A modified procedure for staining roots to detect VA mycorrhizas

R. E. KOSKE
Department of Botany, University of Rhode Island, Kingston, Rhode Island, U.S.A. 02881

J. N. GEMMA
Pacific Tropical Botanic Garden, P.O. Box 340, Lawai, Kauai, Hawaii, U.S.A. 96765

A modified procedure for staining roots to detect VA mycorrhizas is described. It eliminates as many toxic compounds as possible from root fixation/staining procedures without reducing the resolution of the staining. Success has been achieved with angiosperm, fern, lycopod and psilophyte roots and bryophyte.

Key words: Techniques, Staining, VA mycorrhizas.

The method most often employed to stain roots for the detection of vesicular-arbuscular mycorrhizas (VAM) is that described by Phillips & Hayman (1970). A frequent modification of this technique is the elimination of phenol from the staining and destaining solutions, e.g. Kormanik & McGraw (1982). However, even with the removal of phenol, the fixing, staining and assessment of roots expose the investigator to compounds that can induce various ill-effects ranging from irritated skin, watery eyes, nausea, dizziness, and headache to extreme hypersensitive reactions. We have sought to eliminate as many as possible of these offensive and toxic compounds from the root fixation/staining procedures without reducing the resolution of the staining. Faced with problems of availability and cost of chemicals while attempting to process roots in a field laboratory, we also sought to reduce the number of ingredients to a minimum.

The following methods have been applied successfully to over 150 species of plants from 65 families, including Angiosperms (mono- and dicotyledons), ferns, lycopods, psilophytes and bryophytes.

Fixation/Preservation. Fresh, rinsed roots are fixed and stored in 50% ethanol or isopropyl alcohol. These two alcohols are interchangeable in many fixatives (Johansen, 1940). On the basis of noxious vapours, these alcohols are ranked: ethanol (least noxious), isopropyl, denatured ethanol (Proctor & Hughes, 1978).

The alcohol solution replaces the FAA (formalin: acetic acid: alcohol) fixative used by many investigators. In preparing roots for assessment of VAM colonization, the function of the fixative is to preserve the roots until they are cleared in KOH. Thus formalin, a compound used to retain cellular fine structure for histological study (Jensen, 1962) is unnecessary. Elimination of formalin is desirable since it is a highly irritating substance and humans frequently develop hypersensitivity to it after repeated exposure (Sterling, 1985). Acetic acid, another ingredient of FAA, is caustic and its vapours may irritate eyes, nose, throat and lungs (Proctor & Hughes, 1978; Settig, 1979).

Tissue clearing. Fresh, rinsed roots or fixed roots are placed in a 2.5% aqueous solution of KOH (w/v). We routinely use ca. 3 g. (f.wt) of roots in 55 cm³ test tubes (25 x 150 mm) containing 40 ml of 2.5% KOH. Roots and KOH are autoclaved at 121 °C for 3 min or heated in a water bath at 90 °C for 10-30 min. Adequate ventilation is important as KOH fumes are caustic.

The 10% KOH solution recommended by Phillips & Hayman (1970) is harsh and may result in the loss of the cortex from much of the root system. In a survey of the mycorrhizal status of a variety of plant species in Hawaii and California, we found that most of the roots treated with 10% KOH for 40 min at 90 °C lost some or much of their cortex. Fern roots were especially sensitive. Earlier, Hepden (1960) had successfully employed lower concentration of KOH (2%) when clearing fern roots prior to staining for mycorrhizas. The sensitivity of roots to KOH varies among species, and is affected by the concentration of the KOH solution, the length and temperature of the heat treatment, and the thickness of the roots.

Rinsing and bleaching. After treatment with KOH, the roots are rinsed in several changes of water. If roots are dark at this
Table 1. Summary of staining procedures to detect vesicular-arbuscular mycorrhizal fungi in roots

(1) Heat roots in 2.5% KOH 3 min at 121° or 10–30 min at 90°.
(2) Rinse roots in water.
(3) If necessary, bleach roots in alkaline H₂O₂ for 10–30 min. Rinse in water.
(4) Soak roots in 20–50 vol. % HCl for 1–24 h.
(5) Stain roots in acidic glycerol/trypan blue for 3 min at 121° or 10–30 min at 90°.
(6) Destain and store roots in acidic glycerol.

Stage, they may be lightened in a freshly prepared solution of alkaline H₂O₂ (3 ml 20% NH₄OH in 30 ml 3% H₂O₂). We typically treat dark roots for 10–45 min. Household ammonia may be substituted for 20% NH₄OH. Other concentrations of H₂O₂ have been used for bleaching (Janos, 1984; Peterson, Ashford & Allaway, 1985), but we prefer the more readily available 3% H₂O₂ (sold in pharmacies). Rinse roots thoroughly in several changes of water.

Weak household bleach solutions, i.e. 0.525% NaOCl, are sometimes used to lighten dark roots before staining (Bevege, 1968). However, as reported by Phillips & Hayman (1970), bleaching of KOH-treated roots with hypochlorite solutions often results in reduced staining intensity of the fungi. Our experience with hypochlorite bleaches confirmed this earlier observation, and we also noted that partial decortication occurred. Thus we do not recommend hypochlorite.

Acidification. After the KOH or H₂O₂ treatments, roots are highly alkaline and must be acidified for the trypan blue dye to bind well to VAM fungal structures (Jensen, 1962). Roots are acidified by soaking in 1% HCl; the duration of the treatment and volume of the HCl solution being dependent on the root mass. We routinely use 40–50 ml/tube (a volume of acid equal to ca 20–50 x the volume of the roots) in an overnight soak. When an insufficient volume of 1% HCl is used (even if roots are submerged), the root tissues may not become acidic enough for good dye retention. The pH of 1% HCl is ca 0.65. After roots have equilibrated in the acid solution, a rise to pH 0.8–1.4 indicates that an adequate volume of acid was used, and VAM should stain well.

Staining. Acidified roots are stained in an acidic glycerol solution (500 ml glycerol, 450 ml H₂O, 50 ml 1% HCl) containing 0.05% trypan blue (Sigma Chemical Co., St Louis, U.S.A.). Roots are heated in this solution in an autoclave at 121° for 3 min or in a 90° water bath for 15–60 min, the trypan blue solution poured off and the roots destained in acidic glycerol. Destaining is normally performed at room temperature, although heating may improve contrast in roots whose cells retain excessive stain.

Stained roots have been stored for 16 months in the dark.

Figs 1–3. Mycorrhizal fungi in roots of Malacothrix incana (Compositae) stained by the procedure outlined in the text. Bar = 100 μm. Fig. 1. Arbuscule and internal hyphae. Fig. 2. Arbuscules and internal and external hyphae. Fig. 3. Internal hyphae, arbuscules and vesicles.
Observations on 'sporocarps' of the VA mycorrhizal fungus

_Rhizophagus litchii_

**R. E. KOSKE, J. N. GEMMA† AND W. C. MUELLER* **

Department of Botany and *Department of Plant Sciences, University of Rhode Island, Kingston, Rhode Island, U.S.A. 02881-0812
†Pacific Tropical Botanical Garden, P.O. Box 340, Lawai, Kauai, Hawaii, U.S.A. 96765


The structure of 'sporocarps' in _Rhizophagus litchii_ is shown to correspond with the stelar anatomy of short lateral roots of _Litchi sinensis_. Samples of the 'species' from Hawaii were also found to contain vesicles, arbuscules, hyphae and coils of VA mycorrhizal fungi. The name _R. litchii_ is considered to be of confused application.

Key words: _Rhizophagus litchii_, _Litchi sinensis_, Stelar anatomy.


Because _R. litchii_ appears to have been reported only once (Pandey & Misra, 1975) since its description, we sought to recollect it. A unique feature ascribed to this species was the formation of chlamydoospores within a sporangium-like structure (termed a 'sporocarp' by the authors). We hoped to re-examine the species using more recent taxonomic criteria that have been applied to other VA mycorrhizal fungi, e.g. Walker (1983).

Roots of _Litchi sinensis_ were collected at the Pacific Tropical Botanical Garden, Kauai, Hawaii on 18 Nov. 1987. They were washed in running water and some were examined immediately while others were cleared and stained using a modification (Koske & Gemma, 1989) of Phillips & Hayman's (1970) technique. Additional roots were fixed in 0.25% glutaraldehyde in 0.4 M cacodylate buffer, pH 7.2, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in acetone, and embedded in Spurr's low-viscosity medium. Sections (1 μm thick) were stained with 0.6% toluidine blue in acidic glycerol without a noticeable decrease in stain intensity and without growth of micro-organisms.

The omission of lactic acid and phenol from the staining and destaining solutions does not lessen the intensity of the staining, but does eliminate offensive and dangerous vapours and reduce the chance of contact with caustic substances that may irritate the skin (Arena, 1986) during the assessment of fungal colonization. Glycerol was a necessary ingredient in the staining process to achieve even deposition of the stain along the length of hyphae in infected roots.

While this staining procedure has been applied mostly to VAM, it has also successfully stained orchid and ericoid mycorrhizas and ectomycorrhizas. The procedure is summarized in Table 1, and examples are shown in Figs 1–3.

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REFERENCES


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