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Effect of xyloglucan and xyloglucanase activity on the development of the arbuscular mycorrhizal *Glomus mosseae*

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The effect of xyloglucan on spore germination, hyphal length and mycorrhizal colonization of alfalfa plants was studied. The presence of high concentrations of xyloglucan in the rooting medium inhibited mycorrhizal colonization in plants inoculated with *Glomus mosseae*. Intermediate xyloglucan concentrations had no effect on arbuscular mycorrhizal (AM) colonization, but a low concentration increased mycorrhization of host plants. The effects of these doses on spore germination and hyphal length of *G. mosseae* were similar to those observed for mycorrhizal colonization. Production of xyloglucanase was assayed during colonization by the AM fungus *G. mosseae* in lettuce and onion. Endoxyloglucanase activity peaked 15 d after inoculation, whereas exoxyloglucanase activity peaked at 30 and 50 d. Extracts from external mycelia of *G. mosseae* showed endo- and exoxyloglucanase activities. Some of the endoxyloglucanase activities detected in AM colonized plant roots may be derived from the AM fungus, as endoxyloglucanase proteins found in the external mycelia of *G. mosseae* and in mycorrhizal root extracts showed similar electrophoretic mobility. These results suggest that xyloglucanase is involved in the process of colonization of plants by *G. mosseae*.

Hydrolytic enzymes produced by plant pathogenic micro-organisms allow their entry and spread through plant host tissues and their acquisition of carbohydrates. 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 (Cooper & Tinker, 1978). The major carbohydrate store in AM fungal spores is trehalose (Bécard *et al.*, 1991; Schubert, Wyss & Wienken, 1992) and low levels of trehalose have also been reported in the external mycelium or mycorrhizal roots (Amijee & Stribley, 1987; Schubert *et al.*, 1992). The incorporation of radiolabelled glucose into trehalose, glycogen and mannitol of the fungus and into sucrose by the host has been shown (Shachar-Hill *et al.*, 1995). One of the aims of the present work was to study the activity of xyloglucanase enzyme of the AM *Glomus mosseae* (T. H. Nicolson & Gerd.) Gerd. & Trappe on the degradation of xyloglucan and the effects of the oligosaccharides produced on the mycorrhizal development.

Microscopic observations suggest that there are wall-degrading enzyme activities (Gianinazzi-Pearson *et al.*, 1981; Bonfante-Fasolo & Gianinazzi-Pearson, 1982; Jacquelinet-Jeanmougin, Gianinazzi-Pearson & Gianinazzi, 1987). Biochemical studies have shown that ectomycorrhizal and ericoid fungi possess cellulolytic and pectolytic activities, but that these are low in comparison to pathogenic fungi (Keon, Byrde & Cooper, 1987; Cairney & Burke, 1994). The occurrence of peaks in pectinase activity during AM infection (García-Romera, García-Garrido & Ocampo, 1991*b*) and the immunolocalization of an endopolygalacturonase on the wall of external hyphae (Bonfante-Fasolo & Perotto, 1992) all provide further evidence for the possible involvement of fungal hydrolytic enzymes in the process of root colonization.

Mycorrhizal fungi usually cross non-encrusted epidermal walls consisting mainly of a cellulose-xyloglucan framework and fibrous protein embedded in a pectin matrix (Carpita & Gibeaut, 1993). Taiz (1984) suggested that xyloglucanase in the primary wall is one of the most likely contributors to the loosening of cellulose microfibril networks, which renders the wall susceptible to turgor-driven expansion. There is evidence that to penetrate the host cell, mycorrhizal fungi use small amounts of enzyme similar to those used to loosen in plant cells to loosen walls (Bonfante-Fasolo & Perotto, 1990).

The possible involvement of xyloglucanase activity of *G. mosseae* in colonization of plant roots was also examined.

MATERIALS AND METHODS

Effect of xyloglucanase enzyme substrate on AM infection

This experiment was carried out in 80 ml pots with a steam-sterilized sand:vermiculite mixture (1:1 v/v). Xyloglucan from nasturtium seed (*Tropaeolum majus* L.) extracted as described by McDougall & Fry (1989) was added to the rooting medium. Seven xyloglucan concentration were

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prepared in diluted Hewitt's nutrient solution (1/2) (Hewitt, 1952) containing 50 mg l^{-1} of P (pH 7·2). Twenty ml of the autoclaved xyloglucan concentration of 0, 0·001, 0·01, 0·05, 0·2, 0·6 or 1 mg ml^{-1} were added to pots before planting. Five replicates per treatment were carried out making a total of 35 pots.

Inocula consisted of *G. mosseae* spores obtained from sporocarps by wet sieving soil (250 μm) and decanting. Resting spores freshly isolated from these sporocarps by excision were placed on damp filter paper in a Petri dish and stored at 4 °C. The spores were surface-sterilized as described by Mosse (1962). In each pot 30 surface-sterilized spores of *G. mosseae* were placed under the seedling. Seeds of *Medicago sativa* cv. Aragon were surface-sterilized with $HgCl_2$ for 10 min and thoroughly rinsed with sterile water. After germination, seedlings were selected for uniformity before planting. Sterilized perlite was added to the top of the pots.

Plants were grown in a chamber with light from Sylvania incandescent and cold-white lamps (400 nmol m^{-2} s^{-1}, 400–700 nm) at a 16–8 h light–dark cycle, 25–17° day–night temperature, and approx. 50% r.h. Pots were regularly watered and given 3 ml wk^{-1} of 1/2 strength Hewitt's nutrient solution. Plants were harvested after 35 d. The root system was washed and rinsed several times with sterilized distilled water and parts of the root system were cleared and stained (Phillips & Hayman, 1970). The percentage of total root length which was colonized by AM fungi was measured (Giovannetti & Mosse, 1980).

The effect of the same concentration of hydrolytic enzyme substrate on spore germination and hyphal growth of *G. mosseae* was also tested. Spores of *G. mosseae* were surface-sterilized and cultivated on 25 mM MES buffer (Na), pH 7, plus 0·05% (w/v) $MgSO_4$ and 0·4% (w/v) Gel-gro (Agar substitute) (ICN-150180). Ten spores were placed in each Petri dish and ten replicates of each treatment were used. Plates were kept at 20° for 2 wk. The spore germination frequency was determined by light microscope examination and hyphal length of the germinated spores was assessed (Marsh, 1971).

Measurement of enzyme activity

Fungi and plants. Plants were grown in 300-ml capacity open pots of soil. Three replicates per treatment were carried out. The soil was a grey loam soil from the garden of the Estación Experimental del Zaidín, Granada, Spain, with pH 8·1 (1:1, soil:water method), and contained (mg kg^{-1}): 6·2 P ($NaHCO_3$-extractable), 0·3 N, and 132 K, and consisted of (%): 35 sand, 43 silt, 20 clay, and 2 organic matter. It was steam sterilized and mixed with sterilized quartz sand at a proportion of 2:3 (v:v). Lettuce (*Lactuca sativa* cv. Romana) and onion (*Allium cepa* cv. Babosa) were used as test plants. Seeds were sown in moistened sand and after 2 wk seedlings were transplanted to the pots and grown under greenhouse conditions as described before. Plants were watered from below by capillarity, and fed with 1/2 strength nutrient solution (Hewitt, 1952).

The AM inoculum consisted of 5 g of rhizosphere soil from alfalfa plant pot cultures of a Rothamsted isolate of *G. mosseae* (BEG No 12), which contained spores (15 sporocarps g^{-1} with 1–5 spores $sporocarp^{-1}$), mycelium and colonized root fragments. Uninoculated plants were given filtered leachings from the inoculum soil. Soil filtrate (Whatman No. 1 filter paper) from the rhizosphere of mycorrhizal plants was added to the AM-non-inoculated treatment. The filtrate contained soil micro-organisms, but no propagules of *G. mosseae*.

External mycelia were isolated from roots of 30-d-old onion plants colonized with *G. mosseae*. The roots were washed and rinsed gently with sterilized water and the external mycelium was collected with forceps under a dissecting microscope.

Plants were harvested at 7, 15, 30, 50 and 80 d. Mycorrhization in parts of the root system was assessed as described above.

Preparation of extracts for enzyme assays. Roots (10 g F.W.) were frozen in liquid nitrogen and finely pulverized in a mortar. The resulting powder was homogenized in 30 ml of 100 mM Tris-HCl buffer (pH 7) plus 0·02 g polyvinyl-poly-pyrrolidone (PVPP), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 10 mM β-mercaptoethanol, 0·15 mM phenylmethyl sulfonyl 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 20000 *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°. 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, five times at 80 W) and centrifuged at 20000 *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. Xyloglucan hydrolysing endoglucanase (endoxyloglucanase) activity was assayed by the viscosity method, using xyloglucan from nasturtium seed as substrate. The reduction in viscosity was determined at 0–90 min intervals as described Rejón-Palomares *et al.* (1996). One unit of enzyme activity was expressed as specific activity (U mg^{-1} protein) (U reciprocal of time in h for 50% viscosity loss × 10^3).

Exoxyloglucanase was quantified by measuring the reducing sugars with a 2,2′-bicinchoninate reagent (BCA) (Waffenschmidt & Jaenicke, 1987) at 0–90 min intervals as described by Rejón-Palomares *et al.* (1996). One enzyme unit was defined as the amount of enzyme releasing 1 nmol of reducing sugar equivalent min^{-1} at 40° and pH 5.

Polyacrylamide gel electrophoresis

Xyloglucanase enzymes were separated by nondenaturing 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, García-Romera & Ocampo, 1992*a*). The electrode tank con-

tained the same Trys-glycine buffer (pH 8·8) as used in the gel. The wells were filled with 175 μg of either root or fungus extract and 3 μl 0·05% bromophenol blue. Electrophoresis was done at 4° 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° for 16 h, after which they were stained with 0·1% Congo red for 15 min. This was followed by washing in 1 M NaCl until bands became visible.

Protein determination

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

Statistical treatments

The results were evaluated statistically with Duncan's multiple-range test.

RESULTS

Percentage germination and hyphal length of *G. mosseae* spores decreased significantly in the presence of 1 mg ml^{-1} xyloglucan, whereas when spores were cultivated with 0·6, 0·2 and 0·05 mg ml^{-1} xyloglucan, no differences in spore germination and hyphal length were observed with respect to the control. Lower concentrations of xyloglucans (0·001 and 0·1 mg ml^{-1}), however, significantly increased germination and hyphal length (Table 1).

Percentage of AM root length colonization in the presence of 0, 0·001, 0·01, 0·05, 0·2, 0·6 and 1 mg ml^{-1} of xyloglucan was 22·9 ± 1, 33·2 ± 3, 23·0 ± 2, 23·3 ± 1, 21·4 ± 2, 18·6 ± 1 and 0 mg ml^{-1} respectively. Plants grown with concentrations of 0·01, 0·05, 0·2 and 0·6 mg ml^{-1} xyloglucan in the rooting medium had no effect on root length colonization in alfalfa plants. The 0·001 mg ml^{-1} xyloglucan treatment increased root length colonization, whereas the 1 mg ml^{-1} xyloglucan treatment significantly decreased mycorrhizal colonization.

Microscopic observations of stained roots showed no fungi in non-mycorrhizal plants and only AM structures in mycorrhizal plants. AM root length colonization in lettuce at 15, 30, 50 and 80 d was 15 ± 1, 31 ± 2, 67 ± 2 and 73 ± 3 and 8 ± 1, 57 ± 1, 78 ± 2 and 79 ± 2% for onion plants respectively. AM root length colonization in both lettuce and onion plants showed the usual logarithmic growth pattern from 15 d after transplanting until 80 d.

Table 1. Germination and hyphal length of *Glomus mosseae* spores in the presence of different concentrations of xyloglucan

Xyloglucan concentration (mg ml^{-1})	Spore germination (%)	Hyphal length (mm)
0	67·5bc	4·6b
0·001	85·9c	11·8d
0·01	79·9bc	9·2cd
0·05	81·1c	6·2bc
0·2	56·1b	2·2b
0·6	51·4b	2·2b
1·0	3·3a	0·2a

Each value is the mean of five pots. Column values followed by the same letter are not significantly different according to Duncan's multiple range test ($P = 0·05$).

Table 2. Endoxyloglucanase activity of mycorrhizal (+M) and nonmycorrhizal (−M) lettuce and onion plants

	Treatment	Specific activities (U mg^{-1} protein) after (d) 7	15	30	50	80
Lettuce	−M	125a	307b	530d	760f	847g
	+M	423c	533d	690e	894h	952i
Onion	−M	80a	138b	156c	303f	493i
	+M	357g	251d	273e	418h	850j

Each value is the mean of three replicates. For each plant, values sharing the same letter were not significantly different according to Duncan's multiple range test ($P = 0·05$).

Table 3. Exoxyloglucanase activity of mycorrhizal (+M) and nonmycorrhizal (−M) lettuce and onion plants

	Treatment	Specific activities (U mg^{-1} protein) after (d) 7	15	30	50	80
Lettuce	−M	4·1a	4·2a	5·3a	4·1a	4·7a
	+M	5·5a	5·8ab	13·2c	17·2d	6·3b
Onion	−M	2·1a	3·2a	5·5b	5·7b	4·5b
	+M	3·6a	3·9a	9·9c	12·6d	5·5b

Each value is the mean of three replicates. For each plant, values sharing the same letter were not significantly different according to Duncan's multiple range test ($P = 0·05$).

Table 4. Endo- and exoxyloglucanase activity in external mycelia of *Glomus mosseae* isolated from lettuce and onion plants

	Plant	Specific activities (U mg^{-1} protein) after (d) 15	30	50	80
Endoxyloglucanase	Lettuce	82a	158b	169b	410c
	Onion	78a	82a	170b	506d
Exoxyloglucanase	Lettuce	3·6b	7·8c	3·4b	3·8b
	Onion	2·1a	5·4b	2·3a	2·4a

Each value is the mean of three replicates. Within data for endo- and exoxyloglucanase, values sharing the same letter were significantly different according to Duncan's multiple range test ($P = 0·05$).

The levels of endoxyloglucanase activities in control and mycorrhizal plants increased with time (Table 2). There were differences in endoxyloglucanase activity between extracts from non-mycorrhizal plants and plants colonized with *G. mosseae* throughout the assay, but the greatest differences in activity were seen at 7 d. Endoxyloglucanase activity in lettuce was higher than onion plants.

Exoxyloglucanase activity was significantly higher in mycorrhizal lettuce and onion plants in comparison to controls at 30 and 50 d (Table 3). Exoxyloglucanase activity in mycorrhizal plants increased at 30 and 50 d, but in non-mycorrhizal plants this activity remained almost constant.

The external mycelia of *G. mosseae* from lettuce and onion also showed endo- and exoxyloglucanase activities (Table 4).

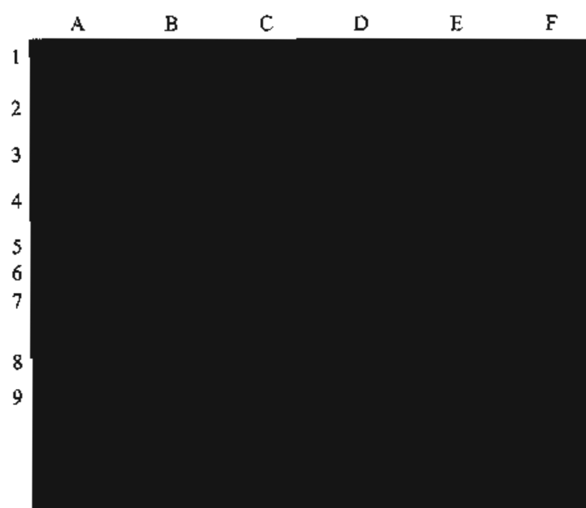

Fig. 1. Non-denaturing PAGE of xyloglucanase. Lane A; extracts from 15-d-old non-mycorrhizal lettuce roots; lane B; extracts from 15-d-old mycorrhizal lettuce roots; lane C; extracts from external mycelium of *Glomus mosseae* isolated from lettuce plants; lane D; extracts from 15-d-old non-mycorrhizal onion roots; lane E; extracts from 15-d-old mycorrhizal onion roots; lane F; extracts from external mycelium of *G. mosseae* isolated from onion plants.

Endoxyloglucanase activity increased throughout the assay, but exoxyloglucanase activity peaked at 30 d.

Several electrophoretic bands of endoxyloglucanase activity were observed in 15-d lettuce and onion root extracts and in external mycelia of *G. mosseae* (Fig. 1). One of the bands found in assays with external mycelia was similar to the one observed in root extracts from mycorrhizal plants, and was not apparent in extracts from non-mycorrhizal lettuce and onion (lane six). There were several bands in non-mycorrhizal plant material (eight and nine for onion; one and two for lettuce) that were absent in mycorrhizal plant samples and in the external mycelia of *G. mosseae*.

DISCUSSION

Increasing evidence of the role of hydrolytic enzymes in the penetration and development of AM fungi in plant roots has been reported (Bonfante-Fasolo & Perotto, 1995). The presence of substrate in an easily accessible form may inhibit enzyme activity due to product accumulation or catabolic repression of enzyme synthesis (Collmer & Keen, 1986). Our results also indicate, however, that the inhibition of mycorrhization caused by high concentrations of xyloglucan in the culture medium may be due to reduced germination and hyphal length of *G. mosseae* spores. Specific oligosaccharides from xyloglucan degradation may also have an inhibitory effect on cell expansion (Fry *et al.*, 1993). Interestingly, low concentrations of xyloglucans in the culture medium increased percentage mycorrhization and spore germination of *G. mosseae*. The low quantity of oligosugars solubilized after glycosidic linkage breakdown may represent a positive stimulus to fungal development (Bonfante-Fasolo & Perotto, 1990), suggesting that the fungus might use sugars as a food source (Dexheimer, Gianinazzi & Gianinazzi-Pearson, 1979; Peretto *et al.*, 1995).

Xyloglucanase enzymes are present in mycorrhizal and non-mycorrhizal roots (Rejón-Palomares *et al.*, 1996). We found that endoxyloglucanase activity increased during growth and development of root. This activity was consistently higher at the beginning of colonization and the logarithmic stage of development of mycorrhizal fungus. The increase in fungal structures which penetrate the cell wall during the logarithmic stage of root colonization may explain the increase in the different activities at this time (Schubert & Wyss, 1995). This does not, however, account for the different rates at which exoxyloglucanase activity increased during AM colonization. We show here that *G. mosseae* is able to produce endoxyloglucanases. As with cellulases and pectinases, however, this activity was extremely low, as might be expected in a mutualistic interaction. The low activity observed in these hydrolytic enzymes make difficult to demonstrate a close relationship between xyloglucanase activities and development of the fungus inside the root (García-Romera, García-Garrido & Ocampo, 1991*a*; García-Garrido, García-Romera & Ocampo, 1992*b*).

The evolution of endoxyloglucanases activities in plants paralleled the changes in the external mycelium. There were, however, bands of xyloglucanase activity in non-mycorrhizal roots which were absent in mycorrhizal roots, that may suggest qualitative inhibition by the fungus of some plant activity. Inhibition of plant protein synthesis by AM fungi has been observed in several plant-AM fungi associations (García-Garrido, Toro & Ocampo, 1993; Dumas-Gaulot *et al.*, 1994).

Several authors have discussed the possibility that hydrolytic enzymes produced by either the plant or the symbiont may be implicated in the process of host wall degradation and cell wall mobilization. Some of the endoxyloglucanase activity can be attributed to the extramatrical phase of the AM fungus, as endoxyloglucanase activity found in the external mycelium and in the mycorrhizal root extracts showed the same electrophoretic mobility. The higher activity shown by mycorrhizal plants may also be the result of an increased plant activity. Colonization of roots by AM fungi can result in significant alterations of plant root system morphology as increases in branching and lengths of individual roots are not entirely due to improved host plant nutrition (Berta *et al.*, 1990; Atkinson *et al.*, 1991; Hooker *et al.*, 1992; Hooker & Atkinson, 1996).

The sequence of endoxyloglucanase activities observed in the AM association suggests that AM fungal xyloglucanases may be involved in the development of mycorrhizal colonization of plant roots but further research is needed to ascertain the role of the higher xyloglucanase activity in mycorrhizal roots.

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