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# Pectinase Activity In Vesicular-Arbuscular Mycorrhiza During Colonization of Lettuce

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## Abstract

The production of pectinases in lettuce (*Lactuca sativa* var. Romana) was examined during the root colonization by vesicular-arbuscular mycorrhizal (VAM fungi *Glomus mosseae* and *G. fasciculatum*. Pectin esterase activity was higher in VAM inoculated plants. Endo-polymethylgalacturonase activity peaked 60 days after inoculation, whereas polymethylgalacturonase activity peaked at 30 days. Pectin lyase activity in lettuce and maize (*Zea mays* var. Calderon) peaked at 30 days when inoculated with *G. mosseae*, but did not appear until 60 days in plants inoculated with *G. fasciculatum*. No significant differences in endopolygalacturonase, polygalacturonase and pectate lyase activities were observed between VAM and control plants. The possible role of the different pectinases in colonization of plant root by VAM fungi is discussed.

Keywords: Pectinase, VAM, lettuce

Abbreviations: PE: PE, PG: polygalacturonase, PL: pectate lyase, PNL: pectin lyase, PMG: polymethylgalacturonase

## 1. Introduction

The establishment of intracellular symbiosis with VAM fungi in plant roots requires penetration of the host cell by fungi (Bonfante-Fasolo, 1984; Bonfante-Fasolo et al., 1990), but the mechanisms by which VAM endophytes enter host tissues are still unknown.

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Pectinases, including pectin esterase, polygalacturonases, and lyases, play a fundamental role in penetration of the plant cell by many microorganisms (Collmer and Keen, 1986; Martinez-Molina et al., 1979). Detection of the presence of pectolytic enzymes in VAM fungi is difficult because of the very low levels of enzyme production (García-Romera et al., 1991a), and the so far unsuccessful attempts to culture VAM fungi in the absence of plant roots (Harley and Smith, 1984). There are biochemical and cytological indications that pectinases may be involved in the VAM colonization process (Bonfante-Fasolo et al., 1990). Moreover, the presence of pectinases (pectin esterase, polygalacturonases, and lyases), detected in the extracts of spores and external mycelium of *G. mosseae* (García-Romera et al., 1991b), may be an indication of the types of enzymes that VA fungi are capable of producing in relation to root colonization.

The aim of this work was to determine whether the different pectinases participate in the colonization of plant roots by VAM fungi.

## 2. Materials and Methods

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 details see García-Romera and Ocampo, 1988) was steam-sterilized and mixed with sterilized sand at a proportion of 1:1 (v/v).

Lettuce (*Lactuca sativa* cv. Romana) and maize (*Zea mays* cv. Calderon) were used as test plants. Seeds were sown in moistened sand; after 2 weeks seedlings were transplanted to pots and grown in greenhouse (natural light was supplemented by Silvania incandescent and cool-white lamps, 400 nmol $m^{-2}s^{-1}$, 400–700 nm; with a 16/8 hr light/dark cycle at 25/19°C and 50% relative humidity). Plants were watered from below using a capillary system, and fed with nutrient solution (Hewitt, 1952), lacking P for VAM inoculated plants.

The VA inoculum consisted of 5 g of rhizosphere soil from either maize or alfalfa plant pot culture of an isolate of *G. mosseae* and *G. fasciculatum* which contained spores (15 sporocarps/g of soil with 1 to 5 spores per sporocarp of *G. mosseae* and 100 spores/g of soil of *G. fasciculatum*), mycelium, and colonized root fragments. Control plants were given filtered leachings from the inoculum soil (Whatman No. 1 filter paper). The filtrate contained common soil microorganisms, but no propagules of *G. mosseae* and *G. fasciculatum.*

Plants were harvested after 30, 60 and 80 days. The root system was washed and rinsed three times with sterilized distilled water, and parts of the roots

system from each of the five replicate groups of pots were cleared and stained (Phillips and Hayman, 1970), and examined under a compound microscope. The percentage of total root length that was colonized by VAM fungi was measured as described by Ocampo et al., 1980. All assays were repeated two times with inoculi from onion and from alfalfa plant pot cultures.

*Enzyme assays*

Roots (20 g) were pulverized in a mortar under liquid nitrogen. The resulting powder was homogenized in 40 ml of 0.25 M NaCl, for pectin esterase extraction, or in 50 mM citrate phosphate (C-P) buffer (pH 7.2), for pectin depolymerases. Sodium azide (0.03%) was added to both extractant solutions. The suspension was centrifuged at 20,000 × g for 15 min, and the pellet was resuspended and washed three times by centrifugation using the same buffer. The supernatant was treated with ammonium sulphate up to 80% of full saturation (to precipitate pectinase protein). The solution was kept for 5 hr at 4°C and centrifuged once more as described above. The sediment was dissolved in a small volume of the same extractant solution and dialyzed against several hundred volumes of the same diluted extractant solutions (1:9, v/v) for 16 hr at 4°C. The samples were then frozen until use.

The extracts were assayed to determine the activities of pectin esterase (PE) (EC 3.1.1.11), endopolygalacturonase (endo-PG) (EC 3.2.1.15), polygalacturonase (PG) (EC 3.2.1.67), pectate lyase (PL) (EC 4.2.2.9) and pectin lyase (PNL) (EC 4.2.2.10). The activities of endo-PG and PG on pectin polymethylgalacturonase (PMG), respectively.

The substrates used in the analyses of pectinase enzymes were Na polygalacturonase and citrus pectin (Sigma).

The activity of PE was assayed by measuring the release of acid groups from pectin (Hancock, 1966). The reaction mixture consisted of 15 ml of 1% pectin (pH 5) and 2.5 ml enzyme solution. After reaction times of 10, 20 and 40 min at 37°C, the enzyme-substrate mixture was titrated with 0.02 N NaOH to the pH recorded at time zero. One unit of PE activity was defined as the amount of enzyme that required the addition of 1 microequivalent of NaOH/hr to maintain the reaction mixture at pH 5.

Endo-PG and endo-PMG activities were assayed by the viscosity reducing method (Bateman, 1963). Viscosity reduction was determined in a Cannon-Fenske viscosimeter (5354/4) at 37°C. Six mililiters of reaction mixture contained 1% substrate, 50 mM C-P buffer (pH 5) and 1 ml enzyme. One unit of enzyme activity was expressed as relative activity (RA: reciprocal of time in hr for 50% viscosity loss $\times 10^3$).

The activities of PG and PMG were measured as the release of reducing groups. Reducing ends were measured with the procedures of Nelson (1944) and Somogyi (1952). The increase in reducing groups was determined in reaction mixtures containing 0.3 ml 1% (w/v) Na polygalacturonate or pectin, 0.3 ml 50 mM potassium phosphate (pH 6.0), 2 mM EDTA (to inhibit lyases) and 0.4 ml enzyme. One unit of enzyme was defined as the amount of enzyme which released 1.0 $\mu$mol of galacturonic acid/hr at 37°C.

The two general methods described by Starr and Moran (1962) and Albersheim and Killias (1962), were employed for the detection of PNL and PL. One enzyme unit was defined as a change of 0.01 absorbance units in 1 hr.

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

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

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

## 3. Results

Microscopic observations of stained roots showed no endophyte fungi in control plants and VAM structures in VA inoculated plants. Percentage VAM root length colonization was similar in lettuce and maize plants after 30, 60 and 80 days of plant growth, and from 30 days after transplanting, VAM colonization showed the usual logarithmic growth pattern until 80 days (Table 1).

PE activity was significantly higher in plants inoculated with either *G. mosseae* or *G. fasciculatum* in comparison to controls (Table 2). PE activity in controls increased in 60 day-old plants, however, this activity in VAM plants remained almost constant.

Endo-PG and PG activities in VAM and control plants were generally decreasing with time (Table 3). The differences in these enzymatic activities between extracts from controls and plants colonized with *G. mosseae* or *G. fasciculatum* were not significant.

The levels of Endo-PMG and PMG activities in nonmycorrhizal roots did not change throughout the assay (Table 4). At 30 days, controls and mycorrhizal plants inoculated with *G. mosseae* or *G. fasciculatum* had similar endo-PMG activities. This activity was higher in 60 day-old mycorrhizal than in control plants, whereas at 80 day, endo-PMG activity was lower in mycorrhizal than in control plants. The only increase in PMG activity was observed in 30 day-old VAM inoculated plants compared with controls, regardless of the endophyte used.

Table 1. Percentage VA root length colonization in lettuce and maize plants inoculated with *G. mosseae* and *G. fasciculatum*

| Plants | Endophyte | % Root length colonization after (days) | | |
|---|---|---|---|---|
| | | 30 | 60 | 80 |
| Lettuce | *G. mosseae* | 27 a | 58 b | 69 c |
| | *G. fasciculatum* | 24 a | 55 b | 70 c |
| Maize | *G. mosseae* | 26 a | 60 b | 73 c |
| | *G. fasciculatum* | 23 a | 58 b | 71 c |

Values of VA root length colonization of lettuce and maize sharing the same letter were not significantly different according to Duncan's multiple range test (P = 0.05).

Table 2. Pectin esterase (PE) activity in root of non-mycorrhizal (−M) and mycorrhizal (+M) lettuce plants inoculated with *G. mosseae* or *G. fasciculatum*

| Endophyte | Treatment | Specific activities (Units/mg prot.) after (days) | | |
|---|---|---|---|---|
| | | 30 | 60 | 80 |
| *G. mosseae* | − M | 3.8 a | 9.3 b | 4.5 a |
| | + M | 15.6 c | 18.6 c | 16.2 c |
| *G. fasciculatum* | − M | 2.3 a | 8.4 b | 2.5 a |
| | + M | 12.4 c | 16.2 c | 15.8 c |

For each endophyte, values sharing the same letter were not significantly different according to Duncan's multiple range test (P = 0.05).

PL and PNL activities of non VAM lettuce and maize plants were constant throughout the assay. Nonsignificant differences in PL activity were noted between control and VA inoculated plants with *G. mosseae* or *G. fasciculatum*. PNL activity in 30 day-old lettuce and maize inoculated with *G. mosseae* and in 60 day-old lettuce and maize inoculated with *G. fasciculatum* was significantly higher than in controls. However, PNL activity in 80 day-old lettuce and maize inoculated with *G. mosseae* was significantly lower than in controls,

Table 3. Endo-polygalacturonase (endo-PG) and polygalacturonase (PG) activities in root of non-mycorrhizal (−M) and mycorrhizal (+M) lettuce plants inoculated with *G. mosseae* or *G. fasciculatum*

| Endophyte | Treatment | Specific activities after (days) | | | (Units/mg prot.) | | |
|---|---|---|---|---|---|---|---|
| | | endo-PG | | | PG | | |
| | | 30 | 60 | 80 | 30 | 60 | 80 |
| *G. mosseae* | − M | 21.1 a | 10.8 b | 6.1 c | 6.7 a | 3.4 b | 1.3 c |
| | + M | 24.2 a | 9.6 b | 5.3 c | 5.4 a | 3.9 b | 1.2 c |
| *G. fasciculatum* | − M | 15.1 a | 11.9 a | 4.2 c | 7.4 a | 6.3 a | 3.1 b |
| | + M | 15.6 a | 12.1 a | 5.4 c | 8.5 a | 7.6 a | 3.6 b |

For endo-PG and PG activities and each endophyte, values sharing the same letter were not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 4. Endo-polymethylgalacturonase (endo-PMG) and polymethylgalacturonase (PMG) activity in root of non-mycorrhizal (−M) and mycorrhizal (+M) lettuce plants inoculated with *G. mosseae* or *G. fasciculatum*

| Endophyte | Treatment | Specific activities after (days) | | | (Units/mg prot.) | | |
|---|---|---|---|---|---|---|---|
| | | endo-PG | | | PG | | |
| | | 30 | 60 | 80 | 30 | 60 | 80 |
| *G. mosseae* | − M | 35.4 a | 30.0 ac | 35.4 a | 3.1 a | 2.4 a | 2.5 a |
| | + M | 34.2 a | 45.6 b | 29.3 c | 7.2 b | 3.2 a | 2.4 a |
| *G. fasciculatum* | − M | 31.8 a | 30.0 ac | 33.6 a | 2.9 a | 2.8 a | 2.5 a |
| | + M | 29.8 a | 43.4 b | 27.3 c | 6.4 b | 2.4 a | 2.3 a |

For endo-PMG and PMG activities and each endophyte, values sharing the same letter were not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 5. Pectate lyase (PL) and pectin lyase (PNL) activities in root of non-mycorrhizal (−M) and mycorrhizal (+M) lettuce plants inoculated with *G. mosseae* or *G. fasciculatum*

| Endophyte | Treatment | Specific activities after (days) | | | (Units/mg prot.) | | |
|---|---|---|---|---|---|---|---|
| | | endo-PG | | | PG | | |
| | | 30 | 60 | 80 | 30 | 60 | 80 |
| *G. mosseae* | − M | 0.17 a | 0.15 a | 0.14 a | 0.15 a | 0.19 a | 0.17 a |
| | + M | 0.14 a | 0.12 a | 0.11 a | 0.45 b | 0.19 a | 0.09 c |
| *G. fasciculatum* | − M | 0.19 a | 0.17 a | 0.15 a | 0.18 a | 0.21 a | 0.22 a |
| | + M | 0.17 a | 0.15 a | 0.13 a | 0.18 a | 0.60 b | 0.21 a |

For PL and PNL activities and each endophyte, values sharing the same letter were not significantly different according to Duncan's multiple range test (P = 0.05).

Table 6. Pectate lyase (PL) and pectin lyase (PNL) activities in root of non-mycorrhizal (−M) and mycorrhizal (+M) maize plants inoculated with *G. mosseae* or *G. fasciculatum*

| Endophyte | Treatment | Specific activities after (days) | | | (Units/mg prot.) | | |
|---|---|---|---|---|---|---|---|
| | | endo-PG | | | PG | | |
| | | 30 | 60 | 80 | 30 | 60 | 80 |
| *G. mosseae* | − M | 0.58 a | 0.47 a | 0.40 a | 0.59 a | 0.60 a | 0.58 a |
| | + M | 0.49 a | 0.45 a | 0.38 a | 1.32 b | 0.57 a | 0.48 c |
| *G. fasciculatum* | − M | 0.60 a | 0.56 a | 0.49 a | 0.56 a | 0.60 a | 0.61 a |
| | + M | 0.57 a | 0.55 a | 0.49 a | 0.54 a | 0.71 b | 0.62 a |

For PL an PNL activities and each endophyte, values sharing the same letter were not significantly different according to Duncan's multiple range test (P = 0.05).

but at this time the difference between plants inoculated with *G. fasciculatum* and controls was not significant (Tables 5 and 6).

## 4. Discussion

In spite of the importance of pectinases in the penetration of the plant cell wall by many microorganisms, little is known about the possible role of these enzymes in the colonization of plant roots by VAM fungi. Experiments based on catabolic repression showed that pectinases may be involved in the colonization of alfalfa root by *G. mosseae* (García-Romera et al., 1990. In addition, it has been reported that extracts of spores and external mycelium of *Glomus mosseae* contain pectinesterase, polygalacturonases and lyases (García-Romera et al., 1991b).

Our results show that PE activity was consistently higher in plants inoculated with either *G. mosseae* or *G. fasciculatum* than in control plants throughout the process of root colonization. This enzyme is thought to facilitate the action of the other pectinase enzymes (Pressey and Avants, 1982). The increase in fungal structures which penetrate the cell wall during the logarithmic stage of root colonization (Hayman, 1983) may explain the increase in endo-PMG, PMG and PNL activities at this time. However, this does not account for the different rates at which endo-PMG, PMG and PNL activities increased during VA colonization, nor does it explain the fact that PNL activity in roots colonized by *G. mosseae* peaked at a different time from that of maximum activity in plants colonized by *G. fasciculatum*, regardless of plant type. However, endo-PG, PG and PL activities in VAM plants were similar to controls throughout the experiment. The lack of differences in these degradative enzymes is not conclusive evidence that they do not participate in the colonization process, in view of the presence of these enzymes in the extracts of spores and external mycelium of *G. mosseae* (García-Romera et al., 1991b). These results suggest that the plant controls the production and/or the activity of these enzymes (Rexova-Benkova and Markovic, 1976), thus avoiding the indiscriminate breakdown of the cell wall. Plant phenolic compounds and ferulic acids have been shown to inhibit pectinase activities (Fielding, 1981; Patil and Dimond, 1967). These compounds negatively affect the development of VA fungus in the plant root throughout root colonization, especially at the end of this period (Allen et al., 1980; Bonfante-Fasolo and Gianinazzi-Pearson, 1986; Wacker et al., 1990).

With the methods used in our experiments it is difficult to decide whether the increase in pectinase activity observed in VA colonized roots is from the fungus or from the plant induced by the presence of the fungus. It is also difficult to

establish the mode of action of the different pectinases produced by *G. mosseae* and *G. fasciculatum* in root colonization. However, our results indicated that PE, endo-PMG and PNL seem to be the main pectinases involved in the colonization of lettuce plants by *G. mosseae* and *G. fasciculatum*. Thus, a system of pectin-degrading enzymes, rather than sodium pectate-degrading enzymes, may play a role in the process of VAM colonization. Further studies with purified VA fungal pectinases will be aimed at developing more sensitive techniques (EM localization using inmunogold complexes with specific antisera to VAM fungal pectinases) to more fully elucidate the role of the different pectolytic enzymes produced by VAM fungi.

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