Rapid assessment of acid phosphatase activity in the mycorrhizosphere and in arbuscular mycorrhizal fungal hyphae

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Abstract A pot experiment has been carried out under controlled conditions to study the possibility of applying the technique of *in vivo* staining for acid phosphatase activity on the roots of mycorrhizal plants and arbuscular mycorrhizal hyphae. The pots had 5 compartments. The central root compartment was separated from the two adjacent hyphal compartments using nylon nets of 30 μm mesh, and the two hyphal compartments were separated from the two outermost compartments with 0.45 μm membranes. Red clover was grown in the root compartment and was either inoculated with the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* or uninoculated.

Sodium phytate was applied to all compartments. The results show that AMF can increase acid phosphatase activity of clover roots. The plant roots acquired deep red "mycorrhizal prints". The external hyphae also had obvious "hyphal prints" on the test papers, indicating the ability of mycorrhizal hyphae to release acid phosphatase.

Keywords: acid phosphatase, arbuscular mycorrhizal hyphae, red clover.

The technique of *in vivo* staining, based on agar as a medium, has been developed to observe chemical changes in the rhizosphere of plants, including changes in pH, oxidation processes, excretion of organic acids, reduction of FeIII and MnIV and the solubilization of various inorganic phosphates. Dinkelaker and Marschner[1] were able to demonstrate rhizosphere acid phosphatase activity at the root-soil interface by modifying the method of Warren and Moss[2] for *in vivo* staining of acid phosphatase in zoological studies. The aim of this experiment was to develop a rapid method for assessing the capacity of extramatrical mycorrhizal hyphae to secrete acid phosphatase.

1 Materials and methods

The experiment used rhizoboxes with 5 compartments (fig.1): one central compartment for root growth, two adjacent compartments for hyphal growth separated from the central root compartment by nylon nets of 30 μm mesh size, and two outer compartments separated from the hyphal compartments by 0.45 μm membranes which neither roots nor hyphae could penetrate. Mycorrhizal hyphae were thus expected to accumulate on the membrane.

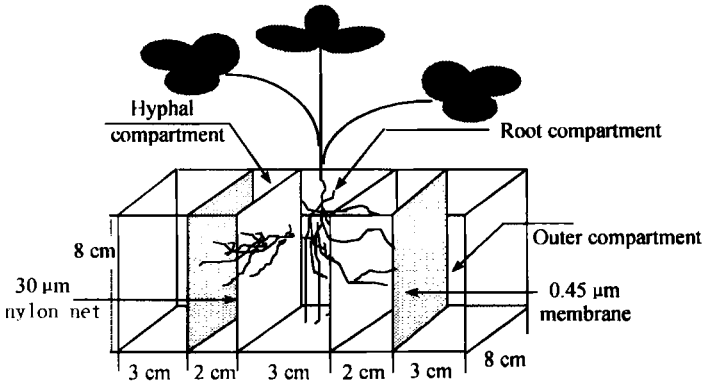

Fig. 1. Diagram of the pot used.

The soil used was from the Changping Long-Term Fertilizer Station of China Agricultural University. Selected physical and chemical characteristics of the soil are presented in table 1. The soil was air dried, sieved (< 2 mm) and stored in plastic containers. The plant species used was red clover (*Trifolium pratense* L) and the mycorrhizal fungus was *Glomus mosseae* propagated on clover plants grown in a glasshouse for three months.

Table 1 Selected physical and chemical properties of the experimental soil

Organic C (%)	Olsen-P/mg • kg^{-1}	1 mol • L^{-1} NaOH extractable N /mg • kg^{-1}	NH_4OAc-K/mg • kg^{-1}	pH ($CaCl_2$)
1.24	2.56	87.2	100.3	7.5

There were two treatments in the experiment, either inoculated with mycorrhizal fungus or uninoculated. The mycorrhizal treatments received 30 g of mycorrizal inoculum mixed with 120 g sterilized soil and placed in the root compartment. Mycorrhizal inoculum consisted of rhizosphere soil, spores and infected root segments. In order to obtain the same microbial population in the control treatment, non-mycorrhizal pots received 10 mL of filtrate from the inoculum together with 30 g sterilized inoculum. In all treatments mineral nutrients were added uniformly to each compartment at the rates of 50 mg P (as Na-phytate), 200 mg N (NH_4NO_3), 200 mg K (K_2SO_4), 50 mg Mg ($MgSO_4$) and 5 mg Zn ($ZnSO_4$) kg^{-1} soil. During the growth of the plants, 50 mg • kg^{-1} N (as NH_4NO_3) was added to ensure an adequate supply of N to the plants. Each hyphal compartment was filled with 100 g sterilized soil, each outer compartment with 150 g sterilized soil.

Red clover seeds were surface sterilized using 10 % (*v*/*v*) H_2O_2 for 10 min and were subsequently washed with distilled water. 70 seeds were planted in the root compartment. The surfaces of all

compartments were covered with a 2-cm layer of sterilized quartz sand to minimize evaporation and contamination. The experiment was carried out in a growth chamber at 20—28℃ and kept in a 15—9 h day-night regime during the growth period.

Plants were harvested after 50 d of growth. The pots were opened by dismantling the nylon net and membrane barriers. Impregnated test papers were placed on the surface of roots of mycorrhizal and non-mycorrhizal plant and on the mycorrhizal hyphae.

(i) Chemical reagents used in the *in vivo* staining method. (a) 50 mmol • L^{-1} pH 5.6 (7.0, 9.0) trisodium citrate buffer: trisodium citrate solution (50 mmol • L^{-1}), adjusted to pH 5.6 with HCl and NaOH (1 mol • L^{-1}); (b) 37.5 mmol • L^{-1} (pH 5.6) 1-naphthyl phosphate disodium salt (Sigma Chemical Co.) in pH 5.6 (7.0, 9.0) citrate buffer; (c) 2.7 mmol • L^{-1} Fast Red TR (Sigma Chemical Co.) in pH 5.6 citrate buffer.

Citrate buffer was stored for up to several weeks at 0—4℃ and the other two solutions were prepared within 2 h before use.

(ii) Procedure. Before staining, the substrate solution and the Fast Red TR solution were mixed at a ratio of 1:10 (*v/v*) and poured into a Petri dish. The volume of solution used depended on the volume of test paper and was found to be suitable when sufficient to submerge the test paper. Whatman MN260 chromatography paper is recommended as a test paper but other fine-textured and inert filter papers might be suitable. The Whatman chromatography papers were placed in the Petri dishes for 10 s, placed on a sheet of Plexiglass and blotted with paper tissues to remove excess solution. The impregnation and air-drying of the test papers were carried out in a fumehood to prevent the inhalation of toxic volatiles and accelerate the drying process). When the test papers were dry, a 5-cm strip of the margin was cut off and discarded because higher concentrations of the reagents tended to accumulate at the margins. Higher concentrations of Fast Red TR were found to result in an enhancement of the indicator reaction[3] on the test papers in this experiment (results not shown). The treated test papers were then placed directly on the root surface. Care was taken to ensure that the test papers were placed on the same part of root surface in the different treatments. In order to ensure good contact between the test paper and the roots, evaporation of water was minimized by covering the papers with plastic film. The whole procedure was carried out in the growth room in the conditions under which the plants had grown to avoid disruption of the physiological activity of the plant roots and fungal hyphae. The test papers were removed after half an hour or one hour and results of staining were observed.

2 Results

The pots were opened by dismantling the nylon net and membrane barriers after 50 d of plant growth. It was observed that red clover roots inoculated with the mycorrhizal fungus grew densely and were distributed widely throughout the root compartment. In contrast, there was less plant root growth in the control treatment and the root distribution was sparse. Acid phosphatase activity of the mycorrhizosphere of clover was visualized as deep red "mycorrhizal prints" on the test papers. All parts of the root system produced a "root and mycorrhizal print" of varying intensity. As expected, the intensity of the colour of the "mycorrhizal prints" depended on the duration of application (fig. 2(a)—(d)). Application for only 5 min resulted in a faint, poorly differentiated colour development which intensified gradually with increasing application time. We observed that the colour of the "mycorrhizal prints" was stronger and clearer following application for one hour (fig. 2(c)) compared with half an hour (fig. 2(a)). However, further increase in the duration of application did not lead to any further intensity in the "root and mycorrhizal prints" (figure not shown). The colour reaction could still occur at pH 7.0 in the experiment, but the reaction was clearly weaker than at pH 5.6. The reaction was strongly inhibited at pH 9.0 with no trace of colour formation on the test papers. Under a dissecting microscope a dense hyphae mat was observed to have formed in the soil near the 0.45 μm membrane (fig. 3). Most of the hyphae were transparent and had obvious mucilage on their surface, suggesting that they still had strong activity. By applying several drops of assay mixture solution directly onto the membrane immediately after opening the culture system, hyphal prints were obtained. This demonstrated that living hyphae produced a deep or light red print on the test paper (fig. 4).

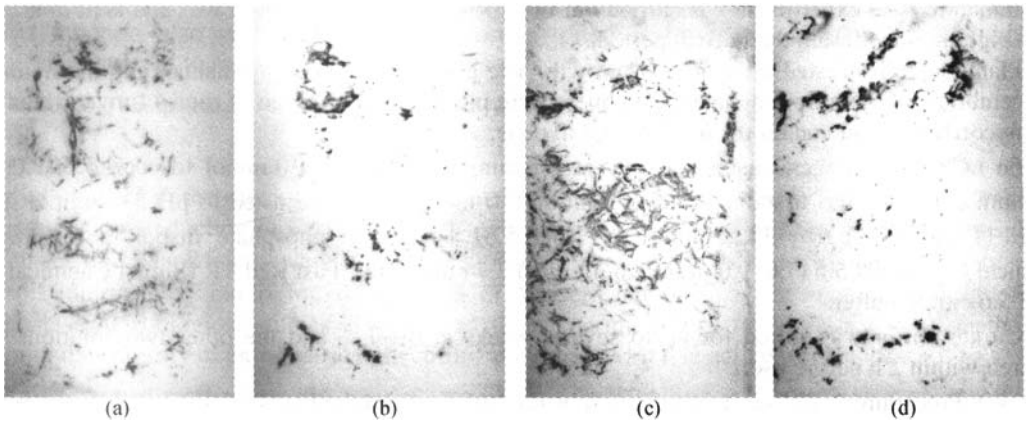

Fig. 2. (a) Mycorrhizal prints formed after 0.5 h in the inoculated treatment; (b) root prints formed after 0.5 h in the control treatment; (c) mycorrhizal prints formed after 1 h in the inoculated treatment; (d) root prints formed after 1 h in the control treatment.

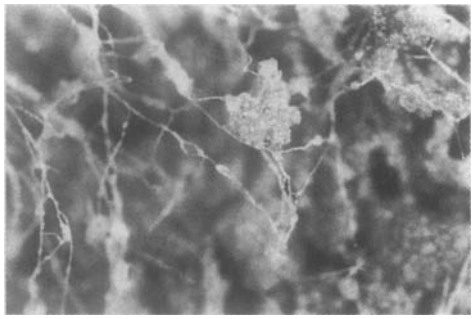

Fig. 3. Mycelium formed in the soil near the 0.45 μm membrane, ×160.

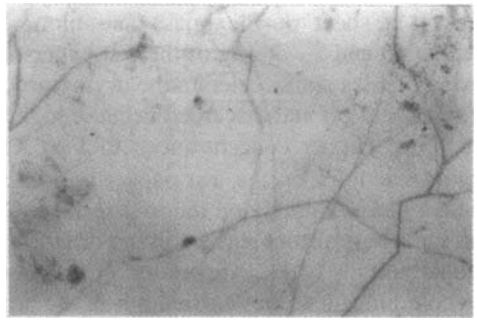

Fig. 4. Hyphal prints formed on the 0.45 μm membrane after dropwise application of assay mixture, ×250.

3 Discussion

In this experiment the mycorrhizal prints originated mainly from the activity of acid phosphatase released by the plant roots. As an ectoenzyme, acid phosphatase could also be associated with root dermal cell walls[4] or released in the mucilage[5]. Some of the acid phosphatase activity may have been derived from soil microorganisms since an open culture system was used, but the contribution from this source was small as indicated by a very low phosphatase activity in the bulk soil (fig. 2(a)—(d)). The deep colour of the root tips (fig. 2(a)—(d)) indicates that there was high phosphatase activity at these root zones, and this is in agreement with the observations of Dinkelaker and Marschner[1] and Shaykh and Roberts[4]. The intensity of the colour of the "mycorrhizal prints" depended on the time of reaction in our experiment using clover.

The test paper reaction at different pH values suggested that it was acid phosphatase and not alkaline phosphatase that was responsible for the hydrolysis of 1-naphthyl phosphate in our experiment because the reaction was strongly inhibited at pH values above 7.0. The colour intensity was weaker at pH 7.0 than at pH 5.6, probably because of the strong buffering capacity of the soil used. As Dinkelaker and Marschner[1] reported, use of a suitable substrate is a key factor for success in this type of experiment. The acid phosphatase activity on the surfaces of plant roots and soil are usually measured with p-nitrophenyl phosphate (pNPP) as the substrate. However, this was not suitable for our experiment because the end product (pNPP) of the colour reaction is soluble. Spraying the filter paper with an alkaline solution at the end of the application period would result in diffusion of the dye and the formation of blurred "root prints". On the other hand, the method with 1-naphthyl phosphate resulted in

an insoluble and diffusive end product[13], thus sharp "root prints" appeared on the test papers. In addition, the method with 1-naphthyl phosphate has the advantage that the reaction between 1-naphthyl phosphate and Fast Red TR takes place at low pH (pH 7.0).

The simple method with 1-naphthyl phosphate permits direct and continuous observation of acid phosphatase activity, but it cannot replace the standard methods *in vitro*. However, the test paper method and the *in vivo* staining are non-destructive and can be applied to soil-grown mycorrhizal plant roots, thus minimizing loss of phosphatase activity. Phosphatase activities of all parts of a mycorrhizal system and the surrounding soil are visualized at the same time. This technique could localize root zones with different phosphatase activity on the basis of differences in colour intensity (fig. 2 (a)—(d)). The method can be used to compare the phosphatase activity of different plant species or genotypes in reflecting their capacity to utilize organic phosphorus depending on growth period, phosphorus nutritional status, temperature and other environmental factors. Another area of application is ectomycorrhizal roots[16]. In our experiment we applied the method to endomycorrhizal roots and succeeded in demonstrating acid phosphatase activity of the surface of endomycorrhizal roots and their extramatrical hyphae growing in rhizoboxes under controlled conditions (figs. 2 and 4). However, the method could not eliminate the effects of microorganisms, and further work is required to determine the specific contribution of mycorrhizal hyphae to the total phosphatase activity.

Acknowledgements This work was supported by the Major State Basic Research Development Program of China (Grant No. G1999011807) and the National Natural Science Foundation of China (Grant No. 39790100).

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(Received September 20, 1999; accepted December 24, 1999)