Physiologia Plantarum 125: 393–404. 2005
Copyright © Physiologia Plantarum 2005, ISSN 0031-9317

TECHNICAL FOCUS

An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots†

Horst Vierheilig[a], Peter Schweiger[b] and Mark Brundrett[c]

[a]Institut für Pflanzenschutz, Universität für Bodenkultur Wien, Peter Jordan-Strasse 82, 1190 Wien, Austria
[b]Institut für Bodenforschung, Universität für Bodenkultur Wien, Peter Jordan-Strasse 82, 1190 Wien, Austria
[c]School of Plant Biology, Faculty of Natural & Agricultural Sciences, The University of Western Australia, Crawley, Western Australia 6009; Science Directorate, Botanic Gardens and Parks Authority, Kings Park and Botanic Garden, West Perth, Western Australia 6005

Correspondence
*Corresponding author,
e-mail: nonhorst@boku.ac.at

Received 19 May 2005; revised 28 June 2005

doi: 10.1111/j.1399-3054.2005.00564.x

One of the most cited papers in arbuscular mycorrhizal (AM) research was published by Phillips and Hayman in 1970 describing an easy standard method to stain AM fungi (AMF) in roots. Since then, a number of other methods (destructive–non-destructive; vital–non-vital) on how to visualize AMF in roots have been published. Our review provides an overview on present techniques used to visualize AMF in roots and gives recommendations on their use. We hope that the present review will help the readers to choose an appropriate method to visualize AMF in roots for their specific experimental set-up.

Introduction

Techniques to detect and quantify arbuscular mycorrhizal fungi (AMF) in roots are essential tools in mycorrhizal research. These methods are primarily used to identify mycorrhizal associations and measure the degree of root colonization. A range of light microscopy-based techniques can be used to detect and quantify AMF in roots including in vivo observations of fungal structures in living roots, non-vital staining methods and vital root-staining methods. Alternative means are biochemical methods that measure characteristic biochemical markers for AMF or the AM symbiosis (Bothe et al. 1994, Frey et al. 1992, Schmitz et al. 1991) and recently developed quantitative and qualitative molecular tools (Alkan et al. 2004, Sanders 2002). Depending on the experimental approach, biochemical and molecular techniques can be reliable tools for the identification and/or quantification of AMF in roots and thus a serious alternative to root staining. However, compared with staining techniques, they are time consuming and costly and therefore currently not practical for routine use. Thus, the staining of the roots and the counting of the stained fungal structures in the root by routine light microscopy still remains the standard technique for the quantification of root colonization by AMF.

Staining and microscopic methods not only provide reliable data on the degree of root colonization but also permit to visualize the presence of key features such as arbuscules, which are the morphological criteria that define AM associations (Brundrett 2004). For the observation of these morphological criteria, it is essential that root material is processed in such a way that these defining anatomical features can be easily detected. The present review provides descriptions and other helpful information on a range of techniques in order to help the reader to choose the most appropriate technique to study AMF in roots for particular purposes. Only light microscopy-based methods are considered,

Abbreviations – ALP, alkaline phosphatase; AMF, arbuscular mycorrhizal fungus; CBE, Chlorazol black E; CLSM, confocal laser-scanning microscopy; DAB, diaminobenzidine; FITC, fluorescein isothiocyanate; SDH, succinate dehydrogenase; WGA, wheat germ agglutinin.

†In commemoration of Marilyn Griffith, editor of Physiologia Plantarum, who initiated this review.

as electron microscopy-based methods provide powerful tools for revealing fine details but are unsuitable for the routine quantification and identification of mycorrhizal associations.

Non-destructive methods for living roots

The visualization of AM fungal structures in roots by non-destructive methods is possible in certain circumstances. Non-destructive methods usually do not include any staining, as this normally requires clearing and thus killing of the root. These methods utilize either visible light, epifluorescence or confocal laser-scanning microscopy (CLSM). Non-destructive methods are unique in that they allow the dynamic processes of fungus activity in roots to be observed to a certain extent. Although excised roots are used in most studies, these methods also allow roots of whole intact plants to be studied. Plants can be repotted after observation and will continue to grow.

Unstained living roots

Different methods are available to visualize AM structures in living roots. The easiest approach is to take a living root and observe it without any pretreatment under the conventional light microscope. However, AM fungal structures are very hard to detect in most living roots. Only very thin and transparent roots with only a few cell layers above the central cylinder warrant observation without processing (Fig. 1). Although this observation technique is not adequate for morphological studies or for the quantification of root colonization, arbuscules could be observed in roots of tobacco (Vierheilig et al. 2001).

Stained living roots

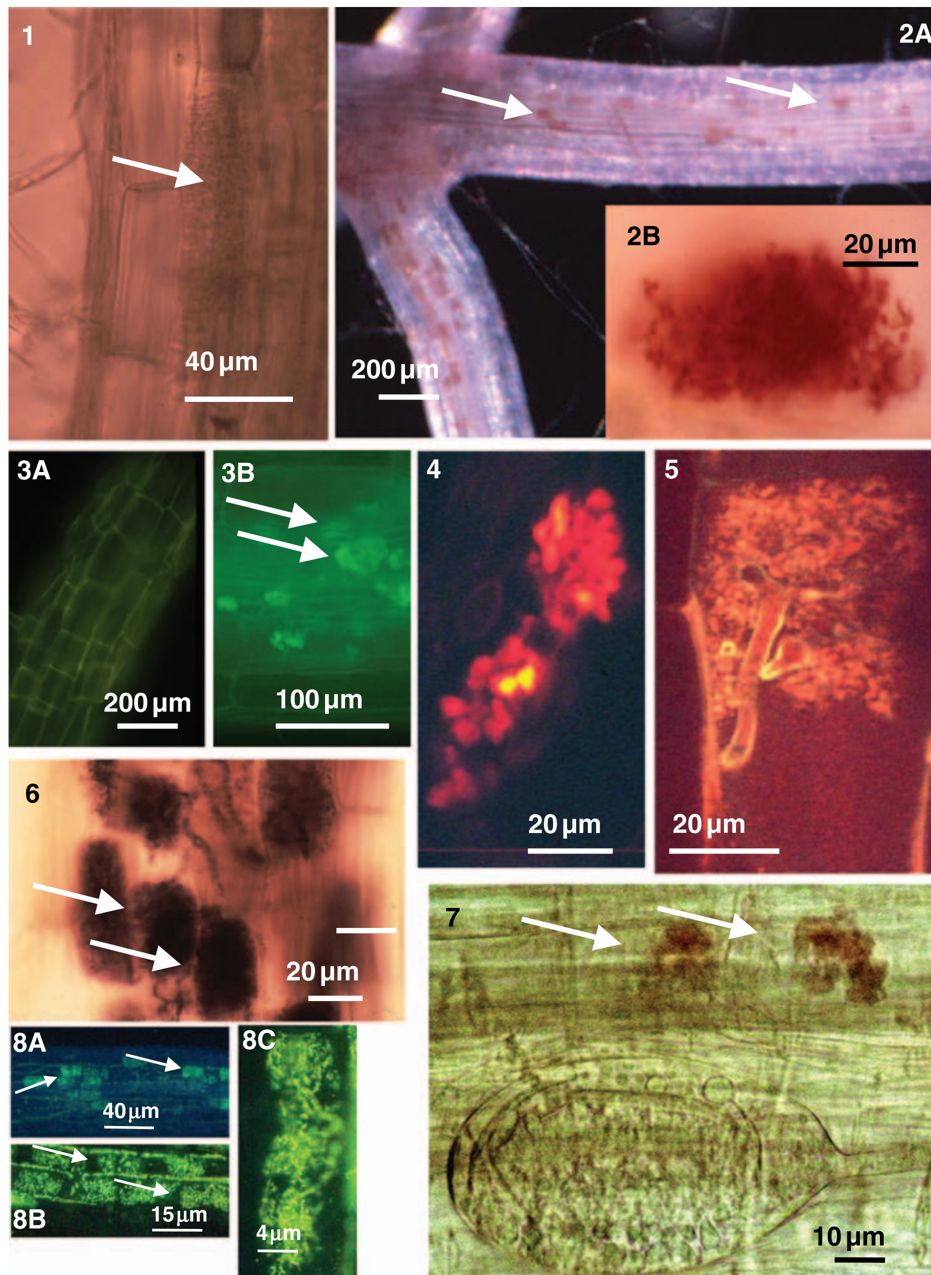
Neutral red can be used as an acidotropic dye to visualize mature arbuscules in living roots (Guttenberger 2000a, 2000b). The staining of these mature arbuscules is based on the ion-trap mechanism, which reveals acidic compartments such as the periarbuscular space. Apart from mature arbuscules, plant vacuoles and a conical zone in the root tip and sometimes fungal hyphae including appressoria are stained. However, these structures are easy to distinguish from the mature arbuscules (Fig. 2A, B).

The time required for staining depends on the complete penetration of the dye into the root and varies with the root diameter (Sorghum 1 h; leek 2–6 h). Arbuscules are not stained in mechanically stressed roots (affected by shearing, twisting and bending during harvest) or in cells close to sectioning planes. A high number of arbuscules is stained in unsectioned roots; however, there is no evidence that all arbuscules are stained with this dye. Consequently, this is an interesting technique to determine pH changes caused by AM structures in the root but would not normally be used to quantify mycorrhizas.

Epifluorescence microscopy of living roots

In 1982, Ames et al. detected autofluorescing spots in roots colonized by AMF (Fig. 3A, B) and identified these spots as arbuscules. Ames et al. (1982) and Jabaji-Hare et al. (1984) proposed the quantification of the autofluorescing 'arbuscules' as a non-destructive method for measuring root colonization by AMF. An extensive study by Gange et al. (1999) compared the level of arbuscular colonization in a range of plants after staining with Chlorazol black E (CBE), acid fuchsin and Trypan Blue with the level of the autofluorescing 'arbuscules'. They concluded that in most plants the number of autofluorescing 'arbuscules' seemed much higher than the number of stained arbuscules. On this basis, the quantification of autofluorescing 'arbuscules' was suggested as a valid method for the quantitative determination of root colonization by AMF.

More recently, the autofluorescing 'arbuscules' have been identified as collapsed arbuscules by observing living roots of tobacco and ryegrass using CLSM (Fig. 4), whereas metabolically active arbuscules exhibited no autofluorescence (Vierheilig et al. 1999, 2001). This would mean that a simple quantification of autofluorescing spots in AM-colonized roots would be an indicator of the degree of collapsed arbuscules in the root but not of the degree of root colonization by AMF. This has been recently confirmed by quantifying the number of autofluorescing spots (collapsed arbuscules) in living barley roots in a time-course study and thereafter the number of stained arbuscules in the same root section (Nell 2003, Vierheilig 2004). This experiment demonstrated a sharp increase in the number of stained arbuscules 2 weeks after inoculation, which culminated 1 week later (3 weeks after inoculation) and was followed by a steady decline in stained arbuscules. The pattern was very different when the collapsed arbuscules (autofluorescing spots) were counted. From 2 weeks on after inoculation, the number of collapsed arbuscules steadily increased until the end of the experiment (7 weeks after inoculation). Interestingly, Vierheilig et al. (2001) also observed that autofluorescing collapsed arbuscules do not become stained by the dyes commonly used to stain mycorrhizal fungi. To



Figs. 1–8. 1: Living tobacco root observed by light microscopy (arrow indicates a highly branched arbuscule). **2:** Living *Allium porrum* roots. (A) Red spots visible in the root are mature arbuscules stained by neutral red. (B) Higher magnification of an arbuscule (both images were kindly provided by M Guttenberger, Tübingen, Germany). **3:** (A) Living tobacco root with no mycorrhizal root colonization, (B) tobacco root with mycorrhizal root colonization observed using epifluorescence microscopy. Fluorescing spots in (B) indicate autofluorescing-collapsed arbuscules (images provided by H Vierheilig). **4:** Autofluorescing collapsed arbuscule observed by confocal laser-scanning microscopy (image provided by H Vierheilig). **5:** Viable, highly branched arbuscule observed by confocal laser-scanning microscopy after labelling the plant with a fluorescent marker (images provided by H Vierheilig). **6:** Staining of metabolically active arbuscules and intraradical hyphae by succinate dehydrogenase (SDH) in tobacco roots. Roots were not cleared after SDH staining (images provided by HVierheilig). **7:** Staining by diaminobenzidine indicating the accumulation of H_2O_2 at clumped arbuscules (arrow indicate collapsed arbuscules) in *Medicago truncatula* roots (kindly provided by P Salzer, Basel, Switzerland). **8:** Fluorescent staining of alkaline phosphatase activity in a *Plantago lanceolata* root. (A) With an epifluorescence microscope, arbuscules and hyphae are easily visible in whole roots. Arbuscules can be clearly distinguished under higher magnifications (B and C) (all images were kindly provided by Elzbieta Orlowska and Katarzyna Turnau, Krakow, Poland).

summarize, we could clearly show that the structures detected by epifluoresence microscopy are not the same as the structures detected after staining with one of the common staining techniques. Thus, epifluoresence

microscopy to study AM fungal structures in roots is of limited use.

CLSM with fluorochromes

As mentioned above, to our knowledge in unstained living roots, the only AM fungal structures which can be observed by epifluorescence microscopy are collapsed arbuscules. However, fluorochromes can be used to visualize all fungal structures in living plant tissues. Specific fluorochromes administered to abraded leaves are transported via the phloem to the roots, where they move into the pericycle, endodermal, cortical and epidermal cells (Oparka et al. 1994, 1995). Thus, structures in the living roots, which exhibit little or no autofluorescence, can be visualized by CLSM (Fig. 5).

Recently, Vierheilig et al. (2001) reported that after the application of the fluorochromes 5(6)-carboxy-fluorescein (CF) or 5(6)-carboxy-seminaphthorhodafluor to tobacco leaves, all AM fungal structures, starting from intraradical hyphae to arbuscules, were clearly visible. Most interestingly, these observations were obtained using living roots. This provides us with an extremely interesting tool to study the in vivo dynamics of the establishment of the AM symbiosis in the root, starting from intraradical hyphal growth and culminating in arbuscule degeneration. This simple and relatively rapid technique allows observations of AMF in roots but requires a confocal laser-scanning microscope and expensive fluorochromes, so it will be restricted to studies requiring in vivo observations.

Destructive vital and non-vital staining techniques

Destructive methods can be divided into vital and non-vital staining techniques. Vital stains use the activity of certain fungus-specific enzymes for the visualization of the metabolically active fungal tissue, whereas non-vital staining techniques are expected to stain both viable and non-viable fungal tissues.

Vital stains

Succinate dehydrogenase

With this method, all metabolically active AM fungal structures such as hyphae, spores, vesicles and arbuscules are stained (Fig. 6) (MacDonald and Lewis 1978). Clumped, collapsed arbuscules are not stained (Vierheilig et al. 2001). Succinate dehydrogenase (SDH), a tricarboxylic acid cycle enzyme in AMF, reacts with nitro blue tetrazolium chloride (NBT) resulting in insoluble formazan, which can be clearly distinguished in roots.

To determine the SDH activity, we incubated the viable mycorrhizal root at room temperature overnight in an NBT-succinate solution (MacDonald and Lewis 1978). The NBT solution (10 ml) consist of 2.5 ml of NBT (4 mg ml^{-1}), 2.5 ml of Tris–HCl buffer (0.2 *M*; pH 7.4), 1 ml of sodium succinate (2.5 *M*), 1 ml of $MgCl_2$ (5 m*M*) and 3 ml of distilled water. In the original article by MacDonald and Lewis (1978), KCN is added to the staining solution; however, it has been reported that omitting KCN gives similar staining results (Smith and Gianinazzi-Pearson 1990). In tobacco roots (these roots are relatively thin and have only a few cell layers above the central cylinder), the insoluble formazan indicating the AM fungal structures with SDH activity can be easily observed without clearing of the root (Vierheilig et al. 2001). However, in general, roots have to be cleared. For clearing, MacDonald and Lewis (1978) suggested the boiling of the roots (approximately 15 min) in chloral hydrate (75%; w/v) dissolved in water. However, after boiling in chloral hydrate roots stay opaque. Roots treated with the SDH stain and thereafter cleared exclusively with KOH turn dark and are nearly completely intransparent (personal observations).

To summarize, in general, best clearing results are obtained by a combination of chloral hydrate and KOH clearing. Roots were first boiled in 20% chloral hydrate (w/v) for 15 min, rinsed three times with distilled water and thereafter cleared overnight in 5% (w/v) KOH at 55°C (drying oven), before three further rinses. After clearing, roots can be visualized mounted in water under the conventional light microscope. This method takes approximately 2 days before you can have a look at the stained and cleared roots. Schaffer and Peterson (1993) compared different clearing methods after SDH staining in a comprehensive study and provided further information on these methods.

Diaminobenzidine

In mycorrhizal roots, clumped mycorrhizal structures are stained by diaminobenzidine (DAB) in root cells (Fig. 7) but not highly branched arbuscules or other AM fungal structures such as hyphae and vesicles (Salzer et al. 1999). Recently, it has been suggested that these clumped structures are collapsed arbuscules (Vierheilig et al. 2001). With the DAB technique, the presence of H_2O_2 can be detected. The induction of H_2O_2 synthesis in plants, the so-called oxidative burst, is a powerful plant defence mechanism against microorganisms.

Mycorrhizal roots are incubated for 12 h (22°C) in a DAB buffer [1 mg ml^{-1} DAB buffered with 10 m*M* Mes-NaOH (pH 5.6) dissolved in a solution containing 0.5 m*M* inorganic potassium phosphate and 2 m*M* KNO_3]. After boiling in 95% ETOH (v/v) for 5 min, roots are observed in 40% (v/v) lactic acid with a microscope. The time required to perform the whole staining process is about 13 h. Roots of *Medicago truncatula* were not cleared before observation (Salzer et al. 1999), thus the detection of the clumped fungal structures was difficult. Possibly, it is easier to visualize the stained structures with thinner and more transparent roots such as roots of Gramineae or tobacco roots (Vierheilig et al. 2001). To our knowledge, this is the only technique that stains exclusively clumped, collapsed arbuscules. However, it is not clear whether all collapsed arbuscules are stained.

Phosphatase assays

Sites of alkaline phosphatase (ALP) activity in AM fungal tissue (e.g. hyphae and arbuscules) are stained in short pieces of root or, alternatively, longitudinally sectioned roots by either a fluorescent (Fig. 8A–C) (Van Aarle et al. 2001) or a non-fluorescent method (Tisserant et al. 1993). AM fungal ALP activity is mainly present in the vacuolar compartment of both arbuscules and intraradical hyphae (Gianinazzi et al. 1979). For the fluorescent method, quickly blotted roots are immediately placed in the reaction medium. All necessary components are part of the 'ELF-97 Endogenous Phosphatase Detection Kit' available from Molecular Probes (Eugene, OR). The staining solution is an enzyme-labelled fluorescence substrate (Solution A), which is diluted in an alkaline detection buffer (Solution B). Dilutions of 20–30 times of the supplied substrate solution have proven most effective.

Roots are stained for 30 min at room temperature in the dark after which they are thoroughly washed with a Tris buffer (30 m*M* Tris, 1.5 *M* NaCl and 0.05% Triton X-100, pH 8.0). Root pieces are then placed in the mounting medium, which is provided with the ELF kit as is the detection buffer. Samples are observed using an epifluorescence microscope with 340–380 nm (UV light) excitation wavelength and a 425-nm-long pass fluorescence emission filter. Bright green fluorescent precipitates are visible against a blackish background.

For the non-fluorescent method, the roots need to be cleared prior to staining. The clearing solution contains 0.05 *M* Tris/citric acid (pH 9.2), 0.05% sorbitol, 15 units ml^{-1} cellulase and 15 units ml^{-1} pectinase (both enzymes are from *Aspergillus niger*). Clearing is done at room temperature for 2 h. Root pieces are subsequently rinsed in deionized water and placed in the staining solution. This solution is prepared in 0.05 *M* Tris buffer (pH 9.2) and contains (in mg ml^{-1}) Fast Blue RR salt 1.0, α-naphthyl acid phosphate 1.0, $MgCl_2.6H_2O$ 1.07 and $MnCl_2.4H_2O$ 0.8. The roots are left in the staining solution overnight at room temperature. Following a thorough rinse with water, the roots are incubated for 5 min in a sodium hypochlorite solution (1% active chlorine). After another thorough rinse, the roots are mounted and viewed under a conventional light microscope. Sites of phosphatase activity are revealed by a dark purple precipitate.

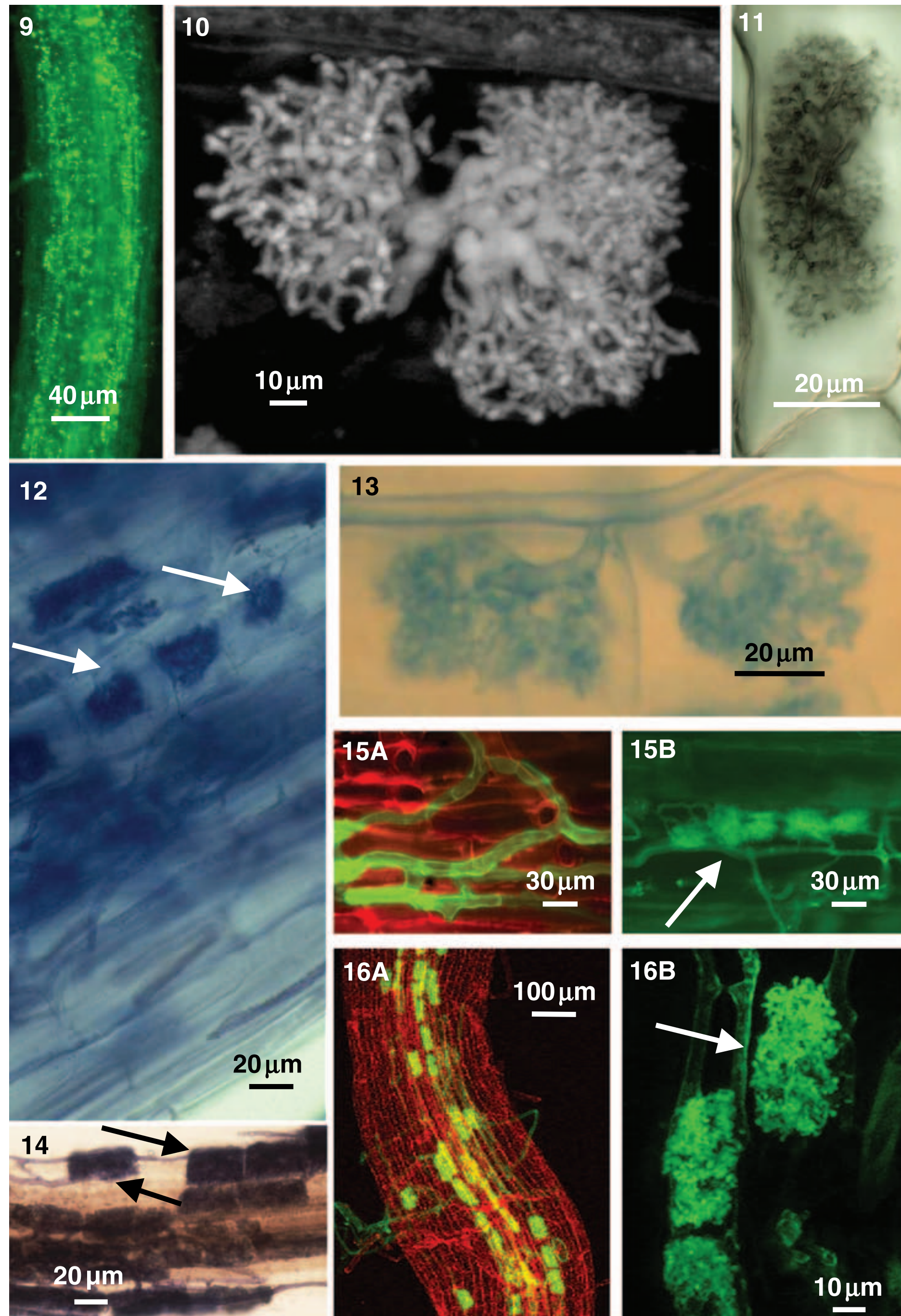
Both methods are suitable for the differentiation of AM fungal structures from root tissue, but the ELF method has been reported to be more sensitive than the Fast Blue RR method (Van Aarle et al. 2001). Staining can be performed directly on microscope slides to save chemicals. Detection of acid phosphatase activity by adjusting the pH of the ELF solution to 4.8 is not recommended, because this results in precipitates throughout the roots so that no fungal structures can be differentiated (Fig. 9).

Non-vital stains

Non-vital staining techniques are the most frequently used methods to visualize AMF in roots. Structures produced by glomeromycotan fungi are usually hidden by the natural pigments and cell contents. Clearing procedures, which use chemical agents to remove these cell contents and cell wall pigments, are routinely used to view internal features in plant tissues (Gardner 1975). Fungal structures in cleared plant tissues are then further revealed by stains which preferentially bind to fungal hyphae without excessive background staining of plant material. These stains are usually applied in an acidic solution containing lactic acid, glycerine and water (Bevege 1968, Brundrett et al. 1984, Kormanik and McGraw 1982, Phillips and Hayman 1970).

Clearing of roots before staining

It is fairly common to see published images of mycorrhizal roots which do not clearly show defining features due to background staining because of the presence of remaining cytoplasm and secondary metabolites. Stains such as Trypan Blue and CBE are believed to bind most strongly to phenolic-like materials (melanins) in hyphal walls but will also stain plant cell walls with encrusted phenolics (suberin and lignin) and any residual cell contents (Brundrett et al. 1984). Improperly cleared roots can result if clearing times are insufficient or too many roots are included relative to the volume of



Figs. 9–16. 9: Fluorescent staining of acid phosphatase activity in a *Plantago lanceolata* root. No fungal structures can be differentiated (kindly provided by Elzbieta Orlowska and Katarzyna Turnau, Krakow, Poland). **10:** Two arbuscules in a linseed root stained with acid fuchsin observed by confocal laser-scanning microscopy (image provided by P Schweiger and S Dickson, Lund, Sweden). **11:** Arbuscule in a root of *Smilacina racemosa* stained with Chlorazol black E (images provided by M Brundrett). **12:** Arbuscules in whole roots of *Tropaeolum majus* stained with Trypan Blue (image provided by H Vierheilig). **13:** Two arbuscules in a pea root stained with aniline blue (images provided by P Schweiger and W Hartl, Vienna, Austria). **14:** Staining of arbuscules and intraradical hyphae by ink in cucumber roots (image provided by H Vierheilig). **15:** Appressoria (A) and arbuscules (B) on *Lotus japonicus*. Fungal wall in sectioned roots was stained for chitin with fluorescein isothiocyanate (FITC)-conjugated wheat germ agglutinin. Red autofluorescence of plant cell walls (A) is due to glutaraldehyde fixation (both images were kindly provided by Andrea Genre, Torino, Italy). **16:** Arbuscules observed by confocal laser-scanning microscopy, stained for chitin with FITC-conjugated wheat germ agglutinin, in whole, unsectioned white clover roots (A) counterstained with acid fuchsin in order to obtain a better contrast. Arbuscules and intraradical hyphae (B) can be clearly distinguished under higher magnifications (both images were kindly provided by A Hirsch, Los Angeles, USA). Arrows indicate arbuscules in whole roots or in sectioned roots.

clearing agent in a sample. Root clearing is fastest in an autoclave, which also provides more uniform clearing of samples than other methods (Bevege 1968, Brundrett et al. 1984). The time required for adequate clearing in a water bath or oven (60–90°C) varies widely (from several minutes up to several days), so experimentation is required to calibrate these methods for different plant species. Many roots have protective outer layers (suberin in the epidermis and exodermis) which prevent maceration even after prolonged clearing, but other roots lack this feature and will fall apart if cleared for too long. Darkly pigmented roots can be cleared by prolonged heating in KOH, but sometimes, not all cell wall-bound secondary metabolites will be removed. Bleaching with alkaline hydrogen peroxide effectively removes the remaining pigments in cleared roots (Bevege 1968, Kormanik and McGraw 1982). However, this procedure should be used cautiously because staining of fungal hyphae may be reduced or prevented. In general, after clearing, the roots are rinsed several times with water and then acidified with diluted HCl (a × 10 diluted concentrated HCl is adequate, i.e. approximately 3.5%). Thereafter roots are stained.

Staining of roots after clearing

Most AMF are stained by the non-vital staining methods described below; however, the total or almost total absence of staining is a problem for certain taxa of AMF such as some species of *Acaulospora* and *Paraglomus* (Morton 1985). Some *Acaulospora* species require careful observation at high magnification to see hyphae which usually contain distinct lipid droplets (Brundrett et al. 1996). Molecular tools have been used to identify *Paraglomus* species in roots (Millner et al. 2001).

Acid fuchsin

Acid fuchsin (CI 42685) is a commonly used dye to stain fungal tissue but also, e.g. proteins, collagen and less commonly mitochondria. It is also used as a general counterstain. In the presented method, all AM fungal structures in roots are stained in cleared or longitudinally sectioned, uncleared roots with 0.01% (w/v) acid fuchsin in lactoglycerol (lactic acid–glycerol–water, 14:1:1 (Kormanik and McGraw 1982) or 1:1:1 (Dickson and Kolesik 1999; Dickson et al. 2003). For root sectioning with a freezing microtome see Dickson et al. (2003). Staining time is dependent on the host plant, but 12–24 h at room temperature should be suitable for most samples. Roots are then rinsed with deionized water and either mounted directly onto slides or stored in 100% glycerol (Fig. 10).

The stained AM fungal structures can be visualized by visible light or by epifluorescence microscopy (Merryweather and Fitter 1991). For the latter, the material should not be intensively stained because fluorescence of the stained fungal structures decreases with the intensity of staining due to self-quenching. Slides are viewed under an epifluorescence microscope equipped with an excitation wavelength of 568 nm and a 590-nm-long pass fluorescence emission filter. Under these filter settings, AM fungal structures show up in bright red and are very well-defined against the continuous pale red background of the root tissue. Changing the excitation wavelength to 510 nm decreases the contrast, with the root tissue becoming yellow and the fungus orange. If both the fungus and the root should be visualized in the same sample, this difference in fluorescence may be exploited in CLSM by the use of two photodetectors set to different wavebands.

Samples may also be viewed by conventional light microscopy, but the contrast between fungal and plant material may be too low for routine quantification of AM root colonization. The use of a higher concentration of acid fuchsin in the staining solution (e.g. 0.1%) or staining with near-boiling staining solution (for 10–60 min depending on the plant species) may increase the contrast. Also, omission of the rinsing step with water between staining and the necessary subsequent destaining in lactoglycerol may improve the result. However, the contrast will most often still be lower than with the other dyes described below.

CBE

A stain solution of 0.03% w/v CBE in lactoglycerol (1:1:1 lactic acid, glycerol and water) can be used to stain roots after clearing in KOH (Brundrett et al. 1984). All AM fungal structures in roots are stained (Fig. 11). The optimum stain concentration will depend on the dye source and microscope procedure used, so it may be necessary to try a range of concentrations (e.g. 0.1, 0.03 and 0.01%) when using a new type of roots, or a new batch of dye. Roots are stained by heating for several hours at 90°C until uniform dye penetration occurs, or 15 min in an autoclave, or by leaving them in staining solution at room temperature for one or more days. Over-staining can occur with CBE, especially if roots were not properly cleared. Staining quality is substantially improved by destaining roots in 50% glycerol for several days prior to observation. Roots stained with CBE can be stored in 50% glycerol. Semi-permanent slides of stained roots can be made with PVLG, glycerol jelly or gum arabic-based mounting media (Cunningham 1972, Koske and Tessier 1983, Widden 2001).

Trypan Blue and similar dyes

Cleared roots can also be stained with Trypan Blue (Fig. 12) (0.05%; w/v), cotton blue (Fig. 13) (0.05%; w/v) or acid fuchsin (Fig. 10) (0.01%; w/v) (Bevege 1968, Kormanik and McGraw, 1982, Phillips and Hayman 1970). All AM fungal structures in roots are stained by these dyes. The method used to clear and stain roots with these other stains is very similar to that described for CBE. Depending on the plant, root size and age, roots are stained by heating for minutes (e.g. cucumber roots 5 min) or several hours at 90°C, or 15 min in an autoclave, or by leaving them in staining solution at room temperature for one or more days.

Trypan Blue staining is not recommended for studies involving morphological observations or photomicroscopy at high magnifications, because the resulting images have lower contrast than those resulting from CBE staining (Brundrett et al. 1994). However, Trypan Blue staining is routinely used for mycorrhizal quantification at low magnifications where colour contrast is beneficial. Roots stained with Trypan Blue are not permanent unless they are stored in lactoglycerol or kept in the staining solution. Acid fuchsin and cotton blue (aniline blue WS) can also be used to stain fungi in roots but destain rapidly and produce relatively low-contrast images (see above and Brundrett et al. 1984).

Ink and vinegar

All AM fungal structures in roots are stained with this method using writing ink as a dye (Fig. 14). The staining solution consists of a 5% ink diluted in vinegar (5% acetic acid) (Vierheilig et al. 1998). After clearing, the roots are boiled (95°C) for at least 3 min in the staining solution. Following staining, roots are rinsed several times during more than 20 min with acidified tap water (add several drops of acetic acid to the water). If the water for rinsing is not acidified and has a high pH (neutral is high), roots will destain. Total time needed for staining including clearing is around 30 min. If desired, the stained fungus can be completely destained by re-incubating the root in KOH.

This is a low-budget, supposingly non-toxic technique, which gives excellent staining results. As it uses mostly non-hazardous and non-toxic chemicals which can be easily purchased (but see *Safety issues* below), it is not only an adequate technique for research but also for teaching purposes. Due to the relatively low cost and small amount of time needed per sample, this technique is very suitable for large sample numbers. When using this technique, it has to be kept in mind that not all inks do stain AMF. Quality of the staining depends on the colour and the brand of the ink. Purple, green and red inks are in general not suitable for staining, whereas nearly all black inks (Shaeffer Jet Black; Cross Black; Pelikan Black) and some blue inks (Pelikan Blue) give good staining results. Thus, before using an ink, which has not yet been reported to stain AMF, it is essential to test for staining quality. Always indicate the brand of the ink which has been used in publications.

When observing the stained structures with a stereomicroscope, structures were clearest with a dark field illumination. Recently, this technique has also been used to double-stain AM fungal structures after β-glucuronidase staining (Hohnjec et al. 2005, Journet et al. 2001)

Dye and vinegar

All AM fungal structures in roots are stained. This method (Vierheilig and Piché 1998) is an adaptation of methods using the dyes Trypan Blue, anilin blue or acid fuchsin and the ink/vinegar method. The staining solution used consists of vinegar (=5% acetic acid) and the respective dye (Trypan Blue 0.05%; cotton blue 0.05%; acid fuchsin 0.01%). Immediately after clearing, the roots are boiled for at least 3 min in the staining solution. Destaining of Trypan Blue and anilin blue is best with tap water, whereas acid fuchsin is better destained with pure vinegar. All fungal structures are stained and clearly visible. The advantage of this technique is that the dyes are predissolved in the inexpensive vinegar and that the acidification step that is usually necessary when using the above mentioned dyes can be omitted. This can be of special advantage when a high volume of samples has to be stained.

Wheat germ agglutinin

AM fungal structures in sectioned roots are labelled with fluorescein-labelled Wheat germ agglutinin (WGA) which binds to chitin, a homopolymer of *N*-acetylglucosamine. After labelling, intercelluar hyphae are weekly fluorescent, whereas active arbuscules are strongly fluorescent (Fig. 15A, B) (Bonfante-Fasolo et al. 1990). The technique is relatively time-consuming, complex and costly in comparison with other staining methods. It is mainly used for cytological and morphological studies. As the method is quite complex, for technical details the reader is referred to the original paper by Bonfante-Fasolo et al. (1990).

A similar method for visualizing the AMF in whole roots by CLSM has been described by Lum et al. (2002) (Fig. 16A, B). Roots are fixed in formalin, acetic acid and alcohol for around 14 h before clearing in 10% KOH (2 h; 50°C). Thereafter, roots are incubated (for around 14 h) in a WGA-Alexa Fluor® 488 conjugate (Molecular Probes). To obtain a better contrast, roots can be counterstained with acid fuchsin (10 μg ml^{-1}) by incubation in phosphate-buffered saline (5 min).

Confocal microscopy at 488 nm is used for observation of the fungus and at 568 nm for observation of the counterstained root.

Comparisons of staining methods

Brundrett et al. (1984) compared Trypan Blue, acid fuchsin, cotton blue and CBE and found that the latter produced higher contrast images than the other stains and was especially effective in combination with interference contrast microscopy. Gange et al. (1999) compared three different stains and found differences in the degree of mycorrhizal colonization between plant species when different stains were used. They found fluorescence microscopy produced higher values for mycorrhizal colonization, provided arbuscules were present (see *Epifluorescence microscopy of living roots* above). However, no images of stained roots were provided, and we cannot be certain that roots were fully cleared in their study and this is the most important factor which impacts on staining contrast in mycorrhizal roots (see above). Moreover, Vierheilig et al. (2001) suggested that by fluorescence microscopy only clumped collapsed autofluorescing arbuscules can be detected, whereas these autofluorescing arbuscules are stained poorly or not at all by the common techniques. This would mean (1) that the root colonization data obtained with fluorescence microscopy and light microscopy after staining are not directly comparable and (2) that with the commonly used staining methods collapsed arbuscules are poorly or not stained so cannot be quantified.

Another key factor which often affects the visual assessment of mycorrhizas is the age of material examined, as key structures such as arbuscules have a short lifespan, so will often not be visible in older roots (Toth et al. 1990). Unfortunately, it is rarely possible to obtain material where fungal associations are of a known age. The use of root organ cultures, transplanted seedlings or soil observation chambers, or sampling roots with a growing apex can overcome this problem (Bécard and Piché 1992, Brundrett et al. 1985, Rosewarne et al. 1997).

Safety issues

A corrosive solution of KOH is used to clear roots, so care must be taken to avoid skin contact with this caustic chemical (avoid also contact with your clothes). A lower concentration of KOH (2.5%) can be used to reduce the risk of injury somewhat (Koske and Gemma 1989), but this may not be practical with roots that are hard to clear. Formalin-based preservatives are toxic and should not be used, as root samples can be stored indefinitely in 70% ethanol or methanol. Phenol, which was historically used in stain preparations, is toxic and can be omitted with little influence on staining quality (Brundrett et al. 1984).

The majority of biological stains are azo dyes, and there is strong evidence that some, including CBE and Trypan Blue, are mutagenic and carcinogenic (Coombes and Haveland-Smith 1982, Weisburger 2002). This necessitates that scientists working with biological stains take great care to avoid direct exposure to staining solution or dye powders. It is believed that contact with skin is much less hazardous than internal exposure, as enzymes are required to activate azo dyes into toxic breakdown products (Weisburger 2002). However, all exposure to these stains should be avoided. The azo dyes most commonly used to stain roots are also widely used by industry (dyeing fabrics and paper, cosmetics etc.), and research to find safer alternatives is currently underway (e.g. Edwards et al. 2004). Many other biological stains not currently listed as carcinogens are azo dyes which may be added to lists of known carcinogens after further safety tests (e.g. SCCNFP 2002).

Grace and Stribley (1991) suggest that methyl blue or cotton blue can be used as less-toxic replacements, however, these azo dyes must also be handled carefully. It is also not safe to assume that the inks used in pens are non-toxic as these are also likely to include azo dyes. In general, it can be said that as ink is used by children, it is subject to strict regulation and should be non-toxic (Colditz et al. 1987, Vierheilig et al. 1998). However, always treat any biological stain with caution and refer to safety data sheets for the latest information on all the chemicals you use. A primary advantage to the use of inks is that the chemicals they contain are already present in diluted form, so exposure to concentrated chemicals is avoided.

The quantification of root colonization

Gridline intersection method and other procedures used to quantify mycorrhizas in cleared roots are explained elsewhere (see Brundrett et al. 1996, Giovannetti and Mosse 1980). Morphometric procedures defined as measurement techniques used to determine the area or length of structures using a microscope provide much more accurate measurements of fungal structures in roots and have been used to measure the lifespan of active mycorrhizal associations (Toth et al. 1990). McGonigle et al. (1990) developed a method which quantifies different mycorrhizal structures separately (a form of morphometrics). This allows the proportion of roots containing arbuscules, vesicles and hyphae to be separately determined. More recently, morphometric analysis has been extended by the use of computerized

Image analysis to measure and quantify mycorrhizal structures in digital images (e.g. Smith and Dickson 1991). This has allowed to measure the area of mycorrhizal interfaces, which comprises the surface area of fungal structures, such as arbuscule branch hyphae in plant cells where exchange processes are considered most likely to occur.

Electron microscopy can reveal minute details of mycorrhizal associations, but these may be misleading in the absence of a low-magnification overview that allow associations to be identified. Problems with the identification of association types may also arise from the use of material of unknown age that lacks ephemeral structures such as arbuscules, or inadequate sampling (e.g. observation of a limited number of sectioned roots). It is preferable to identify mycorrhizal associations in whole-root preparations using a clearing and staining technique that allows sufficient sampling volume and replication. In ecosystem surveys, the degree of mycorrhizal colonization should be expressed as the proportion of susceptible roots that were mycorrhizal, by excluding woody roots. Consequently, any study of mycorrhizas must be based on an understanding of root structure and phenology (Brundrett et al. 1996).

Conclusions

Microscopical methods, which allow details of associations to be clearly seen, are essential to all work with AM associations, as these are defined by morphological criteria and must be identified by the presence of key features, especially arbuscules (Brundrett 2004). Consequently, it is essential to use methods adapted to the question asked in the specific study which allow an easy detection of the defining anatomical features of the AM symbiosis. Although non-destructive observation methods (e.g. fluorescence or confocal microscopy) and vital staining methods (e.g. SDH or ALP staining) have been successfully used in certain studies, the standard techniques for the visualization and later quantification of root colonization by AMF still remain the non-vital staining techniques (e.g. Trypan and cotton blue or CBE and more recently ink).

To summarize, we must choose the staining technique for experimentation that depends on the question and the facilities available to support research. This could mean that for light microscopical studies with a focus on the morphology of the intraradical structures of the AMF, the technique that results in images with the highest contrast (the CBE method in combination with interference contrast microscopy, or one of the methods involving confocal microscopy) could be the most effective. If, by contrast, the degree of root colonization of a large number of samples has to be determined, easy handling and the use of the least-toxic chemical products (vinegar and ink) are most suitable.

References

Alkan N, Gadkar V, Coburn J, Yarden O, Kapulnik Y (2004) Quantification of arbuscular mycorrhizal fungus *Glomus intradices* in host tissue using real-time polymerase chain reaction. New Phytol 161: 877–885

Ames RN, Ingham ER, Reid CPP (1982) Ultraviolet induced autofluorescence of arbuscular mycorrhizal root infections: an alternative to clearing and staining methods for assessing infections. Can J Microbiol 28: 351–355

Bécard G, Piché Y (1992) Establishment of vesicular-arbuscular mycorrhiza in root organ culture: review and proposed methodology. In: Norris, JR, Read, DJ, Varma, AK (eds) Methods in Microbiology, Vol. 24, Techniques for the Study of Mycorrhiza. Academic Press, London, pp 89–108

Bevege DI (1968) A rapid technique for clearing tannins and staining intact roots for detection of mycorrhizas caused by *Endogone* spp. & some records of infection in Australasian plants. Trans Br Mycol Soc 51: 808–810

Bonfante-Fasolo P, Faccio A, Perotto S, Schubert A (1990) Correlation between chitin distribution and cell wall morphology in the mycorrhizal fungus *Glomus versiforme*. Mycol Res 94: 157–165

Bothe H, Klingner A, Kaldorf M, Schmitz O, Esch H, Hundeshagen B, Kernebeck H (1994) Biochemical approaches to the study of plant–fungal interactions in arbuscular mycorrhiza. Experientia 50: 919–923

Brundrett MC (2004) Diversity and classification of mycorrhizal associations. Biol Rev 78: 473–495

Brundrett M, Bougher N, Dell B, Grove T, Malajczuk N (1996) Working with Mycorrhizas in Forestry and Agriculture. ACIAR Monograph 32. Australian Centre for International Agricultural Research, Canberra

Brundrett M, Melville L, Peterson L (1994) Practical Methods in Mycorrhizal Research. Mycologue Publications Ltd*, Waterloo (*see Kendrick, 1992)

Brundrett MC, Piché Y, Peterson RL (1984) A new method for observing the morphology of vesicular-arbuscular mycorrhizae. Can J Bot 62: 2128–2134

Brundrett MC, Piché Y, Peterson RL (1985) A developmental study of the early stages in vesicular-arbuscular mycorrhiza formation. Can J Bot 63: 184–194

Colditz AW, Kunkel E, Bohne KH (1987) Drawing and writing materials. In: Gerhartz W, Yamamoto YS, Kaudy L, Rounsaville JF, Schulz G (eds) Ullmann's Encyclopedia of Industrial Chemistry, Vol. A9.VCH Verlagsgesellschaft, Weinheim, Germany, pp 37–47

Coombes RD, Haveland-Smith RB (1982) A review of the genotoxicity of food, drug and cosmetic colours and other azo, triphenylmethane and xanthene dyes. Mutat Res 98: 101–248

Cunningham JL (1972) A miracle mounting fluid for permanent whole-mounts of microfungi. Mycologia 64: 906–911
Dickson S, Schweiger P, Smith SA, Söderström B, Smith S (2003) Paired arbuscules in the *Arum*-type arbuscular mycorrhizal symbiosis with *Linum usitatissimum*. Can J Bot 81: 457–463
Dickson S, Kolesik P (1999) Visualization of mycorrhizal structures and quantification of their surface area and Volume using laser scanning confocal microscopy. Mycorrhiza 9: 205–213
Edwards LC, Freeman HS, Claxton LD (2004) Developing azo and formazan dyes based on environmental considerations: *Salmonella* mutagenicity. Mutat Res 546: 17–28
Frey B, Buser HR, Schüepp H (1992) Identification of ergosterol in vesicular-arbuscular mycorrhizae. Biol Fert Soils 13: 229–234
Gange AC, Bower E, Stagg PG, Aplin DM, Gillam AE, Bracken M (1999) A comparison of visualisation techniques for recording arbuscular mycorrhizal colonisation. New Phytol 142: 123–132
Gardner RO (1975) On overview of botanical clearing technique. Stain Technol 50: 99–105
Gianinazzi S, Gianinazzi-Pearson V, Dexheimer J (1979) Enzymatic studies on the metabolism of vesicular-arbuscular mycorrhiza. III. Ultrastructural localisation of acid and alkaline phosphatase in onion roots infected by *Glomus mosseae* (Nicol. & Gerd.). New Phytol 82: 127–132
Giovannetti M, Mosse B (1980) An evaluation of techniques for measuring vesicular-arbuscular infection in roots. New Phytol 84: 489–500
Grace C, Stribley DP (1991) A safer procedure for routine staining of vesicular-arbuscular mycorrhizal fungi. Mycol Res 95: 1160–1162
Guttenberger M (2000a) Arbuscules of vesicular-arbuscular mycorrhizal fungi inhabit an acidic compartment within plant roots. Planta 211: 299–304
Guttenberger M (2000b) A rapid staining procedure for arbuscules of living arbuscular mycorrhizas using neutral red as an acidotrophic dye. Plant Soil 226: 211–218
Hohnjec N, Vieweg MF, Pühler A, Becker A, Küster H (2005) Overlaps in the transcriptional profiles of *Medicago truncatula* roots inoculated with two different glomus fungi provide insights into the genetic program activated during arbuscular mycorrhiza. Plant Physiol 137: 1283–1301
Jabaji-Hare SH, Perumalla CJ, Kendrick WB (1984) Autofluorescence of vesicles, arbuscules and intercellular hyphae of a vesicular-arbuscular fungus in leek (*Allium porrum*) roots. Can J Bot 62: 2665–2669
Journet EP, El-Gachtouli N, Vernoud V, de Billy F, Pichon M, Dedieu A, Arnould C, Morandi D, Barker DG, Gianinazzi-Pearson V (2001) *Medicago truncatula* ENOD11: a novel RPRP-encoding early nodulin gene expressed during mycorrhization in arbuscule-containing cells. MPMI 14: 737–748
Kormanik PP, McGraw AC (1982) Quantification of vesicular-arbuscular mycorrhizae in plant roots. In: Schenck NC (ed) Methods and Principles of Mycorrhizal Research. The American Phytopathological Society, St. Paul, pp 37–45
Koske RE, Gemma JN (1989) A modified procedure for staining roots to detect VA mycorrhizas. Mycol Res 92: 486–489
Koske RE, Tessier B (1983) A convenient permanent slide mounting medium. Mycol Soc Am Newsletter 34: 59
Lum MR, Li Y, Larue TA, David-Schwartz R, Kapulnik Y, Hirsch A (2002) Investigation of four classes of non-nodulating white clover (*Melilotus alba* annua Desr.) mutants and their responses to arbuscular-mycorrhizal fungi. Integ Comp Biol 42: 295–303
MacDonald RM, Lewis M (1978) The occurrences of some acid phosphatases and dehydrogenases in the vesicular-arbuscular mycorrhizal fungus *Glomus mosseae*. New Phytol 80: 135–141
McGonigle TP, Miller MH, Evans DG, Fairchild GL, Swan JA (1990) A new method which gives an objective measure of colonization of roots by vesicular-arbuscular mycorrhizal fungi. New Phytol 115: 495–501
Merryweather JW, Fitter AH (1991) A modified procedure for elucidating the structure of the fungal partner in a vesicular-arbuscular mycorrhiza. Mycol Res 95: 1435–1437
Millner PD, Mulbry WW, Reynolds SL (2001) Taxon-specific oligonucleotide primers for detection of two ancient endomycorrhizal fungi, *Glomus occultum* and *Glomus brasilianum*. FEMS Microbiol Lett 196: 165–170
Morton JB (1985) Variations in mycorrhizal and spore morphology of *Glomus occultum* and *Glomus diaphanum* as influenced by plant host and soil environment. Mycologia 77: 192–204
Nell M (2003) The use of fluorescence microscopy to study different aspects in the arbuscular mycorrhizal symbiosis. Master Thesis. Universität für Bodenkultur Wien, Austria
Oparka KJ, Duckett CM, Prior DA, Fisher DB (1994) Real time imaging of phloem unloading in the root tip of *Arabidopsis*. Plant J 6: 759–766
Oparka KJ, Prior DA, Wright KM (1995) Symplastic communication between primary and developing lateral roots of *Arabidopsis thaliana*. J Exp Bot 46: 187–197
Phillips JM, Hayman DS (1970) Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. Trans Br Mycol Soc 55: 158–161
Rosewarne GM, Barker SJ, Smith SE (1997) Production of near-synchronous fungal colonisation in tomato for developmental and molecular analyses of mycorrhiza. Mycol Res 101: 966–970
Salzer P, Corbiere H, Boller T (1999) Hydrogen peroxide accumulation in *Medicago truncatula* roots colonized by

the arbuscular mycorrhiza-forming fungus *Glomus intraradices*. Planta 208: 319–325
Sanders IR (2002) Specificity in the mycorrhizal symbiosis. In: Van der Heijden MGA, Sanders IR (eds) Mycorrhizal Ecology. Studies in Ecology 157. Springer-Verlag, Heidelberg, pp 415–437
SCCNFP (2002) The safety of the use of certain azo dyes in cosmetic products. The Scientific Committee on the Cosmetic Products and Non-food Products Intended for Consumers. http://europa.eu.int/comm/health/ph_risk/committees/sccp/documents/out155_en.pdf
Schaffer GF, Peterson RL (1993) Modifications to clearing methods used in combination with vital staining of roots colonized with vesicular-arbuscular mycorrhizal fungi. Mycorrhiza 4: 29–35
Schmitz O, Danneberg G, Hundeshagen B, Klingner A, Bothe H (1991) Quantification of vesicular-arbuscular mycorrhiza by biochemical parameters. J Plant Physiol 139: 106–114
Smith SE, Dickson S (1991) Quantification of active vesicular-arbuscular mycorrhizal infection using image analysis and other techniques. Aust J Plant Physiol 18: 637–648
Smith SE, Gianinazzi-Pearson V (1990) Phosphate uptake and arbuscular activity in mycorrhizal *Allium cepa* L. effects of photon irradiance and phosphate nutrition. Aust J Plant Physiol 17: 177–188
Tisserant B, Gianinazzi-Pearson V, Gianinazzi S, Gollotte A (1993) In planta histochemical staining of fungal alkaline phosphatase activity for analysis of efficient arbuscular mycorrhizal infections. Mycol Res 97: 245–250
Toth R, Doane C, Bennett E, Alexander T (1990) Correlation between host-fungal surface areas and percent colonization in VA mycorrhizae. Mycologia 82: 519–522
Van Aarle IM, Olsson PA, Söderström B (2001) Microscopic detection of phosphatase activity of saprophytic and arbuscular mycorrhizal fungi using a fluorogenic substrate. Mycologia 93: 17–24
Vierheilig H (2004) Regulatory mechanisms during the plant-arbuscular mycorrhizal fungus interaction. Can J Bot 82: 1166–1176
Vierheilig H, Böckenhoff A, Knoblauch M, Juge C, Van Bel AJE, Grundler MW, Piché Y, Wyss U (1999) *In vivo* observations of the arbuscular mycorrhizal fungus *Glomus mosseae* in roots by confocal laser scanning microscopy. Mycol Res 103: 311–314
Vierheilig H, Coughlan AP, Wyss U, Piché Y (1998) Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. Appl Environ Microbiol 64: 5004–5007
Vierheilig H, Knoblauch M, Juergensen K, van Bel A, Grundler MW, Piché Y (2001) Imaging arbuscular mycorrhizal structures in living roots of *Nicotiana tabacum* by light, epifluorescence and confocal laser scanning microscopy. Can J Bot 79: 231–237
Vierheilig H, Piché Y (1998) A modified procedure for staining arbuscular mycorrhizal fungi in roots. J Plant Nutr Soil Sci 161: 601–602
Weisburger JH (2002) Comments on the history and importance of aromatic and heterocyclic amines in public health. Mutat Res 506–507: 9–20
Widden P (2001) The use of glycerin jelly for mounting stained roots for the observation and quantification of endomycorrhizal fungi. Mycologia 93: 1026–1027

Edited by D. Campbell