ENDOGLUCANASE ACTIVITY IN LETTUCE PLANTS COLONIZED WITH THE VESICULAR-ARBUSCULAR MYCORRHIZAL FUNGUS *GLOMUS FASCICULATUM*

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Summary—We studied the production of endoglucanase (EC 3.2.1.4) enzymes during the process of penetration and development of the vesicular-arbuscular (VA) mycorrhizal fungus *Glomus fasciculatum* in roots of lettuce (*Lactuca sativa*). Mycorrhizal plants showed more endoglucanase activity than non-mycorrhizal plants. Endoglucanse activity in VA-colonized plants increased at the beginning of the logarithmic stage of fungal development, and subsequently declined. The extracts from external mycelia of *G. fasciculatum* showed endoglucanase activity. Some of the endoglucanase activities detected in VA-colonized plant roots can be attributed to the VA fungus, since some of the endoglucanase proteins found in the external mycelia of *G. fasciculatum* and in mycorrhizal root extracts showed the same electrophoretic mobility. However, some of the endoglucanase activities from extracts of mycorrhizal plants had different electrophoretic mobilities than those observed in the external mycelia and in non-mycorrhizal plants.

These results suggest that endoglucanases may be involved in the process of colonization of lettuce roots by *G. fasciculatum*.

INTRODUCTION

The establishment of vesicular-arbuscular (VA) mycorrhizal symbiosis requires penetration of the host cell by fungi (Bonfante-Fasolo, 1984; Bonfante-Fasolo *et al.*, 1990), but the mechanisms by which VA endophytes enter host tissues are still unknown. Cellulolytic enzymes, especially endoglucanases, play a fundamental role in the penetration of phytopathogenic and mutualistic microorganisms into plant cells (Coughlan and Ljungdahl, 1988; Morale *et al.*, 1984). However, research on these enzymes in plant roots, and on their mode of action in the process of penetration and development of VA-mycorrhizal fungi, is scarce (García-Romera *et al.*, 1990). Although extracts of *Glomus mosseae* spores reportedly contain cellulolytic (endoglucanases) enzymes (García-Romera *et al.*, 1990), detection of endoglucanase enzymes in VA mycorrhizal fungi is made difficult by the low levels of enzyme production (García-Romera *et al.*, 1990); attempts to culture VA fungi in the absence of plant roots have so far been unsuccessful (Harley and Smith, 1983).

The aim of this work was to determine whether endoglucanases participate in the colonization of lettuce roots by *Glomus fasciculatum*.

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MATERIALS AND METHODS

Biological material and growth conditions

Plants were grown in 300 ml capacity open pots of soil collected from the Province of Granada, Spain. The soil of "reddish-brown" type, pH 7.6 (for full details see García-Romera and Ocampo, 1988), was steam-sterilized and mixed with sterilized quartz sand at a proportion of 1:1 (V:V). Lettuce (*Lactuca sativa* cv. Romana) seeds were sown in moistened sand, and after 2 weeks seedlings were transplanted to the pots and grown under greenhouse conditions. Natural light was supplemented by Silvania incandescent and cool-white lamps, 400 nmol $m^{-2} s^{-1}$, 400–700 nm; with a 16–8 h light–dark cycle at 25–19°C and 50% r.h. Plants were watered from below using a capillary system, and fed with a nutrient solution (Hewitt, 1952) lacking phosphate for VA-inoculated plants.

The VA inoculum consisted of 5 g of rhizosphere soil from a maize plant pot culture of an isolate of *G. fasciculatum* which contained spores, mycelia and colonized root fragments. Uninoculated plants were given filtered leachings from the inoculum soil. The VA uninoculated (control) treatment consisted of soil filtrate (Whatman No. 1 filter paper) from the rhizosphere of mycorrhizal plants containing common soil microorganisms, but no propagules of *G. fasciculatum*.

Table 1. Percentage VA root length colonization, entry points, vesicles and arbuscules in lettuce plants inoculated with *G. fasciculatum*

	Days after inoculation			
	15	30	50	80
Root length colonization (%)	6.9a	20.1b	45.1c	42.4c
No. of vesicles cm^{-1} of root	2a	9.5b	57.8c	38.8d
No. of entry points cm^{-1} of root	1.5b	1.8b	3.6c	0.4a
Arbuscules (%)	1a	8.7b	26.8c	10.1b

Row values sharing the same letter were not significantly different according to Duncan's multiple range test ($P = 0.05$).

Plants were harvested after 15, 30, 50 or 80 days. The root system was washed and rinsed three times with sterilized distilled water. The root from each of the five replicate group of pots was divided to record (i) VA mycorrhizal colonization (2 g fresh wt) and (ii) endoglucanase activity (20 g fresh wt).

External mycelia were isolated from roots of 8 week old lettuce colonized with *G. fasciculatum*. The roots were washed and rinsed gently with sterilized water, and the external mycelia collected with forceps under a dissecting microscope.

Mycorrhizal measurements

The roots were cleared and stained (Phillips and Hayman, 1970), cut in 1 cm segments that were mixed and repeatedly subdivided to yield random samples of 40 root segments $replicate^{-1}$, which were mounted on slides and examined under a compound microscope at ×160 magnification. The percentage of total root length which was colonized with VA mycorrhiza, percentage of arbuscules, number of entry points, and vesicles were measured as described by Ocampo *et al.* (1980).

Preparation of extracts for enzyme assays

Roots were frozen in liquid N_2 and finely pulverized in a mortar. The resulting powder was homogenized in 40 ml of 0.1 M Tris–HCl buffer (pH 7) plus 13 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, centrifuged at 20,000 *g* for 15 min, and the pellet resuspended and washed by centrifugation with the same buffer three times. The supernatant was treated with ammonium sulphate up to 80% of full saturation. The solution was kept for 5 h at 4°C and centrifuged once more as described above. The supernatant was discarded, and the sediment was dissolved in a small volume of the same extractant solution and dialysed against several hundred volumes of the same diluted extractant solutions (1:9, V:V) for 16 h at 4°C. The samples were kept frozen until use (García-Garrido *et al.*, unpublished results).

External mycelia were frozen in liquid N_2 and finely pulverized in a mortar. The resulting powder was suspended (30 mg m^{-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 15 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

Endoglucanase activity (EC 3.2.1.4) from the extracts of external mycelia of *G. fasciculatum* was detected with the agar plate method (García-Romera *et al.*, 1990). Samples were incubated in agar (1%) plus 0.1% carboxymethylcellulose (CMC) for 16 h at 30°C. Halos of enzymatic activity were revealed by flooding plates with 0.1% (W/V) Congo Red for 15 min, followed by bleaching with 1 M NaCl (Wood, 1980).

Endoglucanase activity was measured by the viscosity reducing method, with CMC as the substrate. Viscosity reduction was determined in a Cannon–Fenske viscosimeter (5354/2) at 37°C. 6 ml of reaction mixture contained 5 ml 0.5% enzyme substrate in 50 mM citrate phosphate buffer (pH 5) and 1 ml enzyme. One unit of enzyme activity was expressed on a relative activity (RA) basis as the reciprocal of time in min for 50% viscosity loss ×1000 (Bateman, 1963).

Controls for all enzyme assays consisted of autoclaved enzyme extracts and buffers, and 0.03% of sodium azide was added to reaction mixtures.

Polyacrylamide gel electrophoresis

Non-denaturing linear-gradient electrophoresis of cellulolytic enzymes in polyacrylamide gels (4–12%) amended with 0.1% CMC in 50 mM Tris–0.1 M glycine buffer (pH 8.8), was based upon the method described for pectinases (Cruickshank and Wade, 1980).

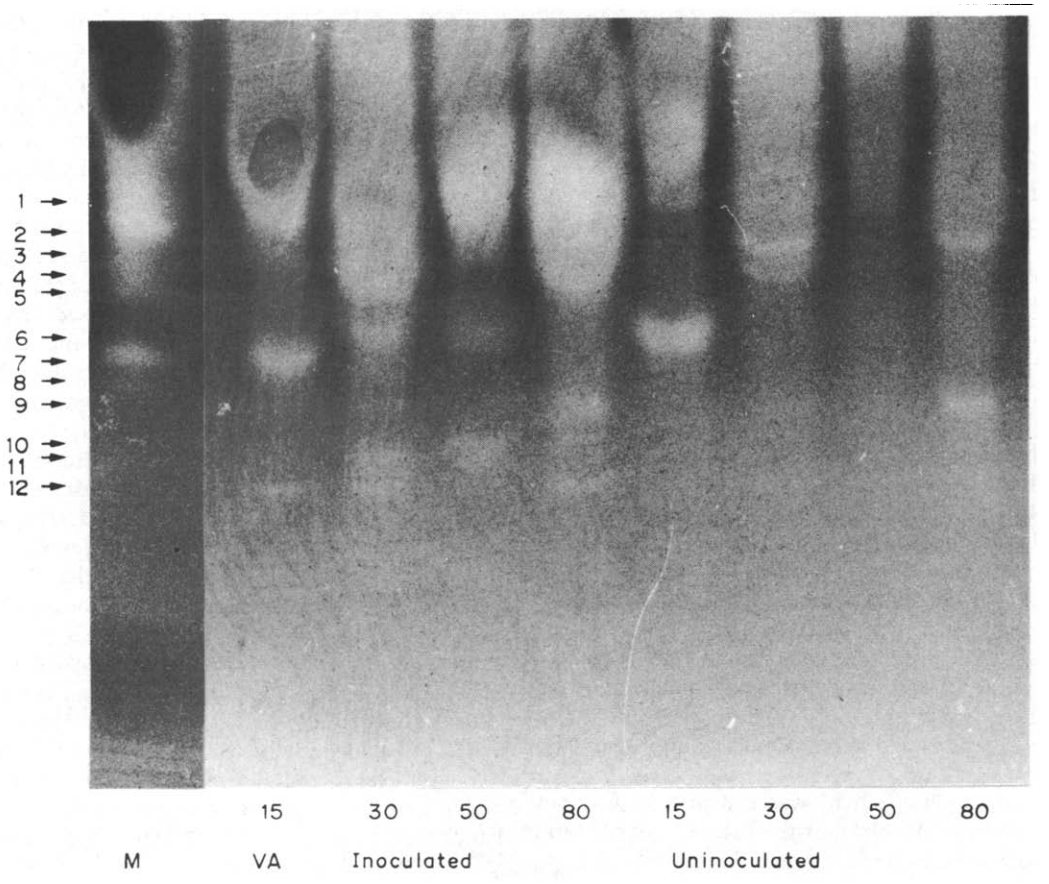
Gels 16 × 18 cm × 25 mm thick were prepared using a gradient gel former (LKB). The electrode tank contained the same Tris–glycine buffer (pH 8.8) as was used in the gel. After pre-electrophoresis of the gel for 30 min, the wells were filled with 75 μl of enzyme samples, and 1 μl 0.05% bromophenol blue in gel buffer was applied to the cathodic side. Electrophoresis was subsequently carried out at 4°C with a constant current of 20 mA gel^{-1} for 7 h.

The gels were immersed in 100 ml of 50 mM citrate–phosphate buffer (pH 5) at 37°C for 15 h, after which

Table 2. Endoglucanase activity in lettuce roots uninoculated and inoculated with *G. fasciculatum*

	Days after inoculation			
Treatments	15	30	50	80
	Units mg^{-1} protein			
Uninoculated	6.1a	6.3a	4.8a	6.4a
VA incoulated	13.2b	24.8c	10.4b	11.6b

Values sharing the same letter were not significantly different according to Duncan's multiple range test ($P = 0.05$).



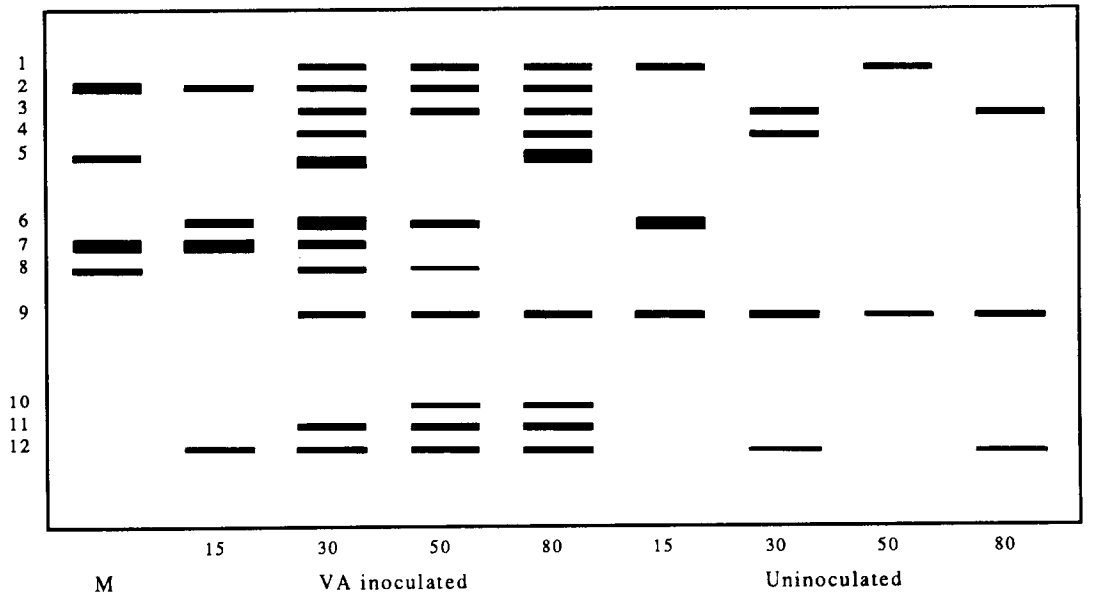


Fig. 1. Bands of endoglucanase activities (lines 1–12) in non-denaturing polyacrylamide gradient gel electrophoresis on 4–12% acrylamide. M: extracts from external mycelium of *G. fasciculatum*; VA Inoculated: extracts from 15, 30, 50 and 80 day old VA-colonized roots; uninoculated: extracts from 15, 30, 50 and 80 day old non-VA-colonized roots. The gel was stained with Congo Red and destained as described in Materials and Methods.

they were stained with 0.1% Congo Red for 30 min. This was followed by washing in 1 M NaCl until the bands became visible.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin (Sigma) as the standard.

Statistical treatment

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

RESULTS

The extracts of external mycelia of *G. fasciculatum* produced zones of hydrolysis in agar plates with CMC.

Microscopic observations of stained roots showed no presence of fungi in uninoculated controls (data not shown). As Table 1 shows, a typical VA colonization curve was observed in lettuce plants; 15 days after transplanting, VA colonization increased, with a peak at 50 days, at which time colonization had reached maximum levels. The numbers of entry points, vesicles and arbuscule development reached their maxima at 50 days.

Endoglucanase activity was evident in lettuce root after 15 days of plant growth (Table 2). Mycorrhizal lettuce plants showed more endoglucanase activity than non-mycorrhizal plants. The amount of this enzymatic activity in non-mycorrhizal plants was constant throughout the experiment. However, in VA-colonized roots, endoglucanase activity increased at 30 days of plant growth, and declined thereafter.

As Fig. 1 shows, the electrophoretic bands of endoglucanase activity in root extracts differed between mycorrhizal and non-mycorrhizal plants. The extracts from external mycelia of *G. fasciculatum* showed several bands of endoglucanase activity (2, 5, 7 and 8). These bands were similar to those observed in the root extracts from mycorrhizal plants, and were not apparent in extracts from non-mycorrhizal lettuce roots. The number of electrophoretic bands of endoglucanase activity was higher in mycorrhizal than in non-mycorrhizal plants at the same age. VA mycorrhizal plants showed several bands of endoglucanase activity (1, 3, 4, 6, 9 and 12) with similar electrophoretic mobilities to those observed in non-mycorrhizal plants. However, there were also several bands in mycorrhizal plant material (10 and 11) that were absent from non-mycorrhizal plant samples and from the external mycelia of *G. fasciculatum*.

DISCUSSION

The presence of endoglucanase activity in extracts of external mycelia of *G. fasciculatum* and *G. mosseae* spores (García-Romera *et al.*, 1990) indicates that VA mycorrhizal fungi were able to produce this enzyme, as noted in other mutualistic microorganisms (Morales *et al.*, 1984). Moreover, our results showed that endoglucanase activity was higher in VA-colonized plants as compared to non-VA-colonized plants. This enzymatic activity increased in VA-colonized roots when the fungus was in its logarithmic stage of growth. The increase in fungal structures that penetrate the cell wall during the logarithmic stage of root colonization (Hayman, 1983) may explain this increase in activity. Endoglucanase activity in mycorrhizal plants decreased at the end of the assay. VA fungal development can be controlled by the plant (Harley and Smith, 1983; Anderson, 1988), and may decrease the production of endoglucanase by the fungus, as is the case with other enzymes (Spanu and Bonfante-Fasolo, 1988). The sequence of endoglucanase activity observed in the VA mycorrhizal association, and the fact that the external mycelia of *G. fasciculatum* showed endoglucanase activity, suggest that this enzyme may be involved in mycorrhizal colonization of plant roots.

Endoglucanases are present in non-VA-colonized roots (Table 2, Fig. 1) during growth and development (Byrne *et al.*, 1975). Several electrophoretic bands of endoglucanase activity observed in VA-colonized plants had the same mobility as in the non-VA-colonized plants, however, some of these bands were present at earlier stages of plant growth in mycorrhizal plants than in non-mycorrhizal plants. This suggests that the presence of the VA fungus in the plant root activated some of the plant's endoglucanase activities. However, some of the endoglucanase activities found in VA-colonized roots can also be attributed to the VA fungus, since endoglucanase proteins found in the external mycelia and in the mycorrhizal root extracts showed the same electrophoretic mobility. The presence of bands different from those observed in non-mycorrhizal roots or external mycelia suggests that some of this activity may be induced by the fungus in the plant. These results indicate that endoglucanases produced by either the plant or the VA fungus may be implicated in the process of host wall degradation and cell wall mobilization during colonization as has been suggested for other symbiotic associations (Dazzo and Hubbell, 1974; Verma *et al.*, 1982).

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REFERENCES

Anderson A. J. (1988) Mycorrhizae-host specificity and recognition. *Phytopathology* **78,** 375–378.

Bateman D. F. (1963) Pectolytic activities of culture filtrates of *Rhizoctonia solani* and extract of *Rhizoctonia*-infected tissues of bean. *Phytopathology* **53,** 197–204.

Bonfante-Fasolo P. (1984) Anatomy and morphology of VA mycorrhizae. In *VA Mycorrhizas* (C. Ll. Powell and D. J. Bagyaraj, Eds), pp. 5–33. CRC Press, Boca-Raton.

Bonfante-Fasolo P., Vian B., Perotto S., Faccio A. and Knox J. P. (1990) Cellulose and pectin localization in roots of mycorrhizal *Allium porrum*: labelling continuity between host cell and interfacial material. *Planta* **180,** 537–547.

Burne H., Christou N. V., Pal D., Verma S. and Maclachlan G. A. (1975) Purification and characterization of two cellulases from auxin treated pea epicotyls. *Journal of Biological Chemistry* **250,** 1013–1018.

Coughlan M. P. and Ljungdahl L. G. (1988) Comparative biochemistry of fungal and bacterial cellulolytic enzyme systems. In *Biochemistry and Genetics of Cellulose Degradation* (J. P. Aubert, P. Beguin and J. Millet, Eds), pp. 11–30, FEMS Symposium 43. Academic Press, London.

Cruickshank R. H. and Wade G. C. (1980) Detection of pectic enzymes in pectin-acrylamide gels. *Analytical Biochemistry* **107,** 177–181.

Dazzo F. and Hubbell D. H. (1974) A quantitative assay of insoluble polyvinylpyrrolidone. *Plant and Soil* **40,** 435–439.

García-Romera I. and Ocampo J. A. (1988) Effect of the herbicide MCPA on VA mycorrhizal infection and growth of *Pisum sativum*. *Zeitschrift Pflanzenernahrung Bodendenkunde* **151,** 225–228.

García-Romera I., García-Garrido J. M., Martinez-Molina E. and Ocampo J. A. (1990) Possible influence of hydrolytic enzymes on vesicular arbuscular mycorrhizal infection of alfalfa. *Soil Biology & Biochemistry* **22,** 149–152.

Harley J. L. and Smith S. E. (1983) *Mycorrhizal Symbiosis*. Academic Press, New York.

Hayman D. S. (1983) The physiology of vesicular-arbuscular endomycorrhizal symbiosis. *Canadian Journal of Botany* **61,** 944–963.

Hewitt E. J. (1952) Sand water culture methods used in the study of plant nutrition. Commonwealth Agriculture Bureau, Technical Communication No. 22.

Lowry O. H., Roserbrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin-phenol reagent. *Journal of Biological Chemistry* **193,** 265–275.

Morales V. M., Martinez-Molina E. and Hubbel D. H. (1984) Cellulase production by *Rhizobium*. *Plant and Soil* **80,** 407–415.

Ocampo J. A., Martin J. and Hayman D. S. (1980) Influence of plant interactions on vesicular-arbuscular mycorrhizal infection I. Host and non-host plant grown together. *New Phytologist* **84,** 27–35.

Phillips J. M. and Hayman D. S. (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.

Spanu P. and Bonfante-Fasolo P. (1988) Cell-wall-bound peroxidase activity in roots of mycorrhizal *Allium porrum*. *The New Phytologist* **109,** 119–124.

Verma D. P. S., Kumar V. and Maclachlan C. A. (1982) β-Glucanases in higher plants: localization, potential function, and regulation. In *Cellulose and Other Natural Polymer Systems: Biogenesis, Structure and Regulation* (R. M. Brown, Ed.), pp. 459–488. Plenum Press, New York.

Wood P. J. (1980) Specificity in the interaction of direct dyes with polysaccharides. *Carbohydrate Research* **85,** 271–287.