Cellulase production by the vesicular–arbuscular mycorrhizal fungus *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe

**By J. M. Garcia-Garrido, I. Garcia-Romera and J. A. Ocampo**

Department of Microbiology, Estacion Experimental del Zaidin, Prof. Albareda 1, E-18008, Granada, Spain

(Received 25 October 1991; accepted 13 February 1992)

**Summary**

Production of endoglucanase (EC 3.2.1.4) and exoglucanase (EC 3.2.1.91) enzymes was studied during penetration of the host and development of the vesicular–arbuscular mycorrhizal (VAM) fungus, *Glomus mosseae*, in roots of lettuce (*Lactuca sativa*) and onion (*Allium cepa*). Endo- and exoglucanase activities increase in VAM plants at the beginning of entry-point formation and arbuscule development. No relationship was found between the number of vesicles and endo- and exoglucanase activities. Extracts from spores and external mycelium of *G. mosseae* had endo- and exoglucanase activities. Some of the cellulase activities detected in VAM roots were attributed to the fungus, since endoglucanase activity found in the external mycelium of *G. mosseae* and in mycorrhizal root extracts showed the same electrophoretic mobility.

Key words: Cellulases, endoglucanases, exoglucanases, *Glomus mosseae*, VA mycorrhiza.

**Introduction**

The colonization of plant roots by vesicular–arbuscular mycorrhizal (VAM) fungi involves the formation of intercellular hyphae, highly branched intracellular arbuscules and vesicles scattered throughout the root (Bonfante-Fasolo, 1984). Arbuscules have a thin fungal wall which is separated from the host cytoplasm by interfacial material of host origin. These observations suggest that the establishment of an intracellular symbiosis between fungus and plant roots requires penetration of the host cell by fungus. Cell wall-hydrolyzing enzymes may be involved in this process (Garcia-Romera et al., 1990).

Infection of roots by pathogenic and mutualistic microorganisms such as *Rhizobium* and *Azospirillum* appears to be mediated by cell wall-hydrolyzing enzymes (Hubbel, Morales & Umali-Garcia, 1978; Umali-Garcia et al., 1980; Colmer & Keen, 1986). In spite of the low production of hydrolytic enzymes by these mutualistic microorganisms (Morales, Martinez-Molina & Hubbel, 1984), endo- and exoglucanase, which belong to the celluose system (Coughlan & Ljungdhal, 1988), seem to be involved in the dissolution of the cell wall which permits *Rhizobium* to enter the host cell (Verma & Zogbi, 1978; Chalifour & Benhamou, 1989). The observation that VAM fungi penetrate the plant cell wall at the site of contact (Bonfante-Fasolo, 1984), and the fact that spore extracts of *Glomus mosseae* contain cellulolytic enzymes (Garcia-Romera et al., 1990), indicate that cellulases may be involved in the colonization process. However, since VAM fungi have not yet been cultured axenically in the absence of plant roots, it is difficult to confirm the production of cellulolytic enzymes by VA mycorrhizas or their possible participation in the colonization of the root.

The aim of this work was to determine whether the VAM fungus, *G. mosseae*, shows endo- and exoglucanase activities, and whether these enzymes participate in the colonization of plant roots.

**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 was a calcereollic xerochrept type, pH 7-6 (for full details see Garcia-Romera & Ocampo, 1988). It was steam-sterilized and mixed 1:1 (v:v) with sterilized quartz sand. Lettuce (*Lactuca sativa*, cv. Romana) and onion (*Allium cepa*, cv. Bobasa) were used as test plants. Seeds were sown in
moistened sand. After 2 weeks, seedlings were transplanted to the pots and grown under greenhouse conditions. Natural light was supplemented by Sylvania incandescent and cool-white lamps providing 400 nmol m⁻² s⁻¹, 400–700 nm, with a 16–8 h light–dark cycle at 25–19 °C and 50% relative humidity. Plants were watered from below using a capillary system, and fed with a nutrient solution (Hewitt, 1952), lacking phosphate for VAM-inoculated plants.

The inoculum consisted of 5 g of rhizosphere soil either from maize or from alfalfa plant pot cultures of an isolate of *Glomus mosseae* (Nicol. & Gerd.) Gerd. and Trappe which contained spores (15 sporocarps per g with 1–5 spores per sporocarp), 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 uninoculated treatment. The filtrate contained common soil microorganisms, but no propagules of *G. mosseae*.

Sporocarps of *G. mosseae* were isolated by wet-sieving the soil (Gerdemann, 1955). Spores were obtained by dissecting the sporocarps, and were then stored in water at 4 °C for use within 1 month. Before enzymatic assay, the spores were surface-sterilized (MacDonald, 1981). External mycelium was isolated from roots of 8-week-old lettuce plants colonized with *G. mosseae*. The roots were washed and rinsed gently with sterilized water, and the external mycelium collected with forceps under a dissecting microscope.

**Mycorrhizal measurements**

Plants were harvested at 15, 35, 50 and 80 d. The root system was washed and rinsed three times with sterilized distilled water. Parts of the root system (2 g f. wt) from each of the five replicate groups of pots were cleared and stained (Phillips & Hayman, 1970). Material from each replicate was cut into 1 cm segments that were mixed and repeatedly subdivided to yield random samples of 40 root segments. These were mounted on slides and examined under a compound microscope at ×160 magnification. The percentage of total root length which was colonized with VAM, percentage of arbuscules, numbers of entry points (appressoria) and vesicles were measured as described by Ocampo, Martin & Hayman (1980). All assays were repeated three times with similar results; in two of them, the source of *G. mosseae* inoculum was from maize and, in the other, inoculum was obtained from alfalfa.

**Preparation of extracts for enzyme assays**

Roots (20 g f. wt) were frozen in liquid nitrogen 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 MgCl₂, 10 mM NaHCO₃, 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 20000 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, which precipitates most of the cellulase proteins (Garcia-Garrido et al., unpublished results). The solution was kept for 5 h at 4 °C and centrifuged once more as described above. The supernatant was discarded, and sediment was dissolved in a small volume of the same extractant solution and was dialyzed 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 (Garcia-Garrido et al., unpublished results).

Spores and 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 (for 1 min, 5 times at 80 W), centrifuged at 20000 g for 15 min. The pellet was resuspended and sonicated again, and washed by centrifugation with the same buffer three times. The supernatant was concentrated by ultrafiltration using PM-10 membranes (Amicon), and used as a crude enzyme extract.

**Enzyme assays**

Endoglucanase activity (EC 3.2.1.4) was assayed viscometrically, using carboxymethylcellulose (CMC) as the substrate. Reduction in viscosity was determined in a Cannon–Fenske viscometer (5354/2) at 37 °C. Six ml of reaction mixture contained 5 ml of 0.5% CMC in 50 mM citrate–phosphate buffer (pH 5) and 1 ml of root or fungus extracts. Enzyme activity was expressed on a relative activity (RA) basis (reciprocal of the time in min for 50% loss of viscosity ×1000) (Bateman, 1963). Exoglucanase activity (EC 3.2.1.91) was determined by measuring the release of p-nitrophenol from p-nitrophenyl-β-D-lactopyranoside (pNLP) (Deshpande, Eriksson & Petterson, 1984). An assay mixture containing 1.8 ml of a 1 mg ml⁻¹ solution of PNLP in 0.05 M citrate–phosphate buffer (pH 5) and 0.2 ml of root or fungus extract was incubated at 37 °C for 0 to 60 min. One unit was defined as the amount of enzyme which released 1 μmol of p-nitrophenol per h.

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

Non-denaturing linear-gradient electrophoresis of cellulolytic enzymes in polyacrylamide gels (4–12 %) amended with 0.1 % CMC in 0.05 M Tris-0.1 M glycine buffer (pH 8.8), was based upon the method described for pectinases by Cruickshank & Wade (1980).

Gels, 16 × 18 cm by 15 mm thick, were prepared using a gradient gel former (LKB). The electrode tank contained the same Tris-glycine buffer (pH 8.8) as used in the gel. After pre-electrophoresis of the gel for 30 min, the wells were filled with 75 μl of root or fungus extract (175 μg protein), and 1 μl 0.05 % bromophenol blue in gel buffer was applied to the cathodic side. Electrophoresis was subsequently carried out at 4 °C and a constant current of 20 mA per gel for 5 h.

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

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

RESULTS

No fungi were detected in microscopic observations of stained roots from uninoculated controls (data not shown). Typical development of VAM colonization was observed in both onion and lettuce; 15 d after transplanting, colonization increased, reaching a peak of 50 days (Fig. 1). Vesicle development reached a maximum at 35 d in lettuce roots, and declined after 50 d (Fig. 2a). There was no apparent decline in entry-point number, which reached a maximum at 80 d. Arbuscule development increased until day 50. In onion roots, the number of entry-points and arbuscule development reached a maximum at 50 d (Fig. 2b). There was an increase in vesicle number, which was more noticeable in the oldest plants.

Endoglucanase activity was evident in lettuce and onion roots after 15 d (Fig. 3a, b). The pattern of enzyme production differed in the two host species. In 35-d-old lettuce plants, endoglucanase activity was higher in mycorrhizal (difference 1.52 units mg⁻¹ protein) than in non-mycorrhizal plants, although these activities were similar after 50 d (Fig. 3a). Endoglucanase activity was greater in 35-d-old mycorrhizal (difference 1.3 units mg⁻¹ protein) than non-mycorrhizal onion plants, but at 50 d this activity declined, reaching lower levels in mycorrhizal than in non-mycorrhizal plants (Fig. 3b). Lettuce plants showed a similar pattern of development of exoglucanase activity in mycorrhizal and non-mycorrhizal plants. This activity was high when the plants were 50 d old, but at 35 and 50 d mycorrhizal plants showed more activity (differences of 0.05 and 0.02 units mg⁻¹ protein, respectively).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1}
\caption{Percentage of root length colonized in lettuce (●) and onion (○) roots inoculated with Glomus mosseae. Each value is the mean of five replicates. For each plant, values within a row sharing the same letter were not significantly different according to Duncan’s multiple-range test (P = 0.05).}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{Entry-points (●), vesicles (○) and arbuscules (×) in lettuce (a) and onion (b) roots inoculated with Glomus mosseae. Each value is the mean of five replicates. For each fungal structure, values within a row sharing the same letter were not significantly different according to Duncan’s multiple-range test (P = 0.05).}
\end{figure}
than non-mycorrhizal plants (Fig. 4a). Mycorrhizal onion plants showed more exoglucanase activity (difference 0.06 units mg\(^{-1}\) protein) than non-mycorrhizal plants at 35 d; thereafter, activity declined, and was similar to that in non-mycorrhizal plants at the end of the experiment (Fig. 4b).

Table 1 shows that spores and external mycelia of *G. mossea* contained endo- and exoglucanase activities. Activities of the enzymes were not significantly different between spores and external mycelium.

Several electrophoretic bands of endoglucanase activity were observed in 35-d-old lettuce and onion

---

**Table 1. Cellulolytic activities in spores and external mycelium of *Glomus mossea***

<table>
<thead>
<tr>
<th>Cellulolytic enzymes</th>
<th>Specific activities (units mg(^{-1}) protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Spores</td>
</tr>
<tr>
<td>Endoglucanase</td>
<td>3.44 a</td>
</tr>
<tr>
<td>Exoglucanase</td>
<td>0.005a</td>
</tr>
</tbody>
</table>

Each number is the mean of three replicates. For each enzyme, values within a row sharing the same letter were not significantly different according to Duncan's multiple-range test (P = 0.05).
plants. The bands in root extracts differed between mycorrhizal and non-mycorrhizal plants (Fig. 5). One band similar to external mycelium was observed in the root extracts from colonized lettuce and onion plants. This band was not apparent in the non-mycorrhizal roots.

DISCUSSION

Considerable attention has been devoted to the study of the role of cellulase enzymes in physiological and pathological changes in shoots (Sexton & Roberts, 1982; Coughlan & Ljungdhal, 1988), but research on these enzymes in plant roots, and their role in penetration and the development of symbiotic relationships is very scarce (Dazzo & Hubbel, 1974). Our results show that endo- and exoglucanase activities increased in 35-d-old VAM plants, as compared to non-VAM plants, when the fungus was in its logarithmic stage of growth. The observation of differences in vesicle production between onion and lettuce (Fig. 2) agrees with that of Lackie et al. (1987), who found differences in vesicle production by the same fungal symbiont in different hosts. However, no relationship between number of vesicles and endo- and exoglucanase activities was found, although in VAM lettuce and onion plants maximum endo- and exoglucanase activities coincided with the beginning of entry-point formation and arbuscule development. The formation of entry-points and arbuscules requires that the fungal hypha passes through the cell wall, after which an interfacial matrix is formed around it (Bonfante-Fasolo, 1984). This matrix is composed of polygalacturonic acid and cellulosic materials of host origin (Bonfante-Fasolo et al., 1990). These cytochemical observations suggest that the fungus produces hydrolytic enzymes which facilitate penetration of the cell wall without affecting host viability (Bonfante-Fasolo, 1984; Bonfante-Fasolo et al., 1990). However, difficulties exist in detecting the activity of cell-wall-degrading enzymes, since breakdown of the walls must be localized during fungal development and, consequently, difficult to detect. Indeed, endo- and exoglucanase activities were extremely low, as might be expected in a mutualistic interaction. Thus, it is difficult to demonstrate a close relationship between cellulase activities and the development of fungal structures.

Cell-wall-hydrolyzing enzymes were present in non-VAM roots (Figs 3 and 4) during growth and development (Byrne et al., 1975). In respect of other symbiotic associations, such as that involving Rhizobium and legumes, several authors (Dazzo & Hubbell, 1974; Verma, Jumar & Maclachlan, 1982), have discussed the possibility that cellulases (mainly endoglucanases) produced by either the plant or the bacterium, may be implicated in the process of host-wall degradation during infection. Some of the endoglucanase activity can be attributed to the extramatrical phase of the VA fungus, since endoglucanase activity found in the external mycelium and in mycorrhizal root extracts showed similar electrophoretic mobility (Fig. 5). However, the presence of bands in extracts from VAM roots, which were absent in non-mycorrhizal roots or external mycelium, suggests that some of this activity may be produced by the fungus inside the root or by the root itself after induction by the fungus. Endo- and exoglucanase activities in mycorrhizal plants decreased at the end of the experiment to levels similar to, or lower than, that of non-mycorrhizal plants. The growth of the VAM fungus inside the root can be controlled by the plant (Harley & Smith, 1983; Anderson, 1988), and this control may reduce the production of these cellulases by the fungus, as is the case with other enzymes (Spanu & Bonfante-Fasolo, 1988).

The sequence of endo- and exoglucanase activities observed in the VAM association suggests that fungal cellulases may be implicated in the mycorrhizal colonization of roots.

ACKNOWLEDGEMENTS

The authors thank Karen Shashok for grammatical correction of the text. Financial support for this study was provided by the Comision Interministerial de Ciencia y Tecnologia, Spain.

REFERENCES


This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.