Ink and Vinegar, a Simple Staining Technique for Arbuscular-Mycorrhizal Fungi

HORST VIERHEILIG,1,2* ANDREW P. COUGHLAN,1 URS WYSS,2 AND YVES PICHÉ1

Centre de Recherche en Biologie Forestière, Université Laval, Québec G1K 7P4, Canada,1 and Institut für Phytopathologie, Christian-Albrecht-Universität Kiel, D-24118 Kiel, Germany2

Received 17 June 1998/Accepted 6 October 1998

We developed a reliable, inexpensive, and simple method for staining arbuscular-mycorrhizal fungal colonizations in root tissues. Apart from applications in research, this nontoxic, high-quality staining method also could be of great utility in teaching exercises. After adequate clearing with KOH, an ink-vinegar solution successfully stained all fungal structures, rendering them clearly visible.

Healthy, fertile soils are characterized by the presence of a diverse population of microorganisms, an important component of which are arbuscular-mycorrhizal (AM) fungi (12). The arbuscular mycorrhiza is a symbiotic association formed between the roots of members of over 80% of all families of land plants and a small group of common soil-borne zygomycete fungi (Glomales). In general, this association is beneficial for both partners. The host plant receives mineral nutrients from outside the root's depletion zone via the extraradical fungal mycelium, while the heterotrophic mycobiont obtains photosynthetically produced carbon compounds from the host (18).

Research into the establishment and role of mycorrhizal associations in natural ecosystems is of fundamental importance. Although data are available from several North American and European ecosystems, few data have been obtained from developing countries where the maintenance of AM fungal populations could be essential for sustainable agriculture. The availability and cost of chemicals used for staining of AM fungi within root tissues, a basic technique in AM research, can be constraints in some countries.

Phillips and Hayman (15) developed a method of staining AM fungal structures in roots that uses trypan blue. Trypan blue is listed by the International Agency for Research on Cancer as a possible carcinogen (9). Another frequently applied technique (3) uses the possibly carcinogenic dye chlorazol black E (10). Acid fuchsin, which also is used to stain AM fungi in roots (7), is also a suspected carcinogen (5). In addition, HCl, although used at a low concentration, is frequently applied for the acidification of roots after clearing with KOH (7, 15).

The use of such chemicals should be reduced for health and safety reasons. Contact with caustic chemicals may cause skin irritation (2), and their vapors may irritate the eyes, nose, throat, and lungs (16, 17). For environmental reasons it is preferable, wherever possible, to find substitutes for harmful chemicals. The “International Directory of Mycorrhizologists” lists more than 1,000 mycorrhizologists in 77 countries worldwide (6); thus, we estimate that tens of thousands of root samples are stained per year. In an attempt to eliminate some of the hazardous compounds, a modified procedure for stain-

**TABLE 1. Comparison of different inks for staining of AM fungi in roots**

<table>
<thead>
<tr>
<th>Color</th>
<th>Company</th>
<th>Staining result</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purple</td>
<td>Waterman</td>
<td>Fungus not stained</td>
<td>Not suitable</td>
</tr>
<tr>
<td>Green</td>
<td>Reynolds</td>
<td>Fungus not stained</td>
<td>Not suitable</td>
</tr>
<tr>
<td>Blue</td>
<td>Shaeffer</td>
<td>Fungus not stained</td>
<td>Not suitable</td>
</tr>
<tr>
<td></td>
<td>Kreuzer</td>
<td>Fungus partially stained</td>
<td>Not suitable</td>
</tr>
<tr>
<td></td>
<td>Pelikan</td>
<td>Fungus stained; good contrast</td>
<td>Suitable</td>
</tr>
<tr>
<td>Red</td>
<td>Lamy</td>
<td>Fungus not stained</td>
<td>Not suitable</td>
</tr>
<tr>
<td></td>
<td>Pelikan</td>
<td>Fungus not stained</td>
<td>Not suitable</td>
</tr>
<tr>
<td></td>
<td>Parker</td>
<td>Fungus stained; roots after destaining</td>
<td>light red; good contrast</td>
</tr>
<tr>
<td>Black</td>
<td>Reform</td>
<td>Fungus not stained</td>
<td>Not suitable</td>
</tr>
<tr>
<td></td>
<td>Carrefour</td>
<td>Fungus stained; roots after destaining</td>
<td>light blue; good contrast</td>
</tr>
<tr>
<td></td>
<td>Pelikan</td>
<td>Fungus stained; roots after destaining</td>
<td>light grey; good contrast</td>
</tr>
<tr>
<td></td>
<td>Shaeffer</td>
<td>Fungus stained; roots after destaining</td>
<td>light brownish; excellent contrast</td>
</tr>
<tr>
<td></td>
<td>Cross</td>
<td>Fungus stained; roots after destaining</td>
<td>light blue; good contrast</td>
</tr>
</tbody>
</table>

* Corresponding author. Mailing address: Centre de Recherche en Biologie Forestière, Université Laval, Pavillon C.-E.-Marchand, Québec G1K 7P4, Canada. Phone: (418) 656-2131, ext. 8129. Fax: (418) 656-7493. E-mail: nonhorst@rsvs.ulaval.ca.
ing of AM fungi in roots has been proposed (11); however, the
carcinogenic dye trypan blue is still used. Recently, a simple
staining technique with an ink–25% acetic acid solution for
screening of *Pseudocercosporella herpotrichoides*
infection in
wheat leaves was developed (14). Our objective was to deter-
mine whether this technique can be adapted for staining of AM
fungi in roots, thus replacing toxic chemicals with nontoxic but
equally effective products.

**Biological materials.** Seeds of plants from different families
having differing root characteristics (bean [*Phaseolus vulgaris*
L.], soybean [*Glycine max* L.], cucumber [*Cucumis sativus* L.],
maize [*Zea mays* L.], wheat [*Triticum aestivum* L.], barley [*Hor-
deum vulgare* L.], and ryegrass [*Lolium perenne* L.]) were sur-
face sterilized by soaking in 0.75% sodium hypochlorite for
5 min, rinsed with tap water, and germinated in vermiculite.
Seedlings (7 days old) were transferred to a steam-sterilized

**VOL. 64, 1998 INK, VINEGAR, AND AM FUNGI 5005**

FIG. 1. *G. mosseae*-colonized roots stained with an ink-vinegar solution. Roots in panels a to e were stained with black ink (Shaeffer). (a) Colonized (dark) and noncolonized ryegrass roots. The colonized root is easily distinguishable from the noncolonized root. Bar = 25 μm. (b) Individual hyphae in both heavily and partially
colonized sections of a bean root are clearly visible. Bar = 20 μm. (c) Arbuscules in ryegrass root tissue. Bar = 20 μm. (d) Vesicles and internal hyphae in ryegrass
root tissue. Bar = 20 μm. (e) Arbuscules and internal hyphae in bean root tissue. Bar = 5 μm. (f) Penetration unit of *G. mosseae* in a ryegrass root stained with blue
ink (Pelikan). Bar = 20 μm.
TABLE 2. Staining of colonized roots with trypan blue (standard method) or with black ink (Shaeffer)

<table>
<thead>
<tr>
<th>Plant</th>
<th>% of roots stained with trypan blue</th>
<th>Ink</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat</td>
<td>43 ± 3</td>
<td>45 ± 5</td>
</tr>
<tr>
<td>Bean</td>
<td>60 ± 6</td>
<td>58 ± 3</td>
</tr>
<tr>
<td>Cucumber</td>
<td>35 ± 4</td>
<td>37 ± 6</td>
</tr>
</tbody>
</table>

* Means (± standard errors) of root samples from five replicate plants per species and treatment.

TABLE 3. Recommended clearing, staining, and destaining procedures for different ink colors

<table>
<thead>
<tr>
<th>Ink color</th>
<th>Procedure*</th>
<th>Staining (min)</th>
<th>Destaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black or blue</td>
<td>Time depends on type of root (e.g. bean, 5 min; ryegrass and cucumber, 3 min); then, rinse several times with tap water</td>
<td>3</td>
<td>Rinse for a minimum of 20 min with tap water acidified with a few drops of vinegar</td>
</tr>
<tr>
<td>Red</td>
<td>Time depends on type of root (e.g. bean, 15 min; ryegrass and cucumber, 5 min); then, rinse several times with tap water</td>
<td>3</td>
<td>Rinse for 10 min with vinegar</td>
</tr>
</tbody>
</table>

* General procedures: clearing, boil in 10% KOH; staining, boil in 5% ink-vinegar.
gar, which is used in human nutrition, is obviously not harmful and ink, because it is used by children, is subject to strict regulations and must be nontoxic in every respect (4).

As not all inks tested were capable of staining AM fungal tissue, and as it might not always be possible to obtain inks from the companies we used in our study, a preassay should be performed with each specific ink considered.

Grace and Stribley (8) and Koske and Gemma (11) proposed modification of the staining method for AM fungi developed by Phillips and Hayman (15) which eliminate as many toxic compounds as possible. Our method, with ink-vinegar solutions, reduces still further the list of potentially hazardous chemicals needed. Moreover, by our method, the acidification procedure that follows the clearing of roots with KOH (15) can be omitted.

Our method provides a simple and safe technique with easily obtainable compounds and also may be applicable for staining of other root-colonizing fungi. For example, on roots of wheat plants inoculated with *Rhizoctonia cerealis*, extensive mycelium was clearly visible with some hyphae penetrating the root (results not shown). This inexpensive staining technique might stimulate AM research in parts of the world where financial resources for scientific studies are highly limited. Moreover, the nontoxic chemicals used make it an excellent technique for teaching situations.

Ryegrass seeds were provided by Yves Desjardins, Centre de Recherche en Horticulture, Université Laval, Quebec, Canada. We thank Maria Gagyi, Bernhard Holtmann, and Holger Klink, Department of Phytopathology, University of Kiel, Kiel, Germany, for helpful advice.

This work was supported by grants from the Deutsche Forschungsgemeinschaft (Germany) and the NSERC.

REFERENCES


