Journal of Experimental Botany, Vol. 51, No. 349, pp. 1443–1448, August 2000

Journal of Experimental Botany

Hydrolytic enzymes and ability of arbuscular mycorrhizal fungi to colonize roots

J.M. Garcia-Garrido, M. Tribak, A. Rejon-Palomares, J.A. Ocampo and I. Garcia-Romera[1]

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

Received 16 February 2000; Accepted 5 April 2000

Abstract

The production of hydrolytic enzymes from external mycelia associated with roots and colonized soybean roots (*Glycine max* L.) inoculated with different arbuscular-mycorrhizal (AM) fungi of the genus *Glomus*, and the possible relationship between these activities and the capacity of the AM fungi to colonize plant roots was studied. There were differences in root colonization and plant growth between the *Glomus* strains, and also between two isolates of *G. mosseae*. Hydrolytic activities in the root and external mycelia associated with roots differed in the AM fungi tested. Correlations were only found between the endoxyloglucanase activity of the external mycelia associated with roots of the AM fungi tested and the percentage root colonization or plant growth. However, hydrolytic activities of roots colonized by the different endophytes correlated with those of external mycelia. The hydrolytic activities were not qualitatively different because the endoxyloglucanase from AM colonized roots and the external mycelia did not show a high degree of polymorphism in the different species of fungus tested. The possible role of the hydrolytic activity of external hyphae of AM fungi was discussed as a factor affecting fungal ability to colonize the root and influence plant growth.

Key words: Arbuscular mycorrhiza, *Glomus* sp., *Glycine max*, hydrolytic enzymes.

Introduction

Although arbuscular mycorrhizal fungi (AM) show no specific variations in their ability to colonize a great range of host plants, they vary considerably in their population biology, ecological specificity and symbiotic activity (Giovannetti and Gianinazzi-Pearson, 1994). There are many reports of inter- and intraspecific differences in the efficiency of AM fungi in terms of plant growth and protection (Harley and Smith, 1983; Sieverding, 1991; Ruiz-Lozano and Azcón, 1995; Ruiz-Lozano *et al.*, 1995). The physiological basis for these variations is poorly known. The ability of mycorrhizas to increase plant growth can in most cases be explained by an increased phosphorus uptake. The hyphal spread of AM fungi is an important factor influencing the phosphorus supply to the host plant (Jakobsen *et al.*, 1992). Mycorrhizal fungi may differ in their capacity to develop an external hyphal system regardless of their capacity to colonize the root cortex (Graham *et al.*, 1982). The morphological properties and spatial distribution of the external hyphae in soil and differences in hyphal uptake, translocation capacities and metabolic activity seem to play an important role in the efficiency of AM fungi (Kothari *et al.*, 1991; Jakobsen *et al.*, 1992).

Differences in the ability of mycorrhizal fungi to enhance phosphorus uptake and growth of the host plant, even between species for which the extent of root colonization is similar, may be due to functional differences in the host–fungal interface. Different band patterns of enzymatic activities in AM fungi belonging to the same genus have been observed (Hepper *et al.*, 1988). It has been suggested that enzymatic polymorphism in different strains of AM fungus may be related to their efficiency in root colonization and influence on plant growth (Hepper *et al.*, 1988; Rosendahl and Sen, 1992).

Hydrolytic enzymes seem to be involved in the penetration and development of AM fungi in plant roots. Cellulase, pectinase and xyloglucanase activities have been found in colonized roots and in the external mycelium of AM fungi (García-Romera *et al.*, 1991*a*; García-Garrido

[1] To whom correspondence should be addressed. Fax: +34 58129600. E-mail: igarcia@eez.csic.es

© Oxford University Press 2000

et al., 1992*b*; Rejón-Palomares *et al.*, 1996*b*). Differences in cellulase and pectinase activities between some *Glomus* isotypes have been observed (García-Garrido, 1991; García-Romera *et al.*, 1991*b*). It is therefore possible that variations in colonization capacities of host tissues may be related to the ability of the fungi to produce hydrolytic enzymes (Gianinazzi-Pearson, 1994). Of the different hydrolytic enzymes, xyloglucanases are the least well known; however, they seem to play an important role in plant cell wall extension (Hoson *et al.*, 1995).

This study tests the possibility that differences in the ability of the fungus to produce hydrolytic enzymes are related to variations in the level of root colonization.

Materials and methods

Growth of plants and inoculation procedures

Inocula of the AM fungi used were two isolates of *G. mosseae* (Nicol. and Gerd.) Gerd. and Trappe (BEG 12) from Rothamsted Experimental Station (UK) and from the University of Kiel (Germany); *G. fasciculatum* (Thax. sensu Gerd.) Gerd. and Trappe (BEG 58) from Dijon (INRA); *G. clarum* (Nicol. and Schenck) and *G. intraradices* (Schenck and Smith) from the Instituto Venezolano de Investigaciones Científicas (IVIC); and *G. deserticola* (Trappe, Bloss and Menge) from the Instituto de Investigaciones Agrobiológicas de Galicia (CSIC). All mycorrhizal inocula consisted of soil, spores, mycelium, and infected root fragments from an open pot culture of soybean plant *(Glycine max* L.). Ten grams of inoculum with similar characteristics (an average of 30 spores g^{-1} and 75% of roots infected) of one of the six *Glomus* isolates was added to each pot at sowing just below the soybean seeds. This saturating amount of the soil inoculum was enough to result in optimal root colonization (Ruiz-Lozano, 1995).

Soybean plants were obtained from surface-sterilized seeds (5 min in 0.75% NaOCl). Seeds were sown in moistened sand, and after 2 weeks, ten seedlings were transplanted to each pot, with three replicate pots per treatment. Plants were inoculated with 10 g of inoculum, and uninoculated plants were given filtered leaching (Whatman No. 1 filter paper) from the inoculum soil (20 ml pot^{-1}, 1 : 2 w:v, soil : water). Plants were grown in 300 ml capacity open pots of soil collected from the province of Granada, Spain. The pots were filled with a gray loam soil obtained from the garden of the Estación Experimental del Zaidín (Granada, Spain). The soil (pH 8.1, 1 : 1, soil : water method) contained (mg kg^{-1}): 6.2 P ($NaHCO_3$-extractable), 0.3 N, and 132 K, and consisted of: 35.8% sand, 43.6% silt, 20.5% clay, and 1.8% organic matter. It was steam-sterilized and mixed with sterilized quartz sand at a proportion of 2 : 3 (v : v).

The plants were kept in a controlled-climate glasshouse, and were watered regularly and given 10 ml Hewitt's nutrient solution per week (Hewitt, 1952). The solution used for AM-inoculated plants lacked phosphate. Natural light was supplemented by Sylvania incandescent and cold-white lamps, 400 μmol m^{-2} s^{-1}, 400–700 nm; with a 16/8 h light/dark cycle. Air temperature was 25–19 °C, and relative humidity was 50%.

Plants were harvested after 30 d. The root system was separated from the shoot, and dry weight of the shoot was determined. The root system was washed and rinsed several times with sterilized distilled water and parts of the root system were cleared and stained (Phillips and Hayman, 1970). The percentage of total root length that was colonized by AM fungi was measured as described previously (Giovannetti and Mosse, 1980).

External mycelia associated with the roots were isolated from roots of 30-d-old soybean plants colonized with the different *G. mosseae* isotypes by rubbing the roots while submerged in sterile water and passing this water through a 50 μm mesh sieve (Benabdellah *et al.*, 1998). The external mycelium was collected with forceps under a dissecting microscope and mycelium dry weight was determined. Viability of the harvested hyphae was assessed by determining succinate dehydrogenase activity (Hamel *et al.*, 1990).

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 (PVPP), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM β-mercaptoethanol, 0.15 mM phenylmethyl sulphonyl fluoride (PMSF) and 0.3% (w : v) 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 was dialysed against several hundred volumes of the same diluted extractant solutions (1 : 9, v : v) for 16 h at 4 °C. The samples were then frozen until used.

External mycelia were frozen in liquid nitrogen and finely pulverized in a mortar. The resulting powder was suspended (30 mg ml^{-1}) in the same extractant solution as for roots. The suspension was briefly sonicated (1 min, 5 times at 80 W) and centrifuged at 20 000 *g* for 20 min; the pellet resuspended and sonicated again, and washed by centrifugation with the same buffer three times. The supernatant was used as a crude enzyme extract.

Enzyme assays

The extracts were assayed to determine the activities of endoxyloglucanase (endo-XG), endoglucanase (endo-GN) (EC 3.2.1.4), endopolymethylgalacturonase (PMG), and endopoly-galacturonase (endo-PG) (EC 3.2.1.15).

All hydrolytic activities were assayed by the viscosity method (Rejón-Palomares *et al.*, 1996*a*) using xyloglucan from nasturtium seed (*Tropaeolum majus* L.) extracted as described previously (McDougall and Fry, 1989), carboxymethylcellulose (CMC), citrus pectin, and Na polygalacturonase as substrates. The reduction in viscosity was determined at 0–30 min intervals. Approximately 0.5 ml of the reaction mixture was sucked into a 1 ml syringe and the time taken for the meniscus to flow from the 0.70 ml to 0.20 ml mark was recorded. The reaction mixture 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^{-1} protein) (U reciprocal of time in hours for 50% viscosity loss $\times 10^3$) (Rejón-Palomares *et al.*, 1996*b*).

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*a*). 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 either root or fungus 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. Washing in 1 M NaCl followed this until the bands became visible.

Protein determination

Total proteins were determined by the method of Bradford (Bradford, 1976) using a Bio-Rad kit with BSA as the standard.

Statistical treatments

Data were subjected to one way ANOVA and Tukey test ($P = 0.05$) evaluated differences in treatment means. Percentage data were subjected to arcsine transformation before analysis by linear regression.

Results

Microscopic observations of stained roots showed no presence of AM fungi in uninoculated controls. The percentage of root length colonization of soybean root ranged from 22% by *G. mosseae* (BEG 12) to 50% by *G. deserticola*. Higher dry weights of the external mycelia associated with the roots were observed in roots colonized either with *G. intraradices* or *G. deserticola*. The differences in shoot dry weight between soybean plants inoculated with *G. mosseae* (BEG 12), *G. clarum* or *G. intraradices* and uninoculated controls were not significant. Shoot dry weights of soybean inoculated with *G. fasciculatum* and *G. deserticola* were higher than with the other treatments (Table 1).

As Table 2 shows, endo-XG, endo-PMG and endo-PG activities were higher in mycorrhizal plant roots than in uninoculated controls. Mycorrhizal plants showed higher endo-GN activity than uninoculated controls, except in roots colonized by *G. mosseae* (BEG 12). Plants colonized with *G. deserticola* showed the highest endo-XG and endo-PMG activities. Endo-GN activity was higher in roots colonized by *G. fasciculatum*, *G. intraradices* and *G. deserticola* than in roots colonized with the other *Glomus* strains. However, endo-PG activity was lower in roots colonized by either *G. mosseae* isotypes (Table 2).

Comparison of hydrolytic activities from the external mycelia associated to roots colonized with different *Glomus* strains shows that the external mycelia of *G. deserticola* exhibited the highest endo-XG and endo-PG activities. Endo-GN activity was lowest in the external mycelia of *G. mosseae* (BEG 12). Endo-PMG activity was variable in the external mycelia of the different endophytes tested; however, *G. deserticola* showed the highest and the two *G. mosseae* isotypes the lowest activity (Table 3).

No correlation was observed between shoot dry weight, percentage of root length colonization and dry weight of external mycelia of the different endophytes tested. However, endo-XG activity in the external mycelia correlated with the dry weight of their external mycelia, with the shoot dry weight of colonized plants and the percentage of root colonization. The hydrolytic activities of roots colonized by the different endophytes correlated with the homonymous enzymatic activity of the external mycelia associated to roots (Table 4).

Because pectinase (endo-PMG, endo-PG) and cellulase activities (endo-GN) of AM roots are difficult to detect (García-Romera *et al.*, 1991*b*; García-Garrido *et al.*, 1992*a*) and since endo-XG is the only hydrolytic enzyme whose activity correlates with root colonization and plant growth, xyloglucanase activity was selected for electrophoretic analysis. Several electrophoretic bands of endo-XG activities were observed in 30-d-old soybean plants. The bands in root extracts differed between the endophytes tested. Only one band of endo-XG activity was detected in the external mycelia of each of the different endophytes tested. The electrophoretic band of the external mycelia of *G. mosseae* (BEG), *G. fasciculatum*, *G. clarum G. intraradices*, and *G. deserticola* showed the same electrophoretic mobility (line 4). Only *G. mosseae* (KIEL) produced a xyloglucanase band with different electrophoretic mobility (line 2). Several electrophoretic bands present in non-inoculated plants were absent in some of the mycorrhizal plants (Fig. 1).

Discussion

The inter- and intraspecific differences in the effectiveness of AM fungi for root colonization and plant growth are

Table 1. *Shoot and dry weight of external mycelia associated to roots, and percentage of root length colonization of soybean (*Glycine max *L.) inoculated with different* Glomus *isotypes*

	Root colonization	Mycelia dry wt. (mg)	Shoot dry wt. (mg)
Control			336 (1.8) a
G. mosseae (BEG 12)	22.4 (7.6) a	0.07 (0.007) a	383 (3.3) a
G. mosseae (KIEL)	32.2 (4.4) a	0.11 (0.007) a	480 (5.9) ab
G. fasciculatum	26.1 (1.5) a	0.20 (0.009) ab	573 (4.8) bc
G. clarum	25.3 (5.5) a	0.15 (0.011) a	420 (3.2) b
G. intraradices	32.6 (4.2) a	0.28 (0.012) bc	390 (1.2) a
G. deserticola	50.5 (4.4) b	0.39 (0.015) c	671 (2.8) c

Each value is the mean of three replicates. Column values sharing the same letter were not significantly different according to Tukey test ($P = 0.05$). Standard errors are given in brackets.

Table 2. *Endoxyloglucanase (endo-XG), endoglucanase (endo-GN), endopolymethylgalacturonase (endo-PMG) and endopolygalacturonase (endo-PG) activities (units/mg protein) of root extracts of soybean (*Glycine max *L.) plants colonized by different* Glomus *isotypes*

	Endo-XG	Endo-GN	Endo-PMG	Endo-PG
Control	127 (3.8) a	204 (1.9) a	41 (1.6) a	25 (1.2) a
*G. mosseae (*BEG12)	226 (1.2) b	271 (7.3) ab	71 (3.1) b	62 (3.3) b
*G. mosseae (*KIEL)	298 (4.1) bc	311 (1.2) b	76 (1.6) b	83 (2.4) bc
G. fasciculatum	321 (2.2) bc	536 (8.7) d	91 (3.9) b	90 (4.1) cd
G. clarum	333 (2.2) cd	333 (7.2) b	71 (2.1) b	109 (4.4) de
G. intraradices	420 (3.7) d	452 (9.9) c	98 (3.1) b	113 (5.6) de
G. deserticola	534 (1.3) e	499 (7.1) cd	150 (1.8) c	129 (1.5) e

Each value is the mean of three replicates. Column values sharing the same letter were not significantly different according to Tukey test ($P=0.05$). Standard errors are given in brackets.

Table 3. *Endoxyloglucanase (endo-XG), endoglucanase (endo-GN), endopolymethylgalacturonase (endo-PMG) and endopolygalacturonase (endo-PG) activities (units* mg^{-1} *protein* $\times 10^{-3}$*) of the external mycelia from root of soybean (*Glycine max *L.) plants colonized by different* Glomus *isotypes*

	Endo-XG	Endo-GN	Endo-PMG	Endo-PG
*G. mosseae (*BEG12)	151 (1.7) a	116 (6.5) a	109 (12.5) a	66 (8.4) a
*G. mosseae (*KIEL)	171 (10.4) ab	367 (10.1) b	124 (12.2) a	119 (9.4) ab
G. fasciculatum	152 (30.4) a	336 (5.6) b	143 (1.8) ab	172 (23.2) b
G. clarum	165 (12.6) a	318 (6.8) b	187 (6.8) bc	132 (5.7) ab
G. intraradices	263 (14.3) bc	320 (6.7) b	187 (7.1) bc	85 (26.8) a
G. deserticola	330 (29.8) c	335 (5.8) b	205 (13.1) c	201 (13.7) c

Each value is the mean of three replicates. Values sharing the same letter were not significantly different according to Tukey test ($P=0.05$). Standard errors are given in brackets.

Table 4. *Relationships between hydrolytic enzymes of external mycelia and colonized soybean roots inoculated with different* Glomus *isotypes*

Four response variables (shoot and mycelia dry weight, percentage root length colonization and hydrolytic activities in external mycelia associated to roots and colonized roots) were evaluated by linear regression analysis. Numbers are regression coefficients (*r*) and probability values (*P*).

	Shoot dry wt.		Mycelia dry wt.		Root colonization		Root hydrolytic activities[a]	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Mycelia dry wt.	0.19	0.451						
Root colonization	0.37	0.045	0.35	0.063				
Root hydrolytic activities								
Endoxyloglucanase	0.11	0.672	0.70	0.001	0.35	0.053		
Endoglucanase	0.22	0.378	0.33	0.593	0.37	0.120		
Endopolymethylgalacturonase	0.36	0.045	0.34	0.055	0.36	0.114		
Endopolygalacturonase	0.29	0.238	0.39	0.110	0.33	0.076		
Mycelia hydrolytic activities								
Endoxyloglucanase	0.52	0.042	0.76	<0.001	0.63	0.004	0.85	<0.001
Endoglucanase	0.36	0.114	0.38	0.115	0.11	0.677	0.58	0.043
Endopolymethylgalacturonase	0.30	0.228	0.34	0.123	0.33	0.073	0.76	<0.001
Endopolygalacturonase	0.39	0.112	0.36	0.147	0.37	0.127	0.52	0.027

[a]Correlations are between each enzyme from roots and its homonymous from external mycelia associated to roots.

well known (Harley and Smith, 1983; Sieverding, 1991). Differences were found in root colonization and plant growth not only between the different *Glomus* strains, but also between the isolates of *G. mosseae* BEG-12 and KIEL. However, as has been observed elsewhere, AM fungi differ in their ability to enhance growth of the host plant, regardless of the extent of root colonization (Graham *et al.*, 1982). One of the most important factors that influences the efficiency of different AM fungal strains seems to be their external mycelium. The production of external hyphae may vary considerably between AM fungi (Sanders *et al.*, 1977; Graham *et al.*, 1982; Abbott and Robson, 1985; Kothari *et al.*, 1991). No clear relationship seems to exist between the amount of external hyphae in soil and the growth responses observed in colonized plants (Jakobsen *et al.*, 1992; Frey and Schuepps, 1993). Other factors such as the difference in rate of appresorium formation, in hyphal uptake and translocation capacities of nutrients, and in the metabolic activity of the external hyphae, seem to have more

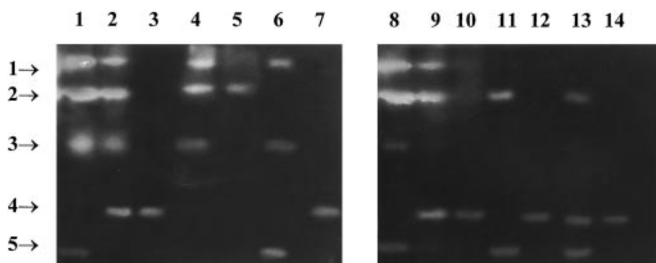

Fig. 1. Bands of xyloglucanase activities (lines 1–5) in non-denaturing polyacrylamide gel electrophoresis. Lanes A and H; extracts of nonmycorrhizal soybean (*Glycine max*) roots; lanes B, D, F, I, K, and M; extracts of *G. mosseae* (BEG 12), *G. mosseae* (Kiel), *G. fasciculatum*, *G. clarum*, *G. intraradices*, and *G. deserticola* soybean roots, respectively; lanes C, E, G, J, L, and N; extracts from external mycelium of *G. mosseae* (BEG 12), *G. mosseae* (KIEL), *G. fasciculatum*, *G. clarum*, *G. intraradices*, and *G. deserticola* isolated from soybean plants.

influence on the efficiency of AM fungi (Jakobsen *et al.*, 1992; Frey and Schuepps, 1993; Giovannetti and Citernesi, 1993). One of the factors involved in the penetration of root by AM fungi is the production of hydrolytic enzymes (García-Garrido *et al.*, 1992*a*; García-Romera *et al.*, 1991*a*). The activities of the hydrolytic enzymes tested were higher in AM colonized than in non-AM colonized root (García-Romera *et al.*, 1991*a*). Quantitative differences in hydrolytic activities of different AM fungi in the root and the external mycelia associated to roots were observed. No correlations were found between most of the hydrolytic activities of the AM fungi and percentage root colonization or plant growth. The low and localized production of hydrolytic enzymes does not allow the establishment of a close relationship between their production and AM root colonization (García-Garrido *et al.*, 1996; García-Romera *et al.*, 1991*b*). However, a correlation between endo-XG activity of the external mycelia and root colonization and plant growth was noted. This result indicates that the activity of this enzyme is an important factor influencing fungal colonization and plant growth (Rejón-Palomares *et al.*, 1996*a*).

It is clear that the external hyphae play an important role in colonization capacity and efficiency of plant growth. These studies shows that the enzymatic activity of the external hyphae associated with roots colonized by AM fungi varies greatly depending on the fungal isolate used. These findings also indicate a correlation between the hydrolytic activities of these external mycelia with those of their colonized roots. Relationships between the metabolic activity of colonized roots and external hyphae have been found (Kothari *et al.*, 1991; Jakobsen *et al.*, 1992; Frey and Schuepps, 1993). The production of hydrolytic enzymes by external hyphae may therefore be a relevant variable that should be considered since colonization of roots requires efficient penetration mechanisms by the external hyphae (García-Romera *et al.*, 1997).

Different isozyme activities in species of the genus *Glomus* show clear variations between species and geographically different isolates (Rosendahl and Sen, 1992). However, the hydrolytic enzyme endo-XG from AM colonized root and external mycelium does not show a high degree of polymorphism between the different species tested. A similar absence of polymorphism was found for other enzymes such as alkaline phosphatase and cellulase (García-Garrido, 1991; Gianinazzi *et al.*, 1992). The presence of one band of xyloglucanase activity in non-mycorrhizal roots which was absent in mycorrhizal roots, suggest qualitative inhibition by the fungus of some enzymatic activity of the plant. Inhibition of plant protein synthesis by AM fungi has been observed in several plant–AM fungi associations (García-Garrido *et al.*, 1993; Dumas-Gaudot *et al.*, 1994).

A time-course study underway in this laboratory will shed further light on the role of hydrolytic enzymes from external mycelia in the mycorrhization of roots.

Acknowledgement

Financial support for this study was provided by the DGICYT, Spain.

References

Abbott LK, Robson AD. 1985. Formation of external hyphae in soil by four species of vesicular-arbuscular mycorrhizal fungi. *New Phytologist* **99,** 245–255.

Benabdellah K, Azcón-Aguilar C, Ferrol N. 1998. Soluble and membrane symbiosis-related polypeptides associated with the development of arbuscular mycorrhizas in tomato (*Lycopersicon esculetum*). *New Phytologist* **140,** 135–143.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248–254.

Dumas-Gaudot E, Guillaume P, Tahiri-Alaoui A, Gianinazzi-Pearson V, Gianinazzi S. 1994. Changes in polypeptide patterns in tobacco roots colonized by two *Glomus* species. *Mycorrhiza* **4,** 215–221.

Frey B, Schuepps H. 1993. Acquisition of nitrogen by external hyphae of arbuscular mycorrhizal fungi associated with *Zea mays* L. *New Phytologist* **124,** 221–230.

García-Garrido JM. 1991. *Estudio de factores bioquímicos responsables de la infección por parte de hongos formadores de micorrizas VA: enzimas celulolíticos*. PhD thesis, Universidad de Granada, Spain.

García-Garrido JM, García-Romera I, Ocampo JA. 1992*a*. Cellulase production by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd) Gerd. and Trappe. *New Phytologist* **121,** 221–226.

García-Garrido JM, García-Romera I, Ocampo JA. 1992*b*. Cellulase activity in lettuce and onion plants colonized by the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. *Soil Biology and Biochemistry* **25,** 503–504.

García-Garrido JM, Toro N, Ocampo JA. 1993. Presence of specific polypeptides in onion roots colonized by *Glomus mosseae*. *Mycorrhiza* **2,** 175–177.

García-Garrido JM, García-Romera I, Parra-García MD,

Ocampo JA. 1996. Purification of an arbuscular mycorrhizal endoglucanase from onion roots colonized by *Glomus mosseae. Soil Biology and Biochemistry* **25,** 1443–1449.

García-Romera I, García-Garrido JM, Ocampo JA. 1991*a*. Pectolytic enzymes in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. *FEMS Microbiology Letters* **78,** 343–346.

García-Romera I, García-Garrido JM, Ocampo JA. 1991*b*. Pectinase activity in vesicular-arbuscular mycorrhiza during colonization of lettuce. *Symbiosis* **12,** 189–198.

García-Romera I, García-Garrido JM, Rejón-Palomares A, Ocampo JA. 1997. Enzimatic mechanisms of penetration and development of arbuscular mycorrhizal fungi in plant In: Pandalai SG, ed. *Recent research developments in soil biology and biochemistry*. India: Research Signpost, 121–136.

Gianinazzi S, Gianinazzi-Pearson V, Tisserant B, Lemoine MC. 1992. Protein activities as potential markers of functional endomycorrhizas in plants. In: Read DJ, Lewis DH, Fitter AH, Alexander IJ, eds. *Mycorrhizas in ecosystems*. Oxon: CAB International, UK, 333–339.

Gianinazzi-Pearson V. 1994. Morphofunctional compatibility in interactions between roots and arbuscular endomycorrhizal fungi: molecular mechanisms, genes expression. In: Kihmito K, Sing RP, Sing US, eds. *Pathogenesis and host-parasite specificity in plant diseases: histological, genetic, biochemical and molecular basis*. Oxford: Pergamon Press, UK, 251–263.

Giovannetti M, Citernesi AS. 1993. Time-course of appresorium formation on host plants by arbuscular mycorrhizal fungi. *Mycological Research* **97,** 1140–1142.

Giovannetti M, Gianinazzi-Pearson V. 1994. Biodiversity in arbuscular mycorrhizal fungi. *Mycological Research* **98,** 705–715.

Giovannetti M, Mosse B. 1980. An evaluation of techniques for measuring vesicular-arbuscular mycorrhizal infection in roots. *New Phytologist* **84,** 489–500.

Graham JH, Linderman RG, Menge JA. 1982. Development of external hyphae by different isolates of mycorrhizal *Glomus* sp. in relation to root colonization and growth of *Troyer citrange*. *New Phytologist* **91,** 183–189.

Hamel C, Fyles H, Smith DL. 1990. Measurement of development of endomycorrhizal mycelium using three different vital strains. *New Phytologist* **115,** 297–302.

Harley JL, Smith SE. 1983. *Mycorrhizal symbiosis*. New York: Academic Press.

Hepper CM, Sen R, Azcón-Aguilar C, Grace C. 1988. Variation in certain isozymes amongst different geographical isolates of the vesicular-arbuscular mycorrhizal fungi *Glomus clarum*, *Glomus monosporum* and *Glomus mosseae*. *Soil Biology and Biochemistry* **20,** 51–59.

Hewitt EJ. 1952. *Sand water culture methods used in the study of plant nutrition*. Commonwealth Agricultural Bureaux, Technical Communication 22. Farnham Royal, Commonwealth Agricultural Bureaux, Buckinghamshire, UK.

Hoson T, Wakabayashi K, Masuda Y. 1995. Inhibition of the breakdown of xyloglucan in azuki bean epicotyls by concavalin A. *Plant Cell Physiologist* **36,** 897–902.

Jakobsen I, Abbott IK, Robson AD. 1992. External hyphae of vesicular-arbuscular mycorrhizal fungi associated with *Trifolium subterraneum* L. Spread of hyphae and phosphorus inflow into roots. *New Phytologist* **120,** 371–380.

Kothari S, Marschner H, Romheld V. 1991. Contribution of the VA mycorrhizal hyphae in acquisition of phosphorus and zinc by maize grown in a calcareous soil. *Plant and Soil* **131,** 177–185.

McDougall GJ, Fry SC. 1989. Structure–activity relationships for oligosaccharides with anti-auxin activity. *Plant Physiology* **89,** 883–887.

Phillips JM, Hayman DS. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Transactions of the British Mycological Society* **55,** 158–161.

Rejón-Palomares A, García-Romera I, Ocampo JA. 1996*a*. Xyloglucan-specific endoglucanase activities in onion plants colonized by the arbuscular mycorrhizal fungus. In: Azcón-Aguilar C, Barea JM, eds. *Mycorrhizas in integrated systems from genes to plant development*. Luxembourg: Official Publications of the European Communities, 256–259.

Rejón-Palomares A, García-Garrido JM, Ocampo JA, García-Romera I. 1996*b*. Presence of xyloglucan-hydrolyzing glucanases (xyloglucanases) in arbuscular mycorrhizal symbiosis. *Symbiosis* **21,** 249–261.

Rosendahl S, Sen R. 1992. Isozyme analysis of mycorrhizal fungi and their mycorrhiza. In: Varma AK, Read DJ, Norris JR, eds. *Methods in microbiology*, Vol. 24: *Experiments with mycorrhizae*. London: Academic Press, UK, 169–194.

Ruiz-Lozano JM. 1995. *Estudio de mecanismos fisiológicos y bioquímicos implicados en la tolerancia de las plantas micorrizadas ante situaciones de estrés*. PhD thesis, Universidad de Granada, Spain.

Ruiz-Lozano JM, Azcón R. 1995. Hyphal contribution to water uptake in mycorrhizal plants as affected by the fungal species and water status. *Physiologia Plantarum* **95,** 472–478.

Ruiz-Lozano JM, Azcón R, Gómez M. 1995. Effects of arbuscular-mycorrhizal *Glomus* species on drought tolerance: physiological and nutritional plant responses. *Applied Environmental Microbiology* **61,** 456–460.

Sanders FE, Tinker PB, Black RLB, Palmerley SM. 1977. The development of endomycorrhizal root systems. I. Spread of infection and growth-promoting effects with four species of vesicular-arbuscular endophytes. *New Phytologist* **78,** 257–268.

Sieverding E. 1991. *Vesicular-arbuscular mycorrhiza management*. Deutsche Gesellschaft für Technische Zusammenarbeit, Eschborn, 371.