Visual demonstration of *in vivo* acid phosphatase activity of VA mycorrhizal fungi

J. C. Tarafdar
Central Arid Zone Research Institute, Jodhpur 342 003, India

For nondestructive and visual demonstration of the release of acid phosphatase by vesicular–arbuscular (VA) mycorrhizal fungi, filter papers treated with a mixture of 1-naphthyl phosphate as substrate and the diazonium salt Fast Red TR as an indicator were used. Wheat (*Triticum aestivum* L.) was grown for 6 weeks in sterilized soils in specially designed pots with five compartments with or without hyphal and root barriers. Plants were inoculated with *Glomus mosseae* (Nicol & Gerd) Gerd & Trappe and the treated filter paper was placed at the outer surface of the hyphal compartment. Acid phosphatase activity was visualized as a red-coloured 'hyphal print' on the filter paper.

PHOSPHATASE activity in the rhizosphere may originate from the plant roots[1], from fungi such as *Aspergillus*[2], ectomycorrhizas[1] or from bacteria[3]. Vesicular–arbuscular (VA) mycorrhizas are widespread in soils, and their beneficial effect on plant growth in P-deficient soils is attributed to uptake and translocation of P by external hyphae[4]. VA mycorrhizal storage of polyphosphates in the hyphae has also been reviewed[5]. Our recent study indicated[6] the production of phosphatase by external hyphae. Here a nondestructive method for the visualization of acid phosphatase activity by VA mycorrhizal fungal hyphae is described.

A special Plexiglass pot (Figure 1) comprising of five compartments, a central one for root growth, two adjacent compartments separated from the central compartment by a nylon net of 30 μm mesh size, for growth of VA mycorrhizal hyphae (hyphal compartments), and two other compartments, separated by a 0.45 μm membrane, through which neither roots nor hyphae could penetrate, has been described by Tarafdar and Marschner[6]. It was modified by placing a Plexiglass barrier (2 cm × 2 cm) between hyphae and outer compartment on one side of the pot to prevent hyphae to pass into that side. Wheat plants (cv. Star) were grown in sterilized soils of Bavendorf under controlled conditions. The soil used was a cambisol of pH 6.9 and sieved (2 mm) prior to sterilization (48 h each at 120°C, on two alternate days). Water was supplied directly into the root compartment using PVC tubes with string wicks inserted in bottles containing sterilized distilled water.

The mycorrhizal fungus used was *Glomus mosseae*. Each pot received about 1500 surface-sterilized (with 0.2% chloramin T and 0.02% streptomycin sulphate) infective propagules of 90–250 μm in size. The inoculum was mixed uniformly with the whole soil, under aseptic conditions, and added to the central (root) compartment of the pots. Wheat seeds were surface-sterilized and planted, four plants in each pot, into the root compartment. The top openings of all compartments were covered with 2 cm layer of sterilized quartz sand. All operations were performed in a laminar flow chamber. The pots were then transferred to a growth chamber (25–18°C and 15–9 h day–night regime, 305 μmol m^{-2} s^{-1} photon flux density).

For demonstration of phosphatase activity the method used for determination of acid phosphatase in blood serum has been modified by soaking filter papers with the reagents. In this method enzymatic hydrolysis of 1-naphthyl phosphate at pH 5.6 liberates 1-naphthol which reacts with the diazonium salt Fast Red TR (diazotized 2-amino-5 chlorotoluene 1,5-naphthalene disulphonate), forming a red dye. The suitability of 1-naphthyl phosphate as a substrate for phosphatase activity from plant tissues was tested[1] with acid phosphatase isolated from wheat germ.

For the assay the following solutions were prepared – citrate buffer: trisodium citrate solution (50 mM), adjusted to pH 5.6 with HCl (1 M); substrate solution: 37.5 mM 1-naphthyl phosphate sodium salt in citrate buffer; Fast Red TR solution: 2.7 mM Fast Red TR solution in citrate buffer. The substrate solution and the Fast Red TR solution were mixed in a ratio of 1:10 (v/v) and poured into a Petri dish. After soaking in filter papers in the assay mixture for some seconds, they were placed on Plexiglass and the excess solution was removed with paper tissues.

The impregnation and air drying of the filter papers should be carried out in a fumehood to prevent the inhalation of toxic volatiles and also to accelerate the drying process. Thereafter, a 5 mm margin of the filter papers is cut off, as it contains higher concentrations of reagents than the remaining filter paper. The filter papers should be prepared no longer than 2 days before application and stored by wrapping in an aluminium foil in a desiccator. Ashless filter paper (Blue ribbon, No. 589, Schleicher & Schüll, Dassel/Germany) was used.

For the visualization of acid phosphatase activity the pots were opened and the outer compartments separated, and an impregnated filter paper was placed on the surface of the mesh outside the hyphal compartment on both the sides (Figure 1). All the tests were carried out in the growth chamber at 25°C. The filter papers were removed after different times of application and dried.

Acid phosphatase activity by VAM hyphae is visualized as dark red (black on the photographs) 'hyphal print' on the test papers (Figure 2 *b–c*). All parts of the hyphae produced a more or less intensive hyphal print. When hyphae were not allowed to pass by introducing a block, no hyphal print in that area could be

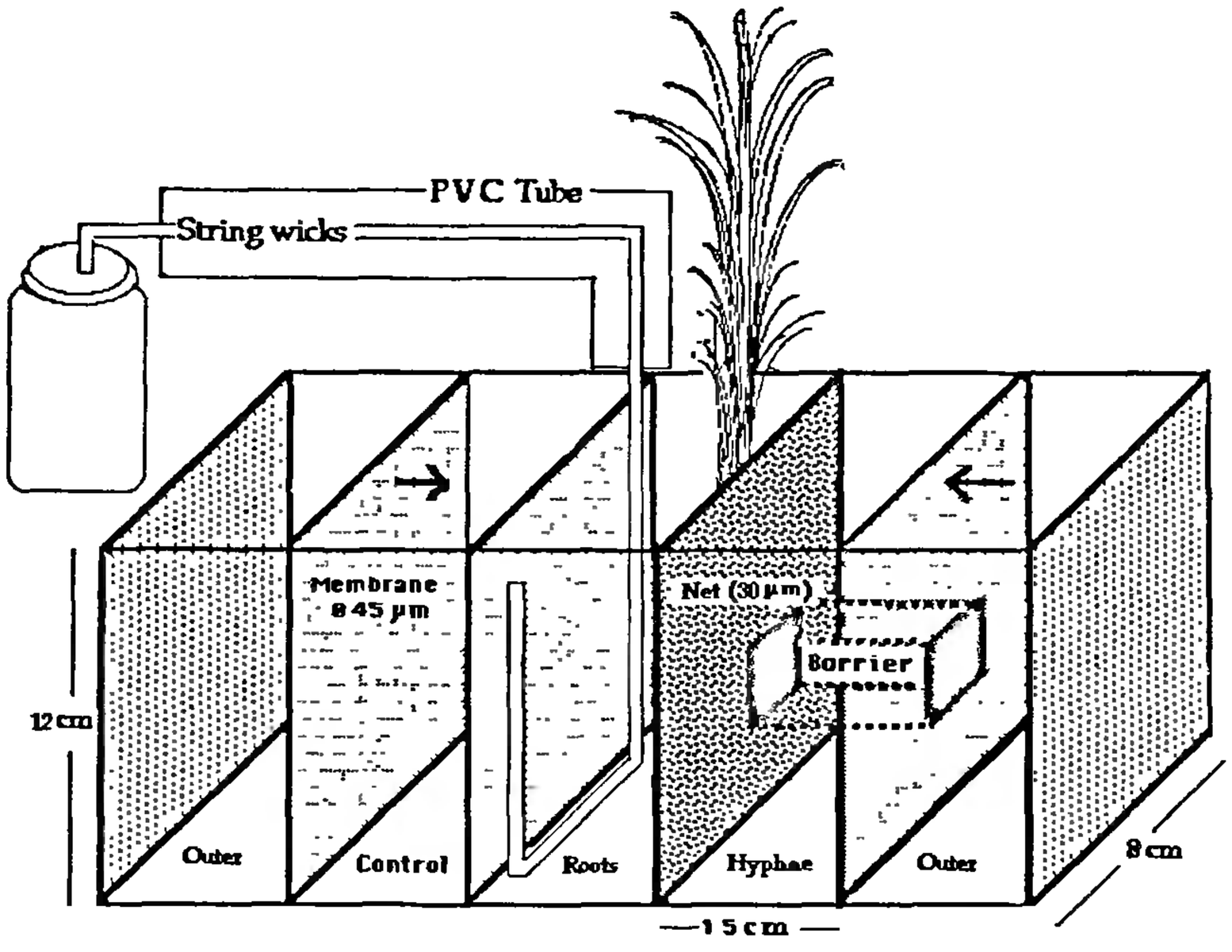

Figure 1. Details of the pots used in the experiment (modified after ref 6) Arrow denotes the position where the filter paper was put

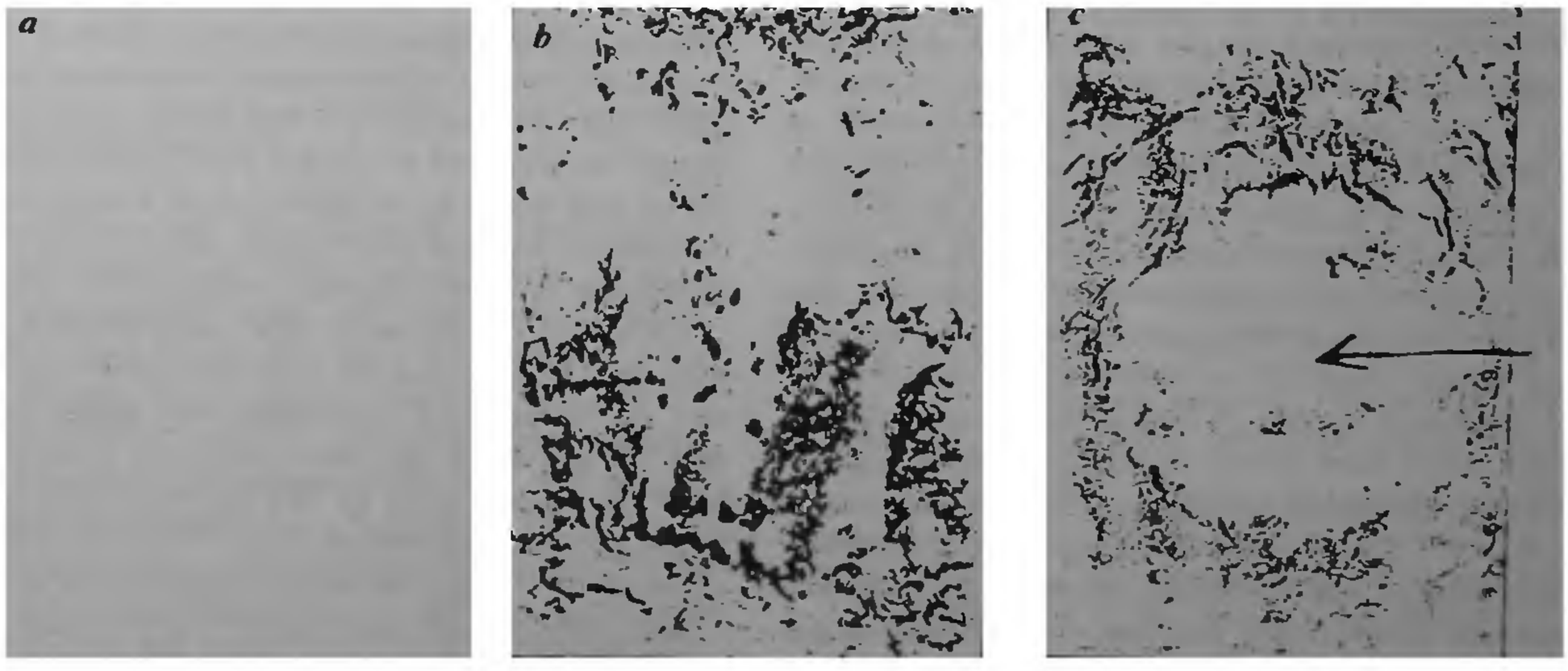

Figure 2. Fingerprints of VA mycorrhizal hyphae *a*, control, *b*, +VAM, *c*, +VAM with barrier Arrow denotes the position where the barrier was put on the hyphal path

seen (Figure 2 *c*). Similarly, no impression was noticed in control (uninoculated pots, Figure 2 *a*). Therefore, these hyphal prints mainly originated from the activity of hyphal surface phosphatase. The dark colour indicates a high phosphatase activity of these hyphal zones. As expected, the intensity of the colour of the hyphal prints depends on the duration of application. Application for only 10 min resulted in a faint, not well-differentiated colouration, whereas it got intensified gradually, with maximum intensity showing when exposed for 60 min.

Acid phosphatase activity in plant roots and soils is usually measured with *p*-nitrophenyl phosphate (pNPP) as the substrate. Since the end product, *p*-nitrophenol is soluble, spraying the filter paper with an alkaline solution at the end of the application period may result in diffusion of the dye and the formation of blurred hyphal prints. In contrast, 1-naphthyl phosphate results in an insoluble end product when no detergent is used. Thus, sharp hyphal prints appear on the filter paper. In addition, the method with 1-naphthyl phosphate has the advantage that the reaction between 1-naphthol and Fast Red TR takes place at low pH. Since acid phosphatases are characterized by maximal activities at low pH values[7], the method with 1-naphthyl phosphate permits a direct and continuous measurement of acid phosphatase activity at its optimal pH.

While 1-naphthyl phosphate is frequently used for the detection of acid phosphatase in medical media[7], only a few reports exist on the use of this substrate for the determination of acid phosphatase from soils[8,9] and from plant roots[1,9]. *In vitro* assay with acid phosphatase from wheat germ also shows that 1-naphthyl phosphate is a very suitable substrate. The present experiment confirmed the production of phosphatase by VA mycorrhizal hyphae. The method may be used for the comparison of phosphatase activity of different VA myocorrhizal hyphae as well as plant species or genotypes in relation to their capacity to utilize organic phosphorus depending upon plant age, phosphorus nutritional status, temperature and other environmental factors.

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In vitro flowering and pod formation in cauliflower (*Brassica oleracea* var. *botrytis*)

Vandana A. Kumar, Atul Kumar and Jitendra Kumar

College of Forestry and Hill Agriculture, G B Pant University of Agriculture and Technology, Hill Campus, Tehri Garhwal 249 199, India

Flowering and pod formation *in vitro* were induced in cauliflower through culture of curd explants in Murashige and Skoog medium supplemented with 3.0 mg l^{-1} indole acetic acid (IAA) and 0.5 mg l^{-1} kinetin after incubation under 16 h photoperiod at 28 ± 2°C temperature during the day and 24 ± 2°C during the night at a light intensity of 3000 lx. Pods were ready to harvest within 90 days of culture initiation and developed small, globular, smooth and brown-coloured seeds. This phenomenon, though gives small amount of seeds, may be helpful when pollination and fertilization *in vivo* are difficult. This way, the long period for flowering and pod formation under field conditions is cut short and the problem of isolation distance in cross-pollinated crops (like cauliflower) is also overcome.

BREEDING programmes in cauliflower rely mainly on raising seed crops and, under field conditions, proper isolation distance between varieties is necessary for hybridization. Besides, curding and flowering are also very sensitive to changes in temperature[1]. Initiation of flowering and complete pod formation *in vitro* may become a valuable research tool for plant breeders ensuring seed purity. This can also facilitate hybridization *in vitro* without any requirement for isolation distance. A protocol for *in vitro* flowering and pod formation in cauliflower is described.

Florets of 1 cm diameter were excised from a mature curd of cauliflower (snowball-type), surface-sterilized with 70% (v/v) ethanol for 90 s, followed by commercial bleach (calcium hypochlorite) solution (5%, w/v) containing 0.1% Tween-20 for 15 min. After three rinses with sterile distilled water, small (approx. 3 mm cubic) pieces of curd surface were dissected and implanted in the