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Presence of Xyloglucan-Hydrolyzing Glucanases (Xylogucanases) in Arbuscular Mycorrhizal Symbiosis

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Abstract

We studied the production of xyloglucan-hydrolyzing glucanases (xyloglucanases) in roots of lettuce (*Lactuca sativa*), onion (*Allium cepa*) and soybean (*Glycine max*) colonized by the arbuscular mycorrhizal (AM) fungus *Glomus mosseae*. Endoxyloglucanase activity in mycorrhizal roots extracted with 100 mM Tris-HClH (pH 7) was higher than in nonmycorrhizal root extracts. There were no significant differences in endoxyloglucanase activity between mycorrhizal and nonmycorrhizal roots when they were extracted with 200 mM Na_2PO_4 (pH 7.2), 100 mM NaCl, 100 mM potassium-phosphate buffer (pH 7.8) or 25 mM MES (Na) (pH 6.6). From the results obtained the most suitable extraction buffer for endoxyloglucanase activity in lettuce plants was 100 mM Tris-HCl (pH 7). Endoxyloglucanase activity was greatest when the reaction was carried out at pH 5 or 8, and activity declined at pH 3, 4, 6, 7 and 9. Maximum endoxyloglucanase activity was observed in a range of temperatures between 37°C and 50°C. Tris extracts of mycorrhizal plants showed more endo- and exoxyloglucanase activity than nonmycorrhizal plants when nasturtium or tamarind xyloglucan was used as the substrate. Extracts from spores and external mycelia of *G. mosseae* also showed endo- and exoxyloglucanase activity. The possible participation of xyloglucanase activity in the colonization of plant roots by AM fungus is discussed.

Keywords: *Allium cepa*, *Glycine max*, *Lactuca sativa*, arbuscular mycorrhiza, *Glomus mosseae*, xyloglucanase

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1. Introduction

Xyloglucan, the major structural hemicellulose in the primary cell walls of dicotyledons and nongraminaceous monocotyledons, also occurs in the primary cell walls of the *Gramineae*, and is a major cell-wall storage component in many dicotyledonous seeds (Reid, 1985). Xyloglucans are characterized by a β (1-4)-glucan with side chains attached at the 6-O position of the glucosyl residues. Xyloglucan chains can hydrogen-bond to cellulosic microfibrils, and may cross-link them, restraining cell expansion. In addition to its structural role, xyloglucan can be hydrolyzed by plant or fungal hydrolytic enzymes, and the oligosaccharides produced may act as a source of signalling molecules (Fry, 1989; Hayashi, 1989).

Plant cell walls contain a set of glucanases and glycosidases that hydrolyze xyloglucan into monosaccharides. Endo-1,4-β-glucanase activity is responsible for the first step of degradation whereby the xyloglucan is endohydrolyzed into large fragments (Fry, 1995). Tomato fruit contains at least three nonspecific carboxymethylcellulases that can also hydrolyze xyloglucan (Maclachlan and Brady, 1992). However, these are not the only enzymes that can cleave xyloglucan: ripening tomato fruit also contains specific xyloglucan-depolymerizing activity (referred to here as xyloglucanase), which reduces the viscosity of the xyloglucan solution. Another xyloglucan-cleaving enzyme has been described in extracts of pea epicotyls and other growing tissues (Fry et al., 1992), as well as in nasturtium cotyledons (Farkas et al., 1992; Fanutti et al., 1993).

Several reports have noted the importance of hydrolytic enzymes in the penetration of the plant cell wall by many microorganisms. The production of hydrolytic enzymes has been observed not only in parasites but also in mutualistic microorganisms such as *Rhizobium* (Mateos et al., 1992) and arbuscular mycorrhiza (AM) (García-Romera et al., 1990). There is evidence that hydrolytic enzymes, including hemicellulases, are involved in the colonization of roots by the fungi (García-Romera et al., 1990). To date, no studies have investigated xyloglucan hydrolytic enzymes in roots and the possible role of these xyloglucanase enzymes in AM infection. Because the production of other hydrolytic enzymes through AM symbiosis is extremely low (García-Romera et al., 1991), various extraction solutions, pH and temperatures were assayed. The aim of this work was to study the presence of xyloglucanases in AM fungal structures, spores and external mycelia, and in plants colonized by *Glomus mosseae*.

2. Materials and Methods

Fungi and plants

Inoculum of the AM fungus *G. mosseae* (Nicol. & Gerd.) Gerd. & Trappe was obtained by inoculating *Medicago sativa* cv. Aragon plants with surface-sterilized spores of the fungus (McAllister et al., 1994), and growing the inoculated plants in the same soil under the conditions described below. After 6 months growth, soil containing fungal sporocarps and mycorrhizal roots were used as inoculum.

Lettuce (*Lactuca sativa*, cv Romana), onion (*Allium cepa*, cv Babosa), and soybean plants (*Glycine max* L, cv) were obtained from surface-sterilized seeds (5 min in 0.75% NaClO). Seeds were sown in moistened sand, and after 2 weeks seedlings were transplanted to pots. Plants were inoculated with 10 g inoculum, and uninoculated plants were given filtered leachate from the inoculum soil. Plants were grown in 300-ml capacity open pots of soil collected from the Province of Granada, Spain. The soil, a "reddish-brown" type, pH 7.6 (for full detail see García-Romera and Ocampo, 1988) was steam-sterilized and mixed with sterilized sand at a proportion of 2:3 (v:v).

Pots kept in a controlled-climate glasshouse, were regularly watered and given 10 ml per week Hewitt's nutrient solution (Hewitt, 1952) lacking phosphate, for AM-inoculated plants. Natural light was supplemented by Sylvania incansdescent and cool-white lamps, 400 nmol/m^2/s, 400–700 nm; with a 16-8 light-dark cycle at 25–19°C and 50% r.h.

Plants were harvested after 30 days. 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 which was colonized by AM fungi was measured as described by Giovannetti and Mosse (1980).

External mycelia were isolated from roots of 30-day-old onion colonized with *G. mosseae*. The roots were washed and rinsed gently with sterilized water and the external mycelium was collected, using forceps, under a dissecting microscope. Spores of *G. mosseae* were obtained by dissecting sporocarps, which were recovered from wet-sieved soil (Gerdemann and Nicolson, 1963), stored in water at 4°C and used within one month. Before enzymatic assay, spores were surface-sterilized (McAllister et al., 1994).

Preparation of extracts for enzyme assays

Roots (10 g) were pulverized in a mortar under liquid nitrogen. The resulting power was homogenized in 30 ml of the following solutions: 100 mM Tris-HCl

buffer (pH 7) plus 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; 100 mM potassium-phosphate buffer (PK) (pH 7.8) plus 15 mM NaCl, 10 mM PMSF, 3 mM KCl and 26 mM β-mercaptoethanol; 25 mM Mes buffer (Na) (pH 6.6) plus 10 mM ascorbic acid and 10 mM $CaCl_2$; 100 mM NaCl and 200 mM Na_2PHO_4 (pH 7.2). Polyvinyl-polypyrrolidone (PVPP) (15%) was added to the above extractant solutions. The ground material was also suspended in 20 mM sodium-phosphate buffer plus 6% PVPP. The liquid was filtered through several layers of cheesecloth. The extract was centrifuged at 20,000 g for 20 min. The supernatant was dialyzed against several hundred volumes of the same diluted extractant solution (1:9, v:v) for 4 h at 4°C. The samples were then frozen until use. Sodium azide (0.03%) was added to all solutions to prevent microbial growth.

External mycelia were frozen in liquid nitrogen and finely pulverized in a mortar. The resulting powder was suspended (30 mg/ml) in 100 mM Tris-HCl buffer (pH 7). The suspension was briefly sonicated (1 min, 5 times at 80 W) and centrifuged at 20,000 g for 20 min, and the pellet resuspended and sonicated again, and washed by centrifugation with the same buffer three times. The supernatant was concentrated by ultrafiltration through PM-10 membranes (AMICON Co.), and used as a crude enzyme extract.

Enzyme assays

Xyloglucan hydrolytic enzymes were detected with a modified agar plate assay (García-Romera et al., 1990). Agarose (1%) was amended with 0.2% substrate. Xyloglucan from nasturtium seed (N) (*Tropaeolum majus* L.) and tamarind (T) (*Tamarindus indica*) extracted as described by McDougall and Fry (1989) were used to detect xyloglucanase activity. One hundred microliter of each enzymatic solution was plated on the surface of the agarose. Autoclaved enzyme extract and buffer were used as controls. Plates were kept at 28°C for 18 h, after which the unhydrolyzed xyloglucan was stained by flooding plates with 0.1% Congo Red for 20 min followed by bleaching with 1M NaCl (Wood, 1981).

Xyloglucan hydrolyzing endoglucanase (endoxyloglucanase) activity was assayed by the viscosity method, using xyloglucan N and T as substrates. The reduction in viscosity was determined at 0–90 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 prot) (U:

reciprocal of time in h for 50% viscosity loss × 10^3) (García-Romera et al., 1991c).

Exoxyloglucanase was quantified by measuring the reducing sugars with a 2,2'-bicinchoninate reagent (BCA) (Waffenschmidt and Jaenicke, 1987). Reaction mixtures at 40°C contained 400 µl of 0.5% substrate in 50 mM citrate-phosphate buffer (pH 5), 25 µl of the enzyme sample diluted to 400 µl with H_2O, and 800 µl of 200 mM potassium phosphate-citric acid buffer (PCA; pH 5). Product formation was measured as described by Mateos et al., 1992. A standard curve for reducing sugars was prepared with glucose in the range of 0–20 nmol. One enzyme unit was defined as the amount of enzyme releasing 1 nmol of reducing sugar equivalent per min at 40°C and pH 5.

The pH dependence and optimum temperature of xyloglucanase activity were assayed by the viscosity reducing method described previously. The substrate (xyloglucan N) was prepared with a different buffer depending on the pH to be assayed: 200 mM PCA buffer for pH 3, 4, 5, 6 and 7, and 200 mM Tris-HCl buffer for pH 8 and 9. To determine the optimum temperature of xyloglucanase activity the substrate was prepared with 200 mM PCA buffer at pH 5, and a range of temperatures was tested (25, 30, 37, 45, 50 and 65°C).

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

3. Results

Microscopic observations of stained roots showed no fungi in uninoculated controls and only AM structures in inoculated plants. In lettuce, onion and soybean plants inoculated with *G. mosseae*, 62±4%, 51±6% and 35±3% of the root length showed AM colonization. Analysis of xyloglucanase activity revealed that the extracts of *G. mosseae* spores and mycelium produced zones of hydrolysis in agar plates with xyloglucan N and T.

Endoxyloglucanase activity from crude extracts of lettuce plants prepared with all the solutions used in our experiments were similar (Table 1). When root extracts were prepared with Tris-HCl buffer, mycorrhizal roots contained more endoxyloglucanase activity than nonmycorrhizal roots. Because of the preceding results, Tris buffer was used as the extractant in all subsequent experiment. However, there were no significant differences in endoxyloglucanase activity between mycorrhizal and nonmycorrhizal roots when they were extracted with 200 mM Na_2PHO_4 (pH 7.2), 100 mM NaCl, 100 mM PK (pH 7.8), or 25 mM MES (Na) (pH 6.6). The substrate used did not influence the differences of activity between extracts from mycorrhizal and nonmycorrhizal roots.

Table 1. Endoxyloglucanase activity in mycorrhizal (+M) and nonmycorrhizal roots (–M) of lettuce plants extracted with different buffers and using nasturtium (N) and tamarind (T) xyloglucan as substrate

Buffer composition	Substrate	Specific activities (U/mg prot)	
		–M	+M
100 mM Tris-HCl (pH 7)	N	161 d	288 b
	T	108 e	536 a
200 mM Na_2PO_4 (pH 7.2)	N	198 cd	210 c
	T	196 cd	223 c
100 mM NaCl	N	268 b	283 b
	T	191 cd	207 c
100 mM PK (pH 7.8)	N	162 d	170 d
	T	150 d	173 d
25 mM Mes (Na) (pH 6.6)	N	90 f	106 ef
	T	128 e	138 de

Each value is the mean of three replicates. Values sharing the same letter were not significantly different according to Duncan's multiple range test (P=0.05).

Tris-extracted endoxyloglucanase activity in lettuce had two maxima at pH 5 and 8, and declined at pH 3, 4, 6, 7 and 9 (Fig. 1). In Tris-extracts of mycorrhizal roots, endoxyloglucanase activity was always higher than in nonmycorrhizal roots at all pH values tested.

When endoxyloglucanase activity was measured as a function of the temperature of the reaction mixture, higher activity was found between 37°C and 50°C (Fig. 2). The lowest endoxyloglucanase activity was seen when reactions were carried out at 25°C and 65°C. In lettuce plants at these temperatures, endoxyloglucanase activity was higher in mycorrhizal than nonmycorrhizal roots.

With both xyloglucans the level of Tris-extracted endoxyloglucanase activity was always higher in mycorrhizal than in nonmycorrhizal roots of all three species (Table 2). With each substrate lettuce plants had higher endoxyloglucanase activity than onion or soybean roots. Values of endoxyloglucanase activity were variable when the substrate used in the reaction mixture was xyloglucan T or N. Exoxyloglucanase activity was similar to endoxyloglucanase activity: mycorrhizal plants had higher activity than control onion, lettuce or soybean plants with xyloglucan N and T (Table 3).

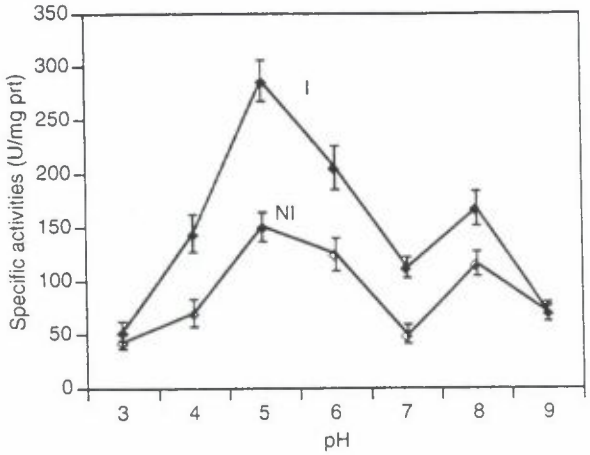

Figure 1. Effect of pH on endoxyloglucanase activity extracted with Tris buffer from mycorrhizal and non-mycorrhizal lettuce roots. I = Plant inoculated with mycorrhiza, NI = Noninoculated plant. Bars show standard errors of the mean.

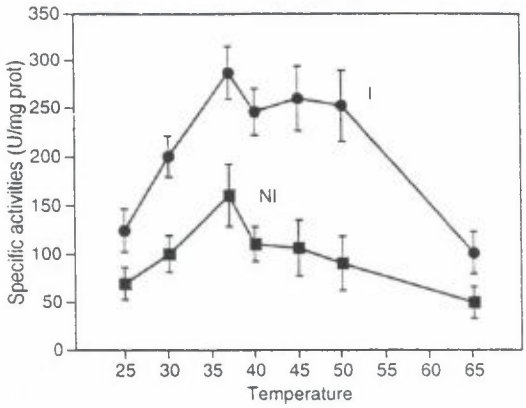

Figure 2. Effect of temperature on endoxyloglucanase activity extracted with Tris buffer from mycorrhizal and nonmycorrhizal lettuce roots. I = Plant inoculated with mycorrhiza, NI = Noninoculated plant. Bars show standard errors of the mean.

The spores and external mycelia of *G. mosseae* also had endo- and exoxyloglucanase activity (Table 4). Enzymes activities were significantly higher in external mycelia than in spores. Lower endo- and exoxyloglucanase activities were found in external mycelia and spores with xyloglucan T than with xyloglucan N.

Table 2. Endoxyloglucanase activity in Tris-extracts of mycorrhizal (+M) and nonmycorrhizal roots (–M) of lettuce, onion and soybean plants using nasturtium (N) and tamarind xyloglucan (T) as substrate

Plant	Substrate	Specific activities (U/mg prot)	
		–M	+M
Lettuce	N	161 d	288 b
	T	108 e	536 a
Onion	N	112 e	160 d
	T	158 d	255 bc
Soybean	N	140 de	199 cd
	T	153 d	214 c

Each value is the mean of three replicates. Values sharing the same letter were not significantly different according to Duncan's multiple range test (P=0.05).

Table 3. Exoxyloglucanase activity in Tris-extracts of mycorrhizal (+M) and nonmycorrhizal roots (–M) of lettuce, onion and soybean plants using nasturtium (N) and tamarind xyloglucan (T) as substrate

Plant	Substrate	Specific activities (U/mg prot)	
		–M	+M
Lettuce	N	6.6 b	20 a
	T	0.2 e	1.4 d
Onion	N	3.9 c	6.0 b
	T	1.8 d	3.2 c
Soybean	N	1.3 d	5.5 b
	T	1.4 d	3.4 c

Each number is the mean of three replicates. Values sharing the same letter were not significantly different according to Duncan's multiple range test (P=0.05).

4. Discussion

The mechanisms by which endomycorrhizal fungi enter and spread through plant host tissues are unknown. Different steps in the infection process

Table 4. Endo- and exoxyloglucanase activity in spores and external mycelia of *Glomus mosseae* using nasturtium (N) and tamarind (T) xyloglucan as substrate

Xyloglucanase	Substrate	Specific activities (U/mg prot)	
		Spores	External mycelium
Endoxyloglucanase	N	63 b	110 a
	T	10 d	21 c
Exoxyloglucanase	N	20 b	32 a
	T	8 c	24 b

Each value is the mean of three replicates. Within data for endo- and exoxyloglucanase, values sharing the same letter were not significantly different according to Duncan's multiple range test (P=0.05).

(formation of entry points, inter- and intracellular colonization) necessitate the growth of hyphae along the middle lamella or through cell walls of the host root. Only localized changes in wall texture have been observed as endomycorrhizal fungi penetrate epidermal cells or develop through the middle lamella of parenchymal tissue, suggesting that wall degrading enzyme activities within host tissues are very limited (Gianinazzi-Pearson et al., 1981; Bonfante-Fasolo and Gianinazzi-Pearson, 1982; Jacquelinet-Jeanmougin et al., 1987). Biochemical studies have shown that endomycorrhizal fungi do possess cellulolytic and pectolytic activities, and that these are low when compared to pathogenic fungi (Pearson and Read, 1975; Cervone et al., 1988; García-Romera et al., 1991a; García-Garrido et al., 1992b). The occurrence of peaks of pectinase activity during AM infection (García-Romera et al., 1991b), the immunolocalization of an endopolygalacturonase on the wall of external hyphae (Bonfante-Fasolo and Perotto, 1992), and the detection of a fungal cellulase isozyme in AM root extracts (García-Garrido et al., 1992a) all provide further evidence for the possible involvement of fungal hydrolytic enzymes in the process of root colonization.

To detect and measure hydrolytic enzymes from plant symbiotic microorganisms, the extraction procedures, pH and temperatures used during the enzymatic reaction are important. The type of extraction solution used influences enzyme recovery (Morales et al., 1984). The most suitable extractants for hydrolytic enzymes in mycorrhizal associations were found to be 250 mM NaCl for pectin esterase, 50 mM citrate-phosphate buffer (pH 7) for other pectinases, and Tris-HCl for cellulases (García-Romera et al., 1991c; García-

Garrido et al., 1992b). From the results obtained the most suitable extraction buffer for xyloglucanase was Tris-HCl. The differences in enzyme extractability between the solutions we used may have been caused by the different localization or properties of binding to the cell wall. Some pectolytic enzymes produced in colonized tissues have been reported to be inactivated by phenol oxidation during extraction (Dazzo and Hubbell, 1974). The insoluble complexes which PVPP forms with phenolic compounds by hydrogen bonding may have been responsible for our success in extracting xyloglucanase enzymes from roots.

Most hydrolytic enzymes from higher plants differ from those of microbial origin in optimum pH: the optimum pH for plant hydrolytic enzymes is around 7, whereas that for microbial hydrolytic enzymes is in the acid range (Rexová-Benková and Markovic, 1976; Jiménez-Zurdo, 1993). Mycorrhizal lettuce roots possess xyloglucanase activity with an optimum at pH 5 which could indicate that this activity is mainly of fungal origin. Another peak in the alkaline region was also observed, suggesting xyloglucanase activity of the plant origin.

The optimum temperature of endoxyloglucanase activity in onion roots ranged between 37°C and 50°C. A similar situation was reported for cellulase and pectinase activities in *Rhizobium* (Jiménez-Zurdo, 1993).

Our results show that endo- and exoxyloglucanase activity was consistently higher in inoculated than in control plants of different species inoculated with *G. mosseae* when either xyloglucan N and T was used as the substrate. Studies in other plant species have also reported higher activity in mycorrhizal plants than in controls for other hydrolytic enzymes such as pectinases and cellulases (García-Romera et al., 1991a; García-Garrido et al., 1992c).

Endoxyloglucanase seems to be the main enzyme necessary for the initial breakdown of xyloglucan molecules, a step that has an immediate impact on their molecular weight. Tamarind seed xyloglucan (Kooiman, 1961) has a structure similar to the nasturtium seed polysaccharide (Le Dizet, 1972); however, of the two enzymes studied, the different plants showed different activities with tamarind and nasturtium xyloglucan. This difference in activity may be attributed to the different accessibilities of the substrates.

The production of xyloglucanase enzymes by *G. mosseae* spores and external mycelia and the fact that mycorrhizal roots possess more xyloglucanase than nonmycorrhizal roots, suggest that these xyloglucanases are involved in the process of colonization. A detailed study under way in our laboratory will shed further light on the role of xyloglucanase in the mycorrhization of roots.

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