfalfa, 5 for pea, 6 for clover and 4 for chickpea), but were not detected in plants inoculated with either microorganism individually. However, band 4 which appeared in pea inoculated with *R. leguminosarum* bv. *viciae* and bands 2 and 4 of bean inoculated with *R. tropici* were absent when these plants were inoculated with both *G. mosseae* and its specific *Rhizobium.*
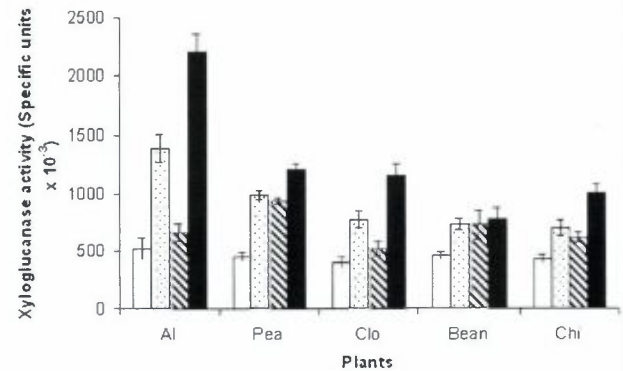

Figure 5. Xyloglucanase activity of alfalfa (Al), pea (Pea), clover (Clo), bean (Bean) and chickpea (Chi) plant roots inoculated with its specific *Rhizobium* alone (*S. meliloti* for alfalfa, *R. leguminosarum* bv. *viceae* for pea, *R. leguminosarum* bv. *trifolii* for clover, *R. tropici* for bean and *M. mediterraneum* for chickpea), AM fungus alone, both symbionts together or non-inoculated control plants. Data are the means ± standard errors of mean of five replicate samples. Control plants (blank); plants inoculated with *G. mosseae* (dotted); plants inoculated with *Rhizobium* (striped); plants inoculated with both symbionts (filled).

4. Discussion

The role of AM fungi and *Rhizobium* in plant growth, as well as the beneficial effect of the joint inoculation of both microorganisms on the growth of leguminous plants, is well known (Bethlenfalvay and Schüepp, 1994; Biró et al., 1993, 2000; Boddey et al., 1991). Our results show that the inoculation of plants with *G. mosseae* or *Rhizobium* alone and with both microorganisms together, increased the shoot dry weights of almost all plants tested. The combined benefit of both symbionts for plants has been described (Barea et al., 1988; Biró et al., 1993, 2000). In fact, the shoot dry weight of plants was higher when both symbionts were inoculated together than when they were inoculated separately, and furthermore there was a concurrent of the increase in the percentage of root length colonization and the number of nodules observed in the joint inoculated plants.

Production of xyloglucanases by *Rhizobium* has been described (Aranda et al., 2005). The bacteria increased, either quantitatively or qualitatively, the xyloglucanase activity in colonized roots. Moreover, all *Rhizobium* increased the number of xyloglucanase isozymes in colonized roots, suggesting that these bacteria were able to produce or induce specific metabolic changes in the root. These results also suggest that xyloglucanase enzymes produced by *Rhizobium,* like other hydrolytic enzymes (Akimova et al., 2000; Chen et al., 2004; Jimenez-Zurdo et al., 1996; Mateos et al., 1992, 2001), may participate in the infection of roots by the bacteria.
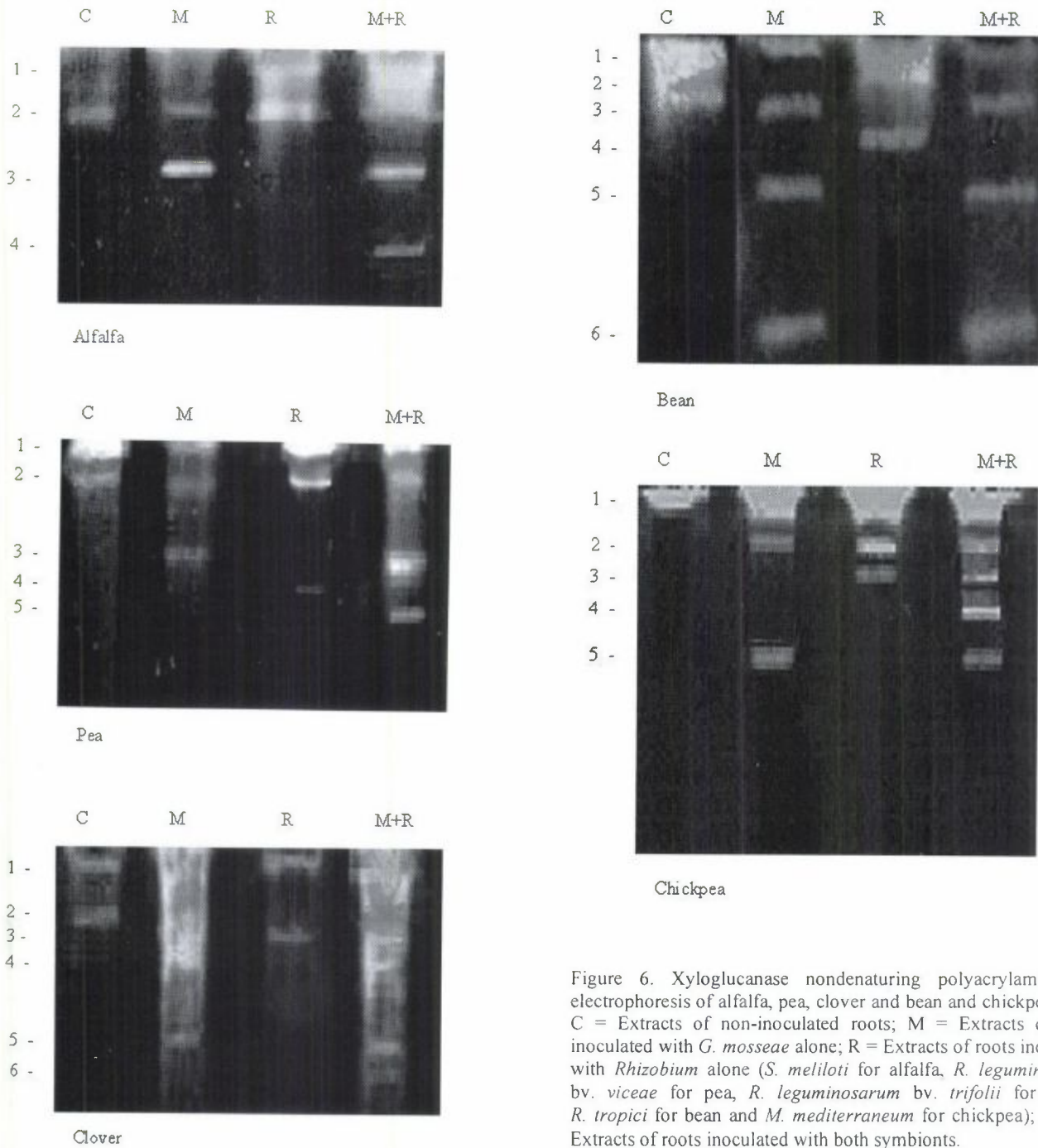
Alfalfa

Bean

Pea

Chickpea

Clover

Figure 6. Xyloglucanase nondenaturing polyacrylamide gel electrophoresis of alfalfa, pea, clover and bean and chickpea roots. C = Extracts of non-inoculated roots; M = Extracts of roots inoculated with *G. mosseae* alone; R = Extracts of roots inoculated with *Rhizobium* alone (*S. meliloti* for alfalfa, *R. leguminosarum* bv. *viceae* for pea, *R. leguminosarum* bv. *trifolii* for clover, *R. tropici* for bean and *M. mediterraneum* for chickpea); M+R = Extracts of roots inoculated with both symbionts.

It is known that AM fungi produce xyloglucanase enzymes which are involved in the penetration and colonization of roots by these fungi (García-Garrido et al., 2002). More xyloglucanase enzymes were observed in all leguminous plants colonized by *G. mosseae* than in the non-inoculated controls, although the level of enzymatic activity and the number of xyloglucanase isozymes was different according to the plant species (García-Garrido et al., 1999). The new xyloglucanolytic isozymes with the same electrophoretic mobility observed in all plant roots colonized with *G. mosseae* indicate the involvement of specific xyloglucanolytic enzymes in the colonization of the root by the AM fungi (García-Garrido et al., 2000).

The level of xyloglucanase activity, the number of xyloglucanolytic isozymes and the percentage of AM colonization of plant roots co-inoculated with *G. mosseae* and most of the *Rhizobium* tested were higher than when each microorganism was inoculated separately. A

correlation between the percentage of AM root colonization and the level of enzymatic activity was found. 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 *Rhizobium* and AM fungi used in our experiments did not increase the root dry weight. The independence of hydrolytic activity from the metabolical status of the plant root was also observed with a xyloglucan endotranshydrolase gene induced by the colonization of root by AM fungi, which was not related to the phosphate status of the root (Maldonado-Mendoza et al., 2005). Moreover, no increase was observed in the level of xyloglucanases activities, the number of xyloglucanolytic isozymes and in the percentage of AM colonization of bean root inoculated with *Rh. tropici* and *G. mosseae*, without an effect on the number nodules of *Rhizobium* and on the root dry weight. These results provide further evidence of the importance of xyloglucanase enzymes in the synergistic effect of *Rhizobium* on the AM colonization of plant roots.

On the other hand, there were more xyloglucanase isozymes and a greater number of nodules on most of the plants co-inoculated with *G. mosseae* and *Rhizobium* than when they were inoculated with *Rhizobium* alone. However, some bands of xyloglucanase activity which appeared in pea and bean inoculated with *Rh. leguminosarum* (Band 4) and *R. tropici* (Bands 2 and 4) respectively were not observed when these plants were inoculated with both symbionts. In addition, the number of nodules on both plants did not increase in presence of *G. mosseae*. A close relationship between the number of bands of xyloglucanase isozymes and the number of nodules was observed and there was also a correlation between the level of enzymatic activity and the nodule numbers in the AM colonized plants. These results suggest that the AM fungus influences the formation of nodules through an effect on xyloglucanase production in roots colonized with *Rhizobium*. Finally, our results suggest that xyloglucan hydrolytic activities may be important in the colonization of roots by these symbiotic microorganisms.

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