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The presence of the arbuscular mycorrhizal fungus *Glomus intraradices* influences enzymatic activities of the root pathogen *Aphanomyces euteiches* in pea roots

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Abstract Fungal enzyme activities were quantified in an interaction study between the fungus *Glomus intraradices* and the pea pathogen *Aphanomyces euteiches*. Fungal and host enzymes were separated by polyacrylamide gel electrophoresis and the activity of *A. euteiches*–specific glucose-6-phosphate dehydrogenase (Gd), phosphoglucomutase and peptidase (PEP) enzymes were quantified by densitometry. The activity of *A. euteiches*–specific enzymes increased until 14 days after inoculation with *A. euteiches*, and then decreased. The plants preinoculated with *G. intraradices* showed no symptoms of severe root rot even though the pathogen was present and active in these plants. Thus, plants preinoculated with *G. intraradices* were more tolerant of infection with *A. euteiches* than non-mycorrhizal plants. This effect was evident even though the *A. euteiches* infection levels of mycorrhizal and non-mycorrhizal plants were the same. *A. euteiches* enzyme activities in the mycorrhizal plants were different to those in non-mycorrhizal plants. The peaks of PEP and Gd enzyme activity of *A. euteiches* were lower and the development of *A. euteiches* PEP activity was later in the mycorrhizal plants than in the non-mycorrhizal plants.

Key words *Aphanomyces euteiches* · Arbuscular mycorrhiza · Isozymes · Activity · Interaction

Introduction

Plants colonized by arbuscular mycorrhizal (AM) fungi are known to suffer less from infection by a wide range of soil-borne pathogens (Dehne 1982; Hooker et al. 1994). In spite of the large number of reports on this subject, the actual mode of action is unknown (Benhamou et al. 1994). A number of hypotheses have been proposed, including enhanced nutritional status of the host plant (Sharma et al. 1992), production of antimicrobial compounds (Morandi and Gianinazzi-Pearson 1986) and changes in the microbial community in the rhizosphere around mycorrhizal roots (Meyer and Linderman 1986).

In most studies the pathogen was quantified by monitoring the symptoms of the host plants (Bärtschi et al. 1981; Kaye et al. 1984; Rosendahl and Rosendahl 1990). However, this does not distinguish direct effects on the pathogen and indirect mycorrhizal effects through the host plant. In a previous study, preinoculation with an AM fungus eliminated the damage caused by the root pathogen *Aphanomyces euteiches* Drechs. (Rosendahl 1985). The pathogen was quantified in the roots after staining with trypan blue, which does not discriminate between active and inactive fungal structures – an important consideration for understanding the protective effect of AM fungi.

Polyacrylamide gel electrophoresis followed by densitometry of specific fungal enzymes has proven successful for quantifying the activity of both fungus and host in AM symbioses (Gianinazzi-Pearson and Gianinazzi 1976; Rosendahl et al. 1989; Rosendahl 1992; Thingstrup and Rosendahl 1994). Measurements of specific enzymes allows selective measurements of different fungi in the same root system (Hepper et al. 1988). The objective of this present study was to determine the effect of preinoculation with the AM fungus *Glomus intraradices* Schenck & Smith on the activity of three different *A. euteiches* enzymes expressed in the roots.

Materials and methods

Plant material and mycorrhizal inoculation

Seeds of *Pisum sativum* L. (cv Ping-Pong) were surface sterilized for 2 min in 70% ethanol, rinsed in distilled water and pregerminated for 4 days on moist filter paper. Two plants each were

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grown in pots containing 612 g of autoclaved soil (pH 5.5, 6.7 mg Pkg^{-1} as Resin P):sand (1:3). The plants were inoculated with 5 ml of a dense *Rhizobium* culture (Risø strain 18a). The *Rhizobium* culture was maintained on yeast glucose agar (YGA; 0.56 g $K_2HPO_4 \cdot 3\ H_2O$, 0.1 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g NaCl, 10 g glucose, 0.4 g yeast extract, 15 g agar in 1 l distilled H_2O), and the inoculum was made by transferring cells from YGA media to YG broth (0.56 g $K_2HPO_4 \cdot 3\ H_2O$, 0,1 g $MgSO_4 \cdot 7\ H_2O$, 0.1 g NaCl, 10 g glucose, 0.4 g yeast extract, in 1 l distilled H_2O) and incubating on a rotary shaker for 2 days at 24 °C. The inoculum of *G. intraradices* (strain 28A) was produced on maize (*Zea mays* L., F1 Spirit) grown in a soil/sand mixture. The inoculum contained colonized roots, spores and external mycelium and was homogeneously mixed with tap water and fine sand before being divided into 20 equal portions for the pots with the mycorrhizal treatment. An equivalent amount of the sand/soil mixture was mixed into the non-mycorrhizal pots. Plants were grown in a growth chamber with a daylight period of 16 h (225–300 $\mu Em^{-2}s^{-1}$) and a temperature regime of 22 °C/16 °C (day/night). The plants were rotated in the chamber in order to obtain a randomized design, and watered to the same weight with distilled water.

Aphanomyces euteiches inoculum

After 19 days growth, the plants were inoculated with 30 000 *A. euteiches* zoospores per pot. *A. euteiches* was maintained on corn meal agar (CMA; 17 g Difco agar to 1 l distilled water) at 4 °C. The zoospore inoculum was made by transferring approximately 10 5 × 5-mm disks from the edge of a CMA culture to maltose-peptone broth (1 g maltose, 3 g peptone in 1 l distilled water) in Petri dishes. After incubating for 5 days at 24 °C, the mycelium was washed twice in autoclaved lake water to induce zoospore production. The zoospore suspension was applied to the base of the plants and the control plants received the same volume of sterile lake water.

Harvest and processing of root material

Plants were harvested 5, 8, 14, 18 and 22 days after inoculation with *A. euteiches*. The shoots were cut off beneath the cotyledons and shoot dry weight determined after drying to constant weight at 70 °C. The roots were washed carefully in cold tap water to remove soil, and kept on ice until processed. The roots were blotted dry with paper towel, cut into 1-cm segments and then divided into two fractions. One fraction was cleared in 10% KOH and stained for fungal structures with trypan blue (Phillips and Hayman 1970). The cleared and stained roots were examined for percentage root length infected with oospores of *A. euteiches* by the grid-line intersect method (Giovannetti and Mosse 1980; Rosendahl 1985). Mycelium of *A. euteiches* does not stain with trypan blue. The other root fraction was freeze-dried and ground to a fine powder with a cold mortar and pestle. The ground material (50 mg) was mixed in a 1.5-ml Eppendorf tube with 1000 µl extraction buffer (20 mM Tris-HCl, 10 mM $NaHCO_3$, 10 mM $MgCl_2$, 0.1 mM Na_2EDTA, 10 mM β-mercaptoethanol, 10% sucrose, 0.1% Triton X-100, pH 8.0) (Rosendahl and Sen 1992). The suspension was mixed for 30 s on a vortex-mixer at maximum speed. The samples were centrifuged twice for 20 min at 20 000 *g* and the supernatant recovered and frozen in aliquots at –80 °C until use. The pellets were frozen at −20 °C for determination of chitin content (see below). Four replicate root samples were extracted at each harvest.

Enzyme activity of *A. euteiches*

Electrophoresis was carried out in a discontinuous, vertical electrophoresis system (Hoefer SE 250-Mighty Small II) with 80 × 70 × 0.7-mm polyacrylamide gels. The separation gel was 7.5% acrylamide, 375 mM Tris-HCL pH 8.8, the stacking gel was 3.75% acrylamide, 124 mM Tris-HCL pH 6.8 and the electrode buffer was 25 mM Tris and 192 mM glycine at pH 8.3. The gels were run at 200 V/15 mA per gel for 90 min with cooling to 4 °C. Aliquots (5–20 µl) of the extract were loaded in each well depending on the enzyme in question. For each root sample, the extract was run twice for each enzyme. Fungal bands were identified in preliminary studies using extracts of *A. euteiches* in comparison with pea roots with or without *A. euteiches* and *G. intraradices*, and cucumber roots with known *G. intraradices* activity. The gels were stained for three enzyme activities: glucose-6-phosphate dehydrogenase (Gd) (EC 1.1.1.49), phosphoglucomutase (PGM) (EC 2.7.5.1), and peptidase (PEP) (EC 3.4.11). The staining procedures for Gd and PGM were as described by Soltis and Soltis (1989), and for PEP as described by Rosendahl and Sen (1992). The Gd and PGM bands were scanned with a densitometer (Hoefer Scientific Instruments model GS 300) to quantify the activity. The enzyme activity was expressed in arbitrary units as the peak height of the densitometric tracings of the gels. Preliminary studies had shown that peak height correlates with the area of the densitometric tracing of the enzymes in the range of protein concentrations used per gel track in this study (data not shown). The PEP bands could not be scanned with the densitometer and the activity of each diagnostic PEP band was divided into four classes: intense, moderate, weak and missing (Hepper et al. 1988). Each class was assigned with a grade from 0–3 where 0 was no band visible and 1, 2 and 3 were weak, moderate and intense activity, respectively.

Mycorrhizal colonization

The mycorrhizal colonization in the roots was determined by chitin analysis based on the colorimetric method for glucosamine (Hepper 1977). *A. euteiches* is an oomycete and does not have chitinous walls. The assay was peformed on the pellets from the enzyme extraction (Hepper et al. 1988). The background readings from the non-mycorrhizal roots at each harvest were substracted from the readings for the mycorrhizal roots.

Results

The plants inoculated with *A. euteiches* were infected and the infection increased during the experiment (Table 1). This increase was similar in mycorrhizal and non-mycorrhizal plants. The plants inoculated with *G. intraradices* were all mycorrhizal and the colonization increased during the experiment. At day 22, the glucosamine concentration was 4 µg/mg dry wt. root, which correspond to approximately 75% colonized root length (Table 1).

Shoot dry weight of the mycorrhizal plants increased during the experiment, whereas that of the non-mycorrhizal plants did not increase beyond 14 days after inoculation with the pathogen (Table 1). The shoot dry weight was significantly higher in the mycorrhizal plants than in the non-mycorrhizal plants at the last two harvests. Root dry weight did not change during the experiment but mycorrhizal plants had a higher root dry weight than non-mycorrhizal plants (Table 1).

Host and fungal bands were easily detected on the gels and on the densitometric tracings as shown for Gd (Fig. 1). The development pattern of the *A. euteiches* Gd activity was almost the same in mycorrhizal and non-mycorrhizal plants. The activity increased until 14 days after inoculation with the pathogen and then de-

Table 1 Plant shoot and root dry weights, *Aphanomyces euteiches* and mycorrhizal colonization of pea plants. The data are means of four replicates. Data followed by the same letter within each harvest were not significantly different ($P \leq 0.05$) by a comparison of means (Student-Newman-Keuls test)

Mycorrhiza inoculation	Harvest (days)	Dry weights (mg)		*A. euteiches* colonization (% root length)[a]	Mycorrhizal colonization (μg glucosamine per mg root dry wt.)
		Shoot	Root		
−	5	569a	199a	0a	−
+	5	569a	238a	0a	1.5
−	8	697b	230b	21b	−
+	8	645b	187c	22b	1.8
−	14	1016c	222d	50c	−
+	14	1050c	289e	43c	2.9
−	18	819d	176f	56d	−
+	18	1228e	290g	56d	3.8
−	22	854f	193h	57e	−
+	22	1223g	253i	55e	4.0

[a] The values for % root length colonized by oospores were arc sin transformed before statistical analysis

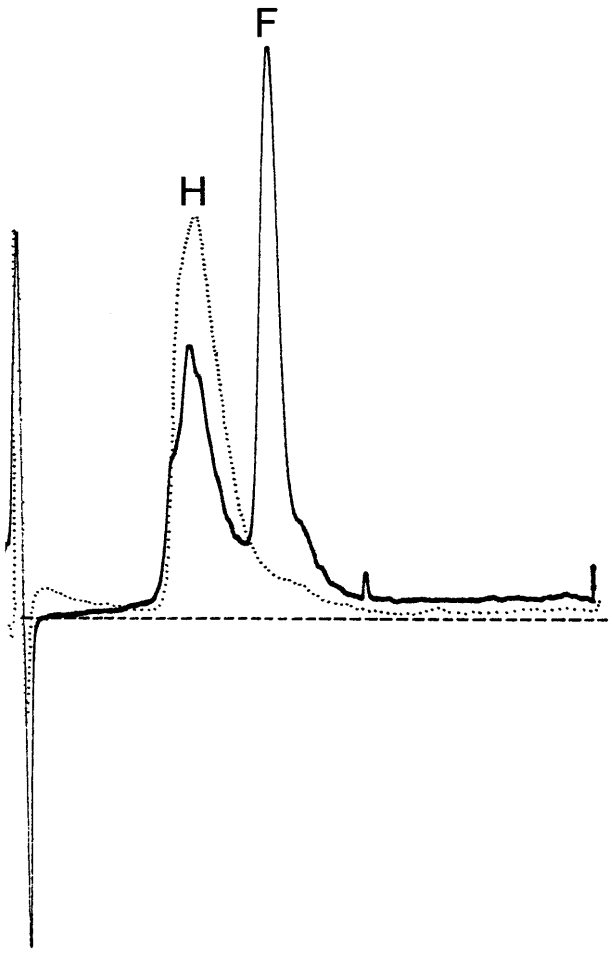

Fig. 1 Densitometric tracing of a gel stained for glucose-6-phosphate dehydrogenase (Gd). At the left of the figure is the interface between the stacking gel and the separation gel. The *full line* is the densitometric reading of an extract of roots colonized by *Aphanomyces euteiches*; the *dotted line* is the densitometric reading of an extract of uncolonized roots. The enzyme activity of *A. euteiches* was quantified as the height of the fungal peak (*F* fungal band peak, *H* lost band peak)

creased to a low level (Fig. 2). The *A. euteiches* Gd activity was significantly higher in non-mycorrhizal than mycorrhizal plants at 14 days after inoculation ($P \leq 0.05$).

The development of the *A. euteiches* PGM activity followed the same pattern as the Gd activity, but the initial activity was slightly higher than the Gd values (Figs. 2, 3). The PGM activity also had a maximal intensity at 14 days after inoculation with the pathogen and then decreased to an almost undetectable level at the last harvest. There was no significant difference in PGM activity between the mycorrhizal and non-mycorrhizal treatments ($P \leq 0.05$). The PEP activity of *A.*

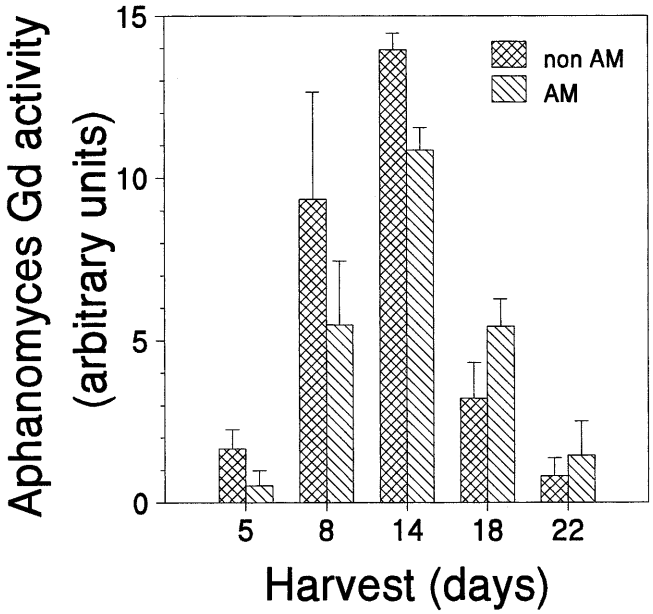

Fig. 2 *A. euteiches* Gd activity in mycorrhizal and non-mycorrhizal pea plants. Each column is the mean of four replicates and two samples of each replicate were run on the gels. Activity is expressed as peak height of the densitometric readings. *Bars* show standard errors

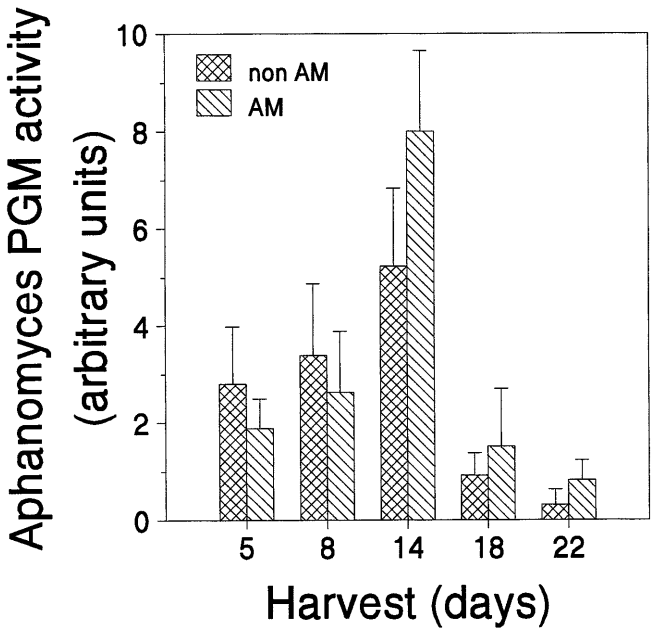

Fig. 3 *A. euteiches* phosphoglucomutase (PGM) activity in mycorrhizal and non-mycorrhizal pea plants. Each column is the mean of four replicates and two samples of each replicate were run on the gels. Activity is expressed as peak height of the densitometric readings. *Bars* show standard errors

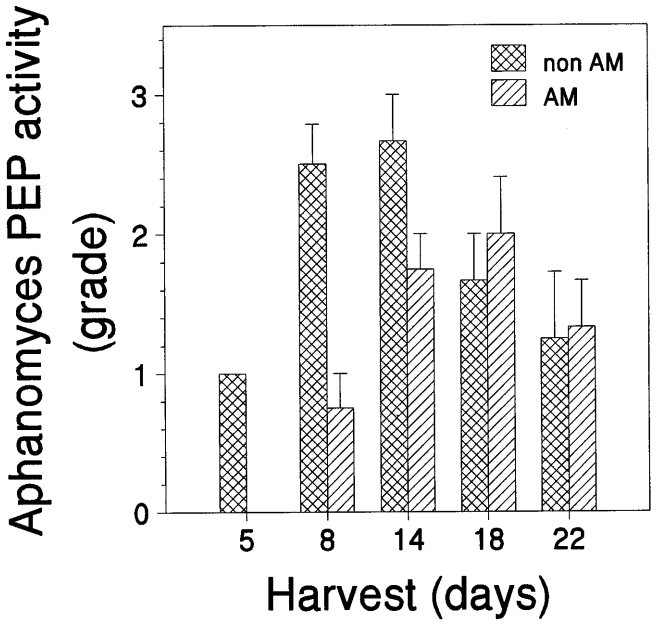

Fig. 4 *A. euteiches* peptidase (PEP) activity in mycorrhizal and non-mycorrhizal pea plants. Each column is the mean of four replicates and two samples of each replicate were run on the gels. Activity is expressed as grades from 0 to 3 where 0 indicates no visible band and 1, 2 and 3 represent weak, moderate and intense bands, respectively. *Bars* show standard errors

euteiches was absent in the mycorrhizal plants at the first harvest, while the activity was already high in the non-mycorrhizal plants 8 days after inoculation. The *A. euteiches* PEP activity was significantly higher in non-mycorrhizal than mycorrhizal plants at 5, 8 and 14 days after inoculation ($P \leq 0.05$). At the later harvests, the PEP activity was almost the same in mycorrhizal and non-mycorrhizal plants (Fig. 4).

An increased yellowing, and later browning, of the roots and epicotyl was seen as the *A. euteiches* infection progressed, and the shoots had started to wilt by the last harvests. This effect was more pronounced in the non-mycorrhizal plants than in the mycorrhizal plants but the symptoms were not quantified.

Discussion

The plants without mycorrhiza showed symptoms of severe root rot with dark, softened roots and epicotyl and wilting of the shoots. This was in contrast to the mycorrhizal plants, which in spite of the active pathogen being present were not damaged to the same degree. *A. euteiches* was shown previously to infect root segments with mycorrhiza to the same degree as uncolonized segments (Rosendahl 1985), and this was also observed although not quantified in the present study. These results suggest that mycorrhiza do not have a local, inhibiting effect on the pathogen, either via activities of the mycorrhizal fungus or a local cellular response by the plant in the presence of the mycorrhizal fungus, but rather a more general influence on root physiology.

The enzyme activities of *A. euteiches* were different in mycorrhizal and non-mycorrhizal plants. This showed the importance of sequential harvests in studies of mycorrhiza-pathogen interactions, and might explain conflicting results of past interactions studies (Dehne 1982). The PEP and Gd activities of *A. euteiches* were suppressed in mycorrhizal plants compared with non-mycorrhizal plants. As the *A. euteiches* infection was similar in mycorrhizal and non-mycorrhizal plants, the lower activities are probably not due to a lower pathogen biomass. This is in contrast to Rosendahl's (1985) observation of a stronger reduction in *A. euteiches* infection in mycorrhizal plants than in non-mycorrhizal plants. However, in that study, only one harvest was taken. The infection with *A. euteiches* was quantified in terms of the presence of oospores in the roots, since trypan blue does not stain mycelium. The enzymes detected probably originated from mycelium and not from oospores, but this can only be clarified using specific stains for *A. euteiches* mycelium.

The patterns of *A. euteiches* enzyme activity suggest that the initial, parasitic phase of the pathogen was relatively short, with oospores formed as the fungus entered its resting state, and that this occurred later in mycorrhizal than in non-mycorrhizal roots. *A. euteiches* may be regarded as a biotrophic pathogen which is only active when the plant is still healthy. This agrees with

the observation that *A. euteiches*-infected pea plants in the field are killed by secondary infections of e.g. *Fusarium solani,* which can enter the vascular cylinder in the weakened plants (Papavizas and Ayers 1974). The short time that *A. euteiches* is metabolically active might also explain the ineffectiveness of fungicides against this pathogen (Pfender 1989), which usually affect pathogens most in active growth. When the symptoms of *A. euteiches* root rot appear, fungal activity is already low as the fungus enteres a resting state.

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