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POSSIBLE INFLUENCE OF HYDROLYTIC ENZYMES ON VESICULAR ARBUSCULAR MYCORRHIZAL INFECTION OF ALFALFA

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Summary—The production of hydrolytic enzymes such as pectinases, cellulases and hemicellulases by extracts of spores of *Glomus mosseae* was observed. The possible involvement of hydrolytic enzymes in the process of root infection by *G. mosseae* in *Medicago sativa* plants grown under axenic conditions was studied. The presence of pectin or Na-pectate substrate in the rooting medium delayed the beginning of mycorrhizal infection and decreased the plateau of the infection curves. Hemicellulose substrate (Locust bean) had no effect on VA infection. Carboxymethyl cellulose at concentrations of 0.2 and 0.6% decreased VA infection from the beginning of the experiment. The possible influence of hydrolytic enzymes in the mechanisms of penetration of VA fungus into plant root cells is discussed.

INTRODUCTION

The role of hydrolytic enzymes (cellulases, hemicellulases and pectinases) in the active penetration of plant cell walls by pathogenic microorganisms is well documented (Collmer *et al.*, 1982). The production of hydrolytic enzymes has been observed not only in parasites but also in mutualistic microorganisms (Martinez-Molina *et al.*, 1979). These enzymes are also thought to be involved in the infection of legumes by *Rhizobium* and in the infection of grasses by *Azospirillum* (Hubbel *et al.*, 1978; Umali-Garcia *et al.*, 1980).

Although vesicular arbuscular (VA) endophytes are classified as mutualistic, they share many features in common with obligatory haustorial parasites (Hepper and Mosse, 1975). The mechanisms by which VA mycorrhizal fungi penetrate host tissues are still unknown, but observations of fungal penetration into roots indicate that these fungi are probably able to degrade cell walls (Kinden and Brown, 1975; Bonfante-Fasolo, 1984). It has been suggested that when the hyphae enter host cells they penetrate the cell wall through a combination of enzymic processes and physical pressure (Jeanmougin *et al.*, 1987). However, attempts to demonstrate cellulase or pectin-degrading enzyme production from ectomycorrhizae in culture or in extracts from VA mycorrhizal tissue have not been successful (Anderson, 1988) and to date no studies on the participation of hydrolytic enzymes in the process of VA mycorrhizal infection have been published.

MATERIALS AND METHODS

Detection of enzyme activity

Sporocarps of *Glomus mosseae* were isolated by wet-sieving the soil (Gerdemann, 1955). Spores were obtained by dissecting the sporocarps and stored in water at 4°C and used within 1 month. Before enzymatic assay, about 2500 spores were surface-sterilized (MacDonald, 1981) and finely pulverized in a mortar under liquid nitrogen. The resulting powder was suspended in 1 ml 50 mM Tris–ClH plus 0.03% sodium azide (pH 7.2). The suspension was 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 used as a crude enzyme extract.

Hydrolytic enzymes were detected with a modified agar plate assay (Martinez-Molina *et al.*, 1979). Agar (1%) was amended with 0.1% substrate. Pectin and Na-pectate were used as substrates for pectolytic activity, carboxymethyl cellulose (CMC) for cellulase and Locust bean (LB, a galactomanan) for hemicellulase activity (all substrates were purchased from Sigma Chemical Co.). The pH of the amended agar was adjusted to 6. After bringing the agar to the boiling point to melt the substrate and sterilize the medium, this was poured into Petri plates and allowed to set. Sterilized stainless-steel cylinders (5 mm dia and 10 mm high) were placed onto the surface of each plate, and 50 μl of crude extract (4 μg protein) was added to each cylinder. Autoclaved enzyme extract and buffer were used as controls. Plates were kept at 28°C for 18 h, after which the unhydrolyzed pectin and pectate were stained with 0.01% Ruthenium Red for 30 min and cleared with distilled water. Activity was recorded as colorless halos on a pink agar-substrate. To detect cellulases and hemicellulases, samples were incubated in agar plus 0.1% of CMC or LB 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, 1981).

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

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Effect of hydrolytic enzyme substrates on VA mycorrhizal infection

This experiment was carried out in 20 × 200 mm glass tubes with 25 g of sand: vermiculite mixture (1:1 v/v) and 12 ml of a diluted (1/2) Hewitt's nutrient solution plus 50 μg ml^{-1} of K_2HPO_4 (pH = 7). Pectin, Na-pectate, CMC and LB were added to the rooting medium at final concentrations of 0, 0.05, 0.2 or 0.6%. Tubes treated with 1% pectin and Na-pectate were also used.

The tubes closed with cotton wool were steam-sterilized three times at 24 h intervals, after that the pH of the rooting medium was 6. To each tube 30 *G. mosseae* spores (surface-sterilized) as described before were placed a few mm under the seedlings. There were also uninoculated controls. 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.

Plants were grown in a chamber with light from Silvania incandescent and cold-white lamps (400 nmol m^{-2} s^{-1}, 400–700 nm), 16–8 h light–dark cycle, 25–17 C day–night temperature and 50% relative humidity. Every 3 weeks during 12 weeks plants were harvested and shoot dry weights were recorded. Roots were cleared and stained (Phillips and Hayman, 1970) and mycorrhizal infection was assessed (Ocampo *et al.*, 1980). Ten replicates tubes per treatment were used and contaminated tubes were discarded (MacDonald, 1981).

The effect of hydrolytic enzyme substrates on spore germination were also tested. Spores of *G. mosseae* were surface-sterilized and cultivated on 1% agar (Difco Bacto) plus 0, 0.05, 0.2 and 0.6% of pectin, Na-pectate, CMC or LB. Concentrations of 1% pectin and Na-pectate were also assayed. Plates were kept at 25 C for 2 weeks, and then the percentage of spore germination was recorded.

RESULTS

The extracts of *G. mosseae* spores produced zones of hydrolysis in agar plates with pectin, Na-pectate, CMC and LB.

Spores of *G. mosseae* reached 70 ± 11% of germination in agar plates after 2 weeks. Non-significant differences in the percentage of spore germination between controls and spores cultivated in the presence of Na-pectate, CMC or LB were observed at all concentrations tested. However, when spores were cultivated in agar with 0.6 and 1% of pectin only, 40 ± 5% and 14 ± 2% germination were observed respectively.

VA root length infection could be assessed after 3 weeks in the VA inoculated controls (Tables 1–4), and in plants grown with CMC (Table 3) and LB (Table 4). Plants grown with pectin and Na-pectate showed infection after 3 weeks only in the 0.05% treatments (Tables 1 and 2).

In plants grown in VA inoculated controls, mycorrhizal infection increased after 3 weeks and reached a maximum after 12 weeks. In the 0.05% pectin and Na-pectate treatments (Tables 1 and 2), mycorrhizal infection increased until 9 weeks, but afterwards both remained almost constant. In 0.2, 0.6 and 1% pectin and Na-pectate (Tables 1 and 2), some mycorrhizal infection commenced slowly.

As Table 3 shows, non-significant differences in the VA root length infection were observed between VA inoculated controls and 0.05% of CMC treatments throughout the experiment. In 0.2 and 0.6% CMC VA root length infection was similar to controls at 3 weeks but remained constant during the entire experiment. Non-significant differences between VA

Table 1. Percentage VA root length infection in alfalfa plants with different pectin concentrations in the rooting medium

Pectin concentration (%)	% Root length mycorrhizal after (weeks)			
	3	6	9	12
0	3.12a	7.4b	9.4d	19.2f
0.05	3.8a	6.1b	10.5d	8.6d
0.2	0	0.4c	1.4e	1.6e
0.6	0	0	0.7c	0.6c
1	0	0	0	0.3c

Each value is the mean of replicate tubes. Values sharing the same letter are not significantly different according to Duncan's multiple range test ($P = 0.05$).

Table 2. Percentage VA root length infection in alfalfa plants with different Na-pectate concentrations in the rooting medium

Na-pectate concentration (%)	% Root length mycorrhizal after (weeks)			
	3	6	9	12
0	2.5a	8.2b	11.3d	16.1f
0.05	2.2a	3.3b	7.8d	8.4d
0.2	0	0.5c	1.5e	2.4e
0.6	0	0	0.4c	0.7c
1	0	0	0	0.5c

Legend as in Table 1.

Table 3. Percentage VA root length infection in alfalfa plants with different CMC concentrations in the rooting medium

CMC concentration (%)	% Root length mycorrhizal after (weeks)			
	3	6	9	12
0	5.1a	7.5b	16.1c	25.2d
0.05	4.5a	8.4b	12.6c	24.8d
0.2	4.5a	4.7a	5.2a	5.4a
0.6	5.9a	3.7a	5.3a	6.5a

Legend as in Table 1.

Table 4. Percentage VA root length infection in alfalfa plants with different LB concentrations in the rooting medium

LB concentration (%)	% Root length mycorrhizal after (weeks)			
	3	6	9	12
0	5.7a	8.2b	17.2c	26.1d
0.05	4.5a	7.5b	16.2c	25.8d
0.2	3.3a	6.2b	13.6c	27.1d
0.6	5.1a	7.7b	15.2c	26.3d

Legend as in Table 1.

Table 5. Number of entry points in mycorrhizal alfalfa plants with different pectin concentrations in the rooting medium

Pectin concentration (%)	Entry points per cm root after (weeks)			
	3	6	9	12
0	0.5a	1.1b	1.6d	3.1f
0.05	0.4a	1.3b	1.6d	1.7d
0.2	0	0.03c	0.2c	0.2c
0.6	0	0	0.1c	0.07c
1	0	0	0	0.1c

Legend as in Table 1.

Table 6. Shoot dry weight (mg plant) of mycorrhizal and non-mycorrhizal alfalfa plants with different pectin concentrations in the rooting medium

Pectin concentration (%)	mg of shoot dry weight after (weeks)							
	3		6		9		12	
	−M	+M	−M	+M	−M	+M	−M	+M
0	100a	110a	191b	262c	232c	280c	400e	390e
0.05	91a	87a	181b	185b	278c	280c	382e	421e
0.2	101a	94a	170b	191b	221c	212c	370e	375e
0.6	87a	92a	184b	195b	240c	245c	372e	389e
1	87a	95a	190b	192b	248c	260c	361e	358e

Legend as in Table 1.

inoculated controls and plants grown in 0.05, 0.2 and 0.6% LB were observed (Table 4).

The number of entry points per cm of root (Table 5) paralleled the root length infection in pectin (Table 1), Na-pectate, CMC and LB treatments (data not shown).

There were no significant differences in shoot dry weight between VA inoculated and uninoculated plants in all treatments tested, with the exception of VA inoculated controls, which increased significantly after 6 weeks. At 12 weeks however, the differences between inoculated and non-inoculated treatments were no longer significant. Pectin (Table 6), Na-pectate, CMC and LB (data not shown) treatments did not affect shoot weight at any concentration used in our experiments.

DISCUSSION

The production of hydrolytic enzymes by *G. mosseae* spores illustrates the possible involvement of these enzymes in the infection process, as suggested by electron microscopic observations (Kinden and Brown, 1975; Bonfante-Fasolo, 1984; Jeanmougin *et al.*, 1987).

Our results indicate that pectolytic enzymes may well be involved in the penetration of VA mycorrhizal fungi into host plant root cells. The delay in the onset of mycorrhizal infection and the decrease in the plateau of VA infection curves paralleled the rising pectin and Na-pectate concentration. Pectin and Na-pectate did not affect plant growth, ruling out the negative effects of insufficient oxygen diffusion as a possible explanation. This is reinforced by the fact that treatments of higher viscosity, e.g. the LB solutions used in our experiments (Table 4) or extracellular polysaccharide from *Rhizobium meliloti* used under conditions similar to those described herein (Azcon-Aguilar and Barea, 1981), failed to inhibit VA mycorrhizal infection. Inhibition of spore germination on agar plates by the higher concentration of pectin used, may explain the delay in mycorrhizal infection by the pectin but not by the Na-pectate treatments.

A certain amount of enzyme production seems to be necessary, for the initial and subsequent development of mycorrhizal infection. Once VA mycorrhizal infection is established in a root, further infection becomes easier (Mosse and Hepper, 1975), but the amount of pectolytic enzymes produced by the inoculated spores or by the mycelium from another part of the infected root seem to be insufficient to produce the same degree of infection in the pectin and Na-pectate added treatments as in the controls.

According to our hypothesis the addition of pectin or Na-pectate to the rooting medium "protects" root walls from pectolytic enzymes produced by the fungus, delaying the expression of enzymatic activity in the root cell walls for a period which parallels the amount of substrate added. The presence of substrate in an easily accessible form inhibits enzyme activity due to accumulation of the product or catabolic repression of enzyme synthesis (Collmer and Keen, 1986).

The effect of cellulases and hemicellulases on the process of infection is not clear from the results obtained in our experiments. In fact the concentration of hemicellulose used on VA infection had no effect and no relationship was noted between the amounts of cellulose added to the rooting medium and the percentage of VA infection.

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