Plant and Soil **144**: 219–226, 1992.

PLSO 9290

Influence of three vesicular-arbuscular mycorrhizal fungi (Glomaceae) on the activity of specific enzymes in the root system of *Cucumis sativus* L.

SØREN ROSENDAHL
Institut for Sporeplanter, University of Copenhagen, Øster Farimagsgade 2D, DK-1353 Copenhagen K., Denmark

Received 18 July 1991. Revised February 1992

Key words: *Cucumis sativus*, electrophoresis, enzymes, isoenzymes, vesicular-arbuscular mycorrhiza

Abstract

The influence of three vesicular-arbuscular mycorrhizal (VAM) *Glomus* species on the activity of enzymes in the roots of *Cucumis sativus* was tested. Cucumber plants were grown in a split-root system, in which colonized and uncolonized roots of a single plant could be separated. The activity of the host root malate dehydrogenase (MDH), glucose 6-phosphate dehydrogenase (Gd), glutamate oxaloacetate transaminase (GOT) and glutamate dehydrogenase (GDH) was measured on a densitometer after separation of the host and fungal enzymes on polyacrylamide gels.

The results showed that only minor changes in the activity of the host root enzymes occurred after VAM inoculation. Gd was stimulated by VAM and phosphorus, and one of the fungi decreased the activity of GDH in the host plant when both parts of the root system were colonized.

Introduction

Vesicular-arbuscular mycorrhizal (VAM) fungi resemble fungal root pathogens and biotrophic leaf pathogens, in that the fungal symbiont drains carbon from the host plant. However, the outcome of this association is very different, as the symbiotic VAM fungi enhance the nutrient uptake of the plant (Abbott and Robson, 1984) and enable the plant to overcome various abiotic and biotic stresses (Dehne, 1987), promoting plant growth and vigour. Changes in respiration and in the activity of specific plant enzymes occur in plants attacked by fungal pathogens (Bollard and Matthews, 1966). Increase in the specific activity of malate dehydrogenase, and in the number of enzyme bands in plants diseased by *Fusarium oxysporum* has been reported (Reddy and Stahmann, 1975). This increase in enzyme activity in diseased plants is believed to be connected to the accumulation of metabolites in the diseased tissue.

In studying the influence of VAM symbiosis on the host plant metabolism Dehne (1986) observed that glycolytic enzymes and various dehydrogenases were stimulated in VAM plants. The enzymes involved in phosphate metabolism (Capaccio and Callow, 1982; Gianinazzi-Pearson and Gianinazzi, 1976 and 1978; Krishna et al., 1983) and nitrogen metabolism (Carling et al., 1978; Oliver et al., 1983; Smith et al., 1985) have also been studied. Direct measurement of the glutamate synthetase activity in the internal mycelium of the fungus suggested that increased activity was at least in part due to the activity of fungal enzymes (Smith et al., 1985). An increase in the activity of nitrate reductase was, however, regarded as an indirect effect of the VAM fungus, and not due to the activity of fungal enzymes (Carling, et al., 1978; Oliver et al., 1983).

Most of the above mentioned studies have been performed with extracts of mycorrhizal roots which included both plant enzymes and enzymes from the fungal symbiont (Dehne,

1986; Dodd et al., 1987). In some studies the fungal component was separated from the plant by degrading the roots enzymatically to measure the activity of the internal mycelium of the fungus (Capaccio and Callow 1982; Smith et al., 1985). However, the enzymatic digestion of the host material resulted in a dramatic decline in activity of the fungal succinate dehydrogenase (McGee and Smith, 1990). In studies of phosphatases, the enzymes of the two organisms were separated by electrophoresis (Gianinazzi–Pearson and Gianinazzi, 1976).

The objective of this study was to investigate whether VAM fungi alter host plant metabolism, or whether changes in enzyme activity from VAM roots can be attributed solely to the presence of fungal enzymes.

Materials and methods

Plant material

Seeds of cucumber plants (*Cucumis sativus* L. cv Aminex F1) were pregerminated on moist filter paper, and the tips of the radicles were cut off to promote growth of lateral roots. The seedlings were grown for one week in vermiculite and the root system of each seedling was then separated into two halves and placed in two adjacent 8 cm pots (split-pots) with a volume of 200 cm^3. Nine treatments with four replicates were included. The plants were grown in a 1:1:2 mixture of autoclaved clay soil (pH 7.0, 9 mg P kg^{-1} as Olsen P), expanded clay and sand supplemented with 100 mg N kg^{-1} as KNO_3 and 100 mg Mg kg^{-1} as $MgSO_4$. The non-VAM phosphorus fertilized treatments received 300 mg P kg^{-1} as KH_2PO_4. The plants were grown for six weeks in a greenhouse at 20–25°C under long day conditions. The daylight was supplemented with TL 33 lamps 24 h a day (130 μE m^{-2} s^{-1}).

VAM inoculum

Three species of *Glomus* isolated in Denmark were used. *G.mosseae* (Nicol. & Gerd.) Gerd. & Trappe strain HEM-01, isolated from strawberry, *G.intraradices* Schenck & Smith strain AR-10, and *G.claroideum* Schenck & Smith strain AL-26, a species isolated from sand dunes. Morphological details of these two latter strains are given by Rosendahl (1989). Inoculum was produced on leeks (*Allium porrum* L.) grown for 4 months. The inoculum consisted of washed infected roots and soil mixed with autoclaved sand. The non-VAM treatment received similarly treated root and soil material from uncolonized leek plants.

Harvest and preparation of root extracts

Shoot dry weights were determined at harvest after drying for 20 h at 80°C. The roots were washed free from soil and rinsed in distilled water to remove salts that could interfere with the enzymes during electrophoresis. The roots were then freeze-dried, weighed, and ground in an ice chilled mortar with 50 mg of insoluble polyvinyl-polypyrroliodone (PVPP). Ground root material (100 mg) was then mixed in 1.5 mL. Ependorph tubes with 1000 μL extraction buffer (Hepper et al., 1986), amended with 10% sucrose and 0.1% Triton X-100. The samples were centrifuged at 9,000 *g* for 30 min and frozen as 25 μL aliquots. The protein concentration in the extracts was determined using the Bio-Rad protein assay kit with bovine serum albumine (BSA) as standard. This assay is based on a modification of the method described by Lowry et al. (1951).

VAM colonization

The mycorrhizal colonization was determined by using the colorimetric method for glucosamine in the residues after protein extraction (Hepper, 1977), and the results are presented as μg glucosamine per mg dry weight. Fungal biomass in mg dry weight was calculated from the values of the relationship between glucosamine and dry weight of VAM fungi given by Hepper (1977). The biomass of *G.mosseae* was estimated using the value for the Rothamsted isolate YV, whereas the biomass of *G.intraradices* and *G.claroideum* was calculated using the value for E3. This was justified on the basis of earlier experiments showing that these fungi have simi-

lar relationships between glucosamine content and percentage rootlength colonized with VAM.

Enzyme activity

Electrophoresis was carried out on 80 × 80 × 0.8 mm vertical polyacrylamide gels. The stacking gel contained 3.75% acrylamide, 0.124 *M* Tris-HCl with pH 6.8. The separation gel was 7.5% acrylamide, 0.375 *M* Tris-HCl, pH 8.8. The electrode buffer contained 3 g Trisma base and 14.4 g glycine per liter (pH 8.3). Each well was loaded with 10 μL of the sample. The gels were run for approximately three hours at 125 V – 15 mA per gel, and then stained for glucose 6-phosphate dehydrogenase (Gd EC.1.1.1.49.), malate dehydrogenase (MDH EC.1.1.1.37.), glutamate dehydrogenase, NAD^+ dependent (GDH EC.1.4.1.2.), and glutamate oxaloacetate transaminase (GOT EC.2.6.1.1.). The staining procedures for Gd, GOT and MDH were as described by Harris and Hopkinson (1978), and GDH as described by Tanksley and Orton (1983).

Fungal bands were identified in previous studies using spores and external mycelium of the three strains. Only fungal MDH and Gd bands were detected. These bands did not overlap the host bands, but had in all cases lower mobility.

Activity of the host and fungal enzymes was measured with a gel scanning densitometer (model GS 300, Hoefer Scientific Instruments). The activities were recorded as heights of the peaks on the densitometric tracings of the gels, or by measuring the area of the densitometric tracing of MDH activity using a ASM 68 K leaf area meter. Within the range of 10–50 μg protein per gel track, peak height of the densitometric tracing is directly proportional to enzyme activity (Rosendahl unpubl.). The area of the densitometric tracings of the host bands of VAM roots was determined by subtracting the area of the fungal band. Data on activity of the four enzymes are given as peak height per mg dry weight of root. As the fungi constituted a part of the biomass of the mycorrhizal roots the plant biomass in these samples was determined by subtracting the estimated fungal biomass.

Statistical treatment

The split-pots with both pots receiving the same treatment (treatments 5–9), were regarded as duplicate samples, and the five treatments were tested with Duncan's Multiple Range test. The split-pots with the adjacent pots receiving different treatments (treatments 1–4) were tested individually with an F-test to determine whether the two halves were different.

Results

The P treatment applied to both parts of the root system increased shoot and root dry weight, whereas none of the VAM treatments influenced dry weights compared to controls (Table 1). The

Table 1. Dry weights (g) of shoots and roots

Treatment		Shoots	Roots	
Left	Right		Left	Right
G.mosseae	Nil	1.45bc[a]	0.19	0.15ns[b]
G.intraradices	Nil	1.34c	0.23	0.18ns
G.claroideum	Nil	1.55bc	0.22	0.17ns
P-fertilized	Nil	1.72b	0.18	0.16ns
G.mosseae	*G.mosseae*	1.52bc	0.17	0.21b[c]
G.intraradices	*G.intraradices*	1.44c	0.19	0.19b[c]
G.claroideum	*G.claroideum*	1.36c	0.13	0.20b[c]
P-fertilized	P-fertilized	2.22a	0.29	0.22a[c]
Nil	Nil	1.51bc	0.16	0.18b[c]

[a] Values in this column followed by the same letter are not significantly different ($p > 0.05$) using Duncan's multiple range test.
[b] ns indicates no significant difference between left and right part of the root system (F-test, $p > 0.05$).
[c] left and right part are regarded as duplicate samples. Values followed by the same letter indicates no significant difference between treatments (Duncan's multiple range test, $p > 0.05$).

root biomass was distributed equally in the split-pots. Even root dry weights of the two adjacent pots that received different treatments were not different.

All inoculated plants became colonized with VAM (Table 2). Cross contamination with *G.mosseae* occurred in some non-VAM treatments. The three fungi varied in the amount of glucosamine found in the roots, with *G.claroideum* having the highest concentration and *G.mosseae* having the lowest. The percentage fungal biomass was calculated from the conversion factors given by Hepper (1977) and represented 2.2, 8.5 and 15.2% of the dry weight of the roots for the three fungi *G.mosseae*, *G.intraradices* and *G.claroideum*, respectively.

The amount of soluble proteins in the extracts did not differ between the treatments, except for a lower concentration in the extracts of roots colonized by *G.claroideum* (Table 3). All host bands were easily detected on the gels and the densitometric tracings (Figs. 1–4). Fungal bands of MDH and Gd were also found (Figs. 1, 2), whereas fungal GOT and GDH bands could not be detected (Figs. 3, 4). Except for a slight stimulation of the activity of MDH from the host plant colonized by *G.intraradices*, the activity of MDH was not affected by any of the treatments (Table 3). A clear correlation was found between peak height of the first and the second host band (Fig. 1), and only measurements of the second host band are shown in Table 3. The correlation between intensity of the two MDH host bands of cucumber measured as peak height and as the area of the peaks was found to be 0.94 and 0.96, respectively, and only activity measured as peak height is shown in the tables. The P treatment and *G.claroideum* increased the activity of Gd in the host plant (Table 3). The presence of *G.mosseae* and *G.intraradices* stimulated this activity further. The activity in the VAM and the non-VAM roots of the VAM plants was not significantly different. The three VAM

Table 2. VAM colonization of the roots measured as μg glucosamine/mg dry weight of roots

Treatment		Colonization (glucosamine mg^{-1})	
Left	Right	Left	Right
G.mosseae	Nil	0.89c[a]	0.34b
G.intraradices	Nil	1.87d	0.05a
G.claroideum	Nil	3.24e	0.0a
P-fertilized	Nil	0.0a	0.0a
G.mosseae	*G.mosseae*	1.02c	0.78c
G.intraradices	*G.intraradices*	1.82d	1.65d
G.claroideum	*G.claroideum*	2.97e	3.35e
P-fertilized	P-fertilized	0.0a	0.0a
Nil	Nil	0.0a	0.0a

[a] Values followed by the same letter are not significantly different ($p > 0.05$) using Duncan's multiple range test.

Table 3. Protein concentrations (P) (mg g^{-1}) and activity of the four enzymes (corrected for the presence of fungal biomass in the root samples) measured as peak height of the densitometric tracings

Treatment	P (mg g^{-1})	MDH	Gd	GOT	GDH
G.mosseae[b]	36.2ab[a]	218c	65a	29ab	69b
G.intraradices	33.7ab	270a	62a	28ab	77b
G.claroideum	29.2c	262ab	50b	25bc	51d
Phosphate	32.9abc	237bc	50b	19c	92a
nil	31.0bc	235bc	34c	34.8a	60c

[a] Values in each column followed by the same letter are not significantly different ($p > 0.05$) using Duncan's multiple range test.

[b] The two halves of the split-pots are regarded as duplicate samples.

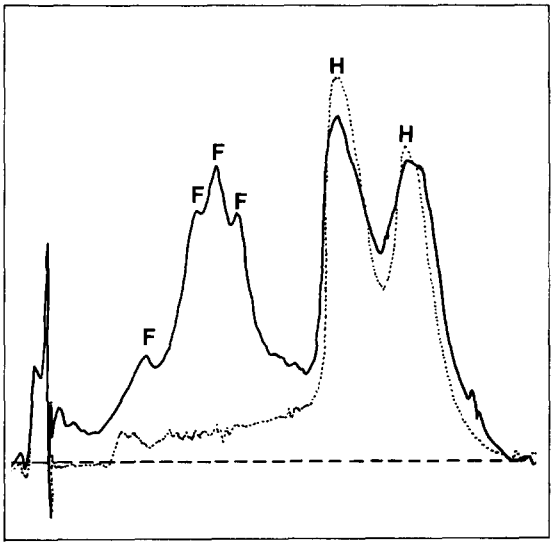

Fig. 1. Densitometric tracing of a gel stained for MDH. Full line is roots colonized by *Glomus intraradices*; dotted line is uncolonized roots of the same plant; F1–F4, peaks from fungal bands; H1–H3, peaks from the host plant.

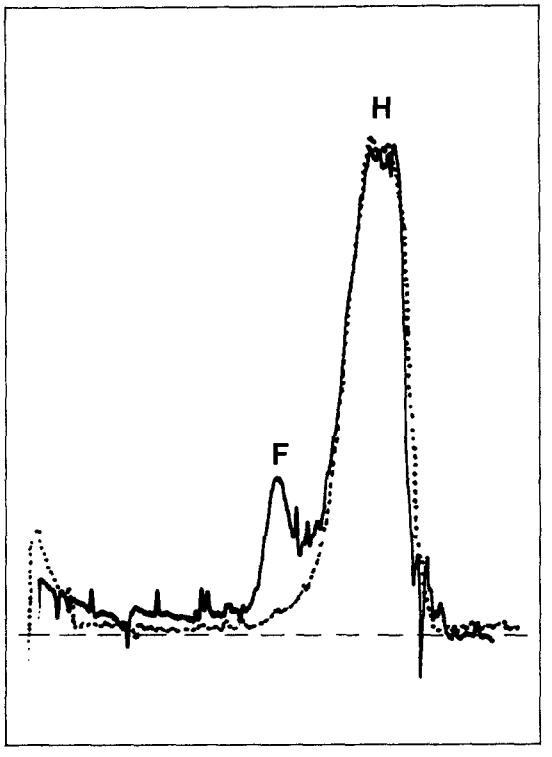

Fig. 2. Densitometric tracing of a gel stained for Gd. Full line is roots colonized by *Glomus mosseae*; dotted line is uncolonized roots from the same plant; F, fungal band; H, host bands.

Fig. 3. Densitometric tracing of a gel stained for GOT. Full line is roots colonized by *Glomus claroideum*; dotted line uncolonized roots from the same plant.

species did not influence the activity of GOT compared to control plants, except for a slightly lower activity in the plants colonized by *G.claroideum*. The P treatment lowered the activity of GOT, whereas this treatment stimulated the activity of GDH compared to control and VAM treatments (Table 3). *G.mosseae* and *G.intraradices* increased the activity compared to control, whereas *G.claroideum* lowered the activity of the host enzyme.

The areas of the densitometric tracings of MDH activity (Fig. 1) from colonized and uncolonized parts of the same plants were compared in order to estimate the percentage of MDH activity for which the fungus was responsible. Although the total area of the densitometric tracing of activity from VAM roots was larger than the area of activity of non-VAM roots, this difference could be explained by the area of activity of the fungal enzymes.

Discussion

VAM had only a limited effect on the activity of the host enzymes. This contrasts with studies of plant pathogens, in which infection by *Fusarium oxysporum* in peas (*Pisum sativum*) resulted in a three times higher activity of MDH (Reddy and Stahmann, 1975). Further, specific bands were induced by the pathogen. No such changes were seen in the present study, suggesting differences in the responses of the plants to infection by pathogens and mycorrhizal fungi.

The activity of Gd was increased in both the VAM treatments and the phosphorus treatment (Table 3). This increase was also seen in non-VAM roots of the VAM plants, confirming that the stimulation was due to improved P nutrition of the host plant. A stimulation of Gd in VAM roots was also found by Dehne (1986), but in his

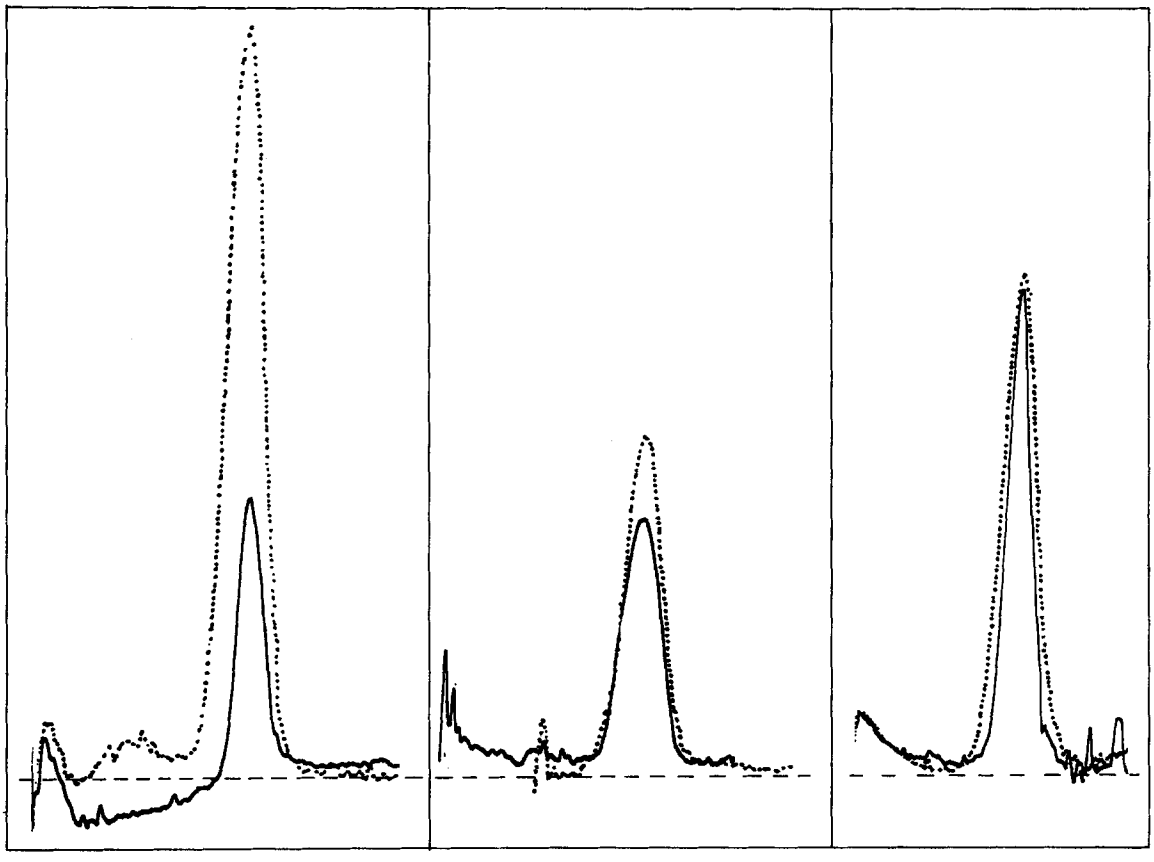

Fig. 4. Densitometric tracings of gels stained for GDH. **a** Full line is roots colonized by *Glomus claroideum*; dotted line is uncolonized roots of the same plant. **b** Plants with both part of the root system colonized by *Glomus claroideum*. Full line is the left part, and dotted line is the right part of the root system. **c** Non-VAM plants. Full line is the left part, and dotted line is the right part of the root system.

study the plant and fungal enzymes were not separated. Gd is the first enzyme in the pentose phosphate pathway (PPP), an alternative pathway by which plants obtain energy. The pathway is of special importance for plants under stress, including plants attacked by pathogens (Bollard and Matthews, 1966). A stimulation of Gd supposedly occurs in these situations to supply the pathogen or the symbiont with energy. In the present study the effect of VAM symbiosis could be achieved by the addition of P, indicating that the effect was not specifically related to the presence of the symbiont. The PP pathway also provides the ribose-5-phosphate used in nucleic acid synthesis, but the importance of this to VAM plants is probably limited since no increase in nucleic acid content has been found (Blair et al., 1988). An interesting function of the PP pathway is the support of erythrose 4-phosphate which is a precursor for synthesis of several phenolic compounds, including lignin, in the plant. Increased lignin content in VAM roots has been reported (Dehne and Schönbeck, 1979). The increase in Gd found in the present study could then be linked with an increased lignification of VAM roots.

Smith et al. (1985) detected low activity of GDH in VAM and non-VAM roots. In the present study activity of GDH was easily detected in the host roots, although fungal bands were not seen (Fig. 4). The most important function of GDH in plants is the catalysis of the oxidative deamination of glutamate (Miflin and Lea, 1977). The presence of VAM in the root system may influence the amino acid metabolism of the host by increasing the specific activity of some

enzymes in the ornithine-cycle (Dehne et al., 1978). In the present study the enzymes involved in amino acid metabolism (GOT and GDH) were not specifically stimulated by VAM. However, the amino acid degradation by GOT and GDH is not directly linked with the ornithine-cycle. The significantly lower activity of GDH in plants inoculated with *G.claroideum* is interesting. However, the lower activity/mg could be due to lower concentration of protein in the extract, but it could also be an artifact due to inaccurate determination of the fungal biomass. The fungal biomass was calculated on basis of the values found for the E3 strain, but the actual biomass could be higher. The fungal GOT and GDH bands were not detected on the gels (Figs. 3, 4), although GOT activity has been detected in both spores and mycelium of a variety of VAM fungi (Hepper et al., 1986; Sen and Hepper, 1986). However, GOT activity has been reported to be rather labile and occasionally absent in extracts, probably due to the extraction procedure (Hepper et al., 1986).

The correlation coefficient between the intensity of the fungal MDH bands and the concentrations of glucosamine was found to be 0.85, confirming that fungal colonization can be quantified on the basis of the enzyme activity (Rosendahl et al., 1989). The activity of the VAM fungal enzyme MDH was up to 40% of the host MDH activity for one of the fungi, *G.claroideum*. This result is similar to findings from VAM onions in which the mycorrhizal specific alkaline phosphatase constituted up to 32% of the total activity (Gianinazzi-Pearson and Gianinazzi, 1978).

The fungi constituted an unknown proportion of the root biomass, and the plant biomass of VAM and non-VAM roots could not be compared directly, without subtracting the estimated fungal biomass. However, this biomass is not easily determined. Hepper (1977) measured the content of glucosamine in external mycelium and found values from 21–40 $\mu g\, mg^{-1}$ dry weight and these values are used in the present study. However, the content of glucosamine per mg hyphae is probably not equal in external and internal mycelium, as the morphology of the hyphae differ. Although the results of the present study indicate that *G.mosseae* has a 10 times higher activity of MDH per biomass, than *G.claroideum*, this difference could both be due different infection anatomy and/or difference in chitin content or difference in activity of the enzyme. Ongoing studies are attempting to elucidate this.

Acknowledgements

The work was supported by a grant from the Danish Natural Science Research Council. I wish to thank Dr John Dodd, University of Kent and Mikako Sasa, University of Copenhagen for their critical comments on the manuscript and to C Walker for help with identification of the fungi.

References

Abbott L K and Robson A D 1984 The effect of mycorrhizas on plant growth. *In* VA Mycorrhizae. Eds. C L Powell and D J Bagyaraj. pp 113–130. CRC Press, Boca Raton, FL.

Blair D A, Peterson R L and Bowley S R 1988 Nuclear DNA content in root cells of *Lotus* and *Trifolium* colonized by the VAM fungus *Glomus versiforme*. New Phytol. 109, 167–170.

Bollard E G and Matthews R E F 1966 The physiology of parasitic disease. *In* Plant Physiology. A Treatise. Vol. IVB: Metabolism: Intermediary Metabolism and Pathology. Eds. F C Steward. pp 417–550. Academic Press, New York, London.

Capaccio C M and Callow J A 1982 The enzymes of polyphosphate metabolism in vesicular-arbuscular mycorrhizas. New Phytol. 91, 81–91.

Carling D E, Riehle W G, Brown M F and Johnson D R 1978 Effects of a vesicular-arbuscular mycorrhizal fungus on nitrate reductase and nitrogenase activities in nodulating and non-nodulating soybeans. Phytopathology 68, 1590–1596.

Dehne H W 1986 Influence of VA mycorrhizae on the host plant physiology. *In* Mycorrhizae: Physiology and Genetics. Eds. V Gianinazzi and S Gianinazzi. pp 431–435. INRA, Dijon, France.

Dehne H W 1987 Zur Nutzung der VA Mykorrhiza als Antistressfaktor. Angew. Bot. 61, 135–143.

Dehne H W, Schönbeck F and Baltruschat H 1978 The influence of endotrophic mycorrhiza on plant diseases. 3. Chitinase-activity and ornithine-cycle. Z. Pflanzenkr. Pflanzenschutz 85, 666–678.

Dehne H W and Schönbeck F 1979 Unterschungen zum Einfluss der endotrophen Mykorrhiza auf Pflanzenkrankheiten. II. Phenolstoffwechsel and Lignifizierung. Phytopath. Z. 95, 210–216.

Dodd J C, Burton C C, Burns R G and Jeffries P 1987 Phosphatase activity associated with the roots and the rhizosphere of plants infected with vesicular-arbuscular mycorrhizal fungi. New Phytol. 107, 163–172.

Gianinazzi-Pearson V and Gianinazzi S 1976 Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. I. Effect of mycorrhiza formation and phosphorus nutrition on soluble phosphatase activities in onion roots. Physiol. Veg. 14, 833–841.

Gianinazzi-Pearson V and Gianinazzi S 1978 Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. II. Soluble alkaline phosphatase specific to mycorrhizal infection in onion roots. Physiol. Pl. Pathol. 12, 45–53.

Harris H and Hopkinson D A 1978 Handbook of Enzyme Electrophoresis in Human Genetics (with supplements). Oxford American Publishing Co., New York.

Hepper C M 1977 A colorimetric method for estimating vesicular-arbuscular mycorrhizal infection in roots. Soil Biol. Biochem. 9, 15–18.

Hepper C M, Sen R and Maskall C S 1986 Identification of vesicular-arbuscular mycorrhizal fungi in roots of leek (*Allium porrum* L.) and maize (*Zea mays* L.) on the basis of enzyme mobility during polyacrylamide gel electrophoresis. New Phytol. 102, 529–539.

Krishna K R, Bagyaraj D J and Papavinsasundaram K G 1983 Acid and alkaline phosphatase activities in mycorrhizal and uninfected roots of *Arachis hypogaea* L. Ann. Bot. 51, 551–553.

Lowry O H, Rosenburg N J, Farr A L and Randall R J 1951 Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265–275.

McGee P A and Smith S E 1990 Activity of succinate dehydrogenase in vesicular-arbuscular mycorrhizal fungi after enzymic digestion from roots of *Allium porrum*. Mycol. Res. 94, 305–308.

Miflin B J and Lea P J 1977 Amino acid metabolism. Annu. Rev. Plant Physiol. 28, 299–329.

Oliver A J, Smith S E, Nicholas D J D, Wallace W W and Smith F A 1983 Activity of nitrate reductase in *Trifolium subterraneum*: Effects of mycorrhizal infection and phosphate nutrition. New Phytol. 94, 63–79.

Reddy M N and Stahmann M A 1975 Malate dehydrogenase in the Fusarial wilt disease of peas. Physiol. Plant Pathol. 7, 99–111.

Rosendahl S 1989 Comparisons of spore-cluster forming *Glomus* species (Endogonaceae) based on morphological characteristics and isoenzyme banding patterns. Opera Bot. 100, 215–223.

Rosendahl S, Sen R, Hepper C M and Azcon-Aguilar C 1989 Quantification of three vesicular-arbuscular mycorrhizal fungi (*Glomus* spp.) in roots of leek (*Allium porrum*) on the basis of activity of diagnostic enzymes after polyacrylamide gel electrophoresis. Soil Biol. Biochem 21, 519–522.

Sen R and Hepper C M 1986 Characterization of vesicular-arbuscular mycorrhizal fungi (*Glomus* spp.) by selective enzyme staining following polyacrylamide gel electrophoresis. Soil Biol. Biochem. 18, 29–34.

Smith S E, St John B J, Smith F A and Nicholas D J D 1985 Activity of glutamine synthetase and glutamate dehydrogenase in *Trifolium subterraneum* L. and *Allium cepa* L.: Effects of mycorrhizal infection and phosphate nutrition. New Phytol. 99, 211–227.

Tanksley S D and Orton T J 1983 Isozymes in plant genetics and breeding, Part A. Elsevier, Amsterdam, Oxford, New York.