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Review Article

Review of Comprehensive Staining Techniques Used to Differentiate Arbuscular Mycorrhizal Fungi from Plant Root Tissues

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Abstract

Arbuscular mycorrhizal fungi are beneficial obligate endophytes that inhabit the root systems of the majority of land plants. Various microscopy techniques are commonly utilized to image these fungi, and the use of differential stains is a critical step in the imaging process. Differential stains are used to provide contrast between the fungal tissues and the host plant cells. Some staining procedures utilize harmful chemicals that are potential health hazards to the microscopist. A comprehensive review of staining procedures has not been assembled for researchers studying AMF, and this paper should offer insight into potential differential stains to image fungal organelles within the roots of the host plant. This review is designed to cover the majority of staining techniques used to image AMF, and to suggest the safest stains for the correct microscopic technique.

Keywords: Mycorrhizae; Fungi; Glomeromycota; Microscopy; Bright Field; Electron Microscopy; Laser Scanning Confocal Microscopy; AMF; Arbuscular Mycorrhizal Fungi; Microscope

Introduction

Arbuscular Mycorrhizal Fungi (AMF) are categorized into the phylum Glomeromycota and are known to inhabit the root systems of many herbaceous plant species (Ahktar., *et al.* 2011, Pozo., *et al.* 2008). An ecological mutualism forms, where an endosymbiotic relationship creates structures that span between the fungus, and the host plant (Ahktar., *et al*. 2011, Liu., *et al*. 2007, Pozo., *et al.* 2008). Ahktar., *et al*. reports that nearly 80% of herbaceous plants form relationships with AMF (Ahktar., *et al*. 2011). The herbaceous plant receives nutrients, water, and protection from pathogenic invaders, where the fungus receives protection and niche in which to survive (Ahktar., *et al*. 2011, Pozo., *et al*. 2008). AMF are identified by the presence of branched haustoria like structures, which are found in association with the plasma membrane of cortical cells in the host plant and are termed arbuscules (Ahktar., *et al*. 2011). These modified hyphal structures are the location where nutrient exchange occurs between the host plant and the AMF symbiont (Ahktar., *et al*. 2011). Once the arbuscule is formed, the AMF will grow from the root surface and penetrate the surrounding soil, expanding the zone in which water and nutrients can be acquired (Ahktar., *et al.* 2011). Dickson., *et al*. states that AMF are dichotomized into two distinct groups based upon the morphology of their arbuscules [1]. The first morphology of AMF is 'Arum' type, which has highly branched arbuscules, where the second variety of AMF is 'Paris' type, which has regions of coiled intracellular hyphae and intercalary arbuscules [1].

Imaging of AMF in root tissues is the first step in verification of a mycorrhizal relationship with a host plant. The most important aspect of capturing an image of AMF is choosing an appropriate stain that allows the microscopist to clearly differentiate between fungal and plant tissues. In previous years, researchers experimented with numerous stains, which have advantages and disadvantages when trying to image AMF for publication. Selection of the best stain for a particular research experiment involving AMF has not been standardized, and a manuscript that encompasses all major staining techniques has not been made available to botanists, plant pathologists, or mycologists.

Stains can be categorized into four main groups, positive stains, negative stains, nucleotide binding stains, and fluorescent proteins. Positive stains chemically bind to the molecules within the sample, where negative stains don't actively bind to the sample. Nucleotide binding stains can be thought of as positive stains, but they are more specific then generalist positive stains, in which they bind only to DNA, RNA, or other nucleic acids.

The main types of microscopy employed for studying root colonization by AMF includes bright field light microscopy, dark field light microscopy, laser scanning confocal microscopy, scanning electron microscopy, and transmission electron microscopy.

Wide field light microscopy is the most common microscopic technique encountered when studying AMF in root systems, due to the lowest cost of owning, maintaining, and servicing the microscope. Staining techniques to image AMF for light microscopy has provided a wide array of chemicals that differentiate between fungal tissue and plant root tissue, although many prove toxic to humans. Common stains for light microscopy include acid fuchsin, chlorazole black e, trypan blue, ink and acetic acid (ink and vinegar), sudan black, sudan IV, nile blue, fast blue, and nitro blue tetrazolium chloride (NBT) [1-10]. Light microscopes can be broken into two groups, wide field, or narrow field, based upon if the image collected is captured at one plain of focus, or at many plains of focus in the Z direction.

Brundentt., *et al.* 1984 described a wide field light microscopy staining technique that would allow for higher contrast than achievable with common differential stains such as acid fuchsin, trypan blue, or aniline blue [2]. Brundentt and colleagues stated that chlorazol black e, provides the highest contrast compared to acid fuchsin, trypan blue, or aniline blue [2]. Chlorazole black e was able to provide excellent contrast for the imaging of arbuscules, intracellular hyphae, intercellular hyphae, and vesicles [2]. McGonigle., *et al*. utilized techniques as described by Brundentt., *et al.* to quantify the percent of root colonization by AMF in Zea mays (McGonigle., *et al*. 1990). In 1991, Grace and Stribley published a review of common AMF differential stains, and arose awareness of the potential health hazards associated with Trypan Blue and Chlorazol Black E [8]. Both Trypan Blue and Chlorazol Black E are registered by the International Agency for Research on Cancer (IARC) as a confirmed animal carcinogen and possible human carcinogen [8]. Grace and Stribley offer alternative stains such as Acid Fuchsin, Azure B, Cotton Blue, Fast Green, Feulgen, Nile Blue, Sudan Black, and Sudan IV [8]. Vierheilig., *et al*. published a paper that suggested using Ink and Vinegar as a differential stain for AMF research [6]. Vierheilig and colleagues tested the major ink brands, including Waterman, Reynolds, Shaeffer, Kreuzer, Pelikan, Lamy, Pelikan, Parker, Reform, Carrefour, Cross, as well as different colors of these brands of ink [6]. Vierheilig and colleagues suggest that the best inks used for this technique are Parker's red ink, as well as Carrefour, Cross, Reform, Pelikan, and Parker's black inks [6]. In particular, Schaeffer's black ink provided the highest contrast of all the inks tested [6]. It is stated that Parker's red ink provides good contrast, and could be an effective AMF viability stain [6]. Ink and vinegar staining methods provides to be the safest differential staining method tested on AMF, since there are no associated health hazards with pen inks or vinegar.

Almost twenty years after Grace and Stribley's manuscript, researchers continue to publish papers using Trypan Blue and Chlorazol Black E despite previous publications sighting health hazards of using these stains. Vierheilig's 1998 publication offered the best differential stain for imaging AMF in host tissues, yet, many 21st century publications still insist on using animal carcinogens and possible human carcinogens.

Hanke and colleagues used wide field light microscopy to visualize AMF associations with the rhizoids of *Marchantia polymorpha* (Hanke., *et al.* 2010). Glomus intraradices was inoculated onto the rhizoids of *Marchantia polymorpha* and allowed to develop for 40 days post inoculation. Rhizoid samples were cleared in 10% KOH and incubated at 95°C for 5 minutes, were then acidified in 5% HCL for 3 minutes at 2°C, and then stained with 1% Trypan blue at 95°C for visualization under a light microscope at 600x magnification (Hanke., *et al*. 2010).

The use of fluorescent proteins as a staining tool has become commonplace for researchers studying AMF. In 1961, Osamu Shimomura first isolated fluorescent proteins from *Aequorea victoria*, a jellyfish that emits a pale green glow via florescent proteins. Other fluorescent proteins have since been isolated from numerous other species of jellyfish (Sanchez., *et al*. 2004). The use of florescent proteins is becoming more commonplace in molecular biology because of its acceptance as a tool to visualize gene expression. Fluorescent proteins gained popularity since the structure and function was identified in 1996, when a Tsien., *et al*. reviled its 3D structure in a paper published in the Journal of Nature. His research on green florescent protein won a Nobel Prize in Chemistry in 2008.

The fluorescent protein is a barrel shaped molecule that has a light emitting chromophore at its center. Fluorescent proteins were originally purified from *Aequorea victoria* and emit a light peaking at 2 wavelengths, a major peak at 395 nm and a minor one at 475 nm and has an overall emission peak at 509 nm (Tsien., *et al.* 1995). This pale green glow is the result of a blue light wave created from the first emission peak that is absorbed and excites the protein causing the second peak. This process results in a green glow that is on the low end of the green photospectrum.

Since the original isolation of green florescent protein from *A. victoria*, researchers have modified this molecule to be more efficient by shifting the second emission peak from 475 nm to 488 nm. This causes an increased photo output of the green fluorescent protein, and the result is a stronger fluorescing molecule. Other modifications have been made to change the overall color from green wavelengths, to yellow, and blue, and even orange (Tsien., *et al.* 1995). This is useful as a research tool because different col-

ors can be utilized as a contrast to differentiate various metabolic processes. Some colors of fluorescent proteins are not stable under certain conditions, so many researchers have modified a specific color fluorescent protein to perform best in conditions of high/low chloride concentrations and in a range of high to low pH (Nagai., *et al.* 2002). Selection of a stain is based solely on the application of the researcher, and what kind of data the stain can provide.

Genre and colleagues used GFP as a probe to visualize hyphopodium and pre-penetration apparatus formation by the AMF *Gigaspora gigantea* in *Medicago truncatula* and *Daucus carota* var. *sativus* [11]. A hyphopodium is a morphological structure that is analogous to an appressoria, produced on lateral hyphael branches, which is created by AMF within the host plant tissue. This structure allows for penetration into subsequent cortex cells. A pre-penetration apparatus is formed to allow for intracellular movement of AMF hyphae through the lumen [11,12]. Genre., *et al.* used *Agrobacterium* mediated transformation techniques to transform *Medicago truncatula* and *Daucus carota* var. *sativus* with GFP-MAN and GFP-HDEL [11]. GFP-HDEL was shown to accumulate in the Endoplasmic Reticulum (ER) of the host plant tissues [11]. GFP-MAN in *Daucus carota* var. *sativus* appeared as bright spots throughout the host cytoplasm in epidermal tissue, and are interpreted as cis-Golgi elements [11]. After *G. gigantea* produced a hyphopodium in the host tissue, GFP-MAN accumulated at the pre-penetration apparatus (PPA) location [11].

Staining is not necessarily required for all microscopic imaging techniques involving arbuscular mycorrhizal fungi, although they may improve contrast and resolution of fungal tissues within a host plant. Genre and colleagues used the autoflourescent properties *G. gigantea*, to image appressoria, hyphae, and arbuscules in host plant tissues with LSCM [11,12]. This approach proved to be an effective method for imaging AMF without any staining procedures.

During the late 1990's, a type of narrow field light microscope called a Laser Scanning Confocal Microscope became a common method for visualizing AMF [13]. Laser scanning confocal microscopy (LSCM) uses optics to create thin sections of a desired specimen, then digitally fuses the individual sections into a three dimensional image [13]. Melville and colleagues state that the use of laser scanning confocal microscopy is good option for studying the inner workings of a plant cell [13].

In Melville's 1998 manuscript, the authors used four xanthene dyes, (4,5,6,7-tetrachlorofluorescein, phloxine B, rose Bengal, and sulforhodamine G) on LR-White resin imbedded plant/fungal tissues, and images were obtained from laser scanning confocal microscopy [13]. Both angiosperms and gymnosperms including leek (*Allium porrum* L.), green alder (*Alnus crispa* (Ait.) Pursh.), arbutus (*Arbutus menziesii* Pursch.), ginseng (*Panax quinquefolius* L.), ponderosa pine (*Pinus ponderosa* Dougl. ex Laws.), Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco), orchid (*Spiranthes sinensis* (Pers.) Ames), corn (*Zea mays* L.), were used as stock root tissues for their study, and the fixing procedure remained the same for the different plant/tree species sampled [13]. Sulforhodamine G was said to be the most effective of the dyes tested in this study. LSCM provided to be an excellent technique to visualize arbuscules, coiled hyphae, and vesicles of AM fungi within a variety of host tissues [13].

Dickson and Kolesik published a manuscript that focused on using LSCM to image the AM fungi Glomus spp. and *Scutellospora calospora* within *Allium porrum* L. and *Lilium* sp. [1]. Dickson and Kolesik used freeze sectioning and staining of fresh plant material to visualize AMF with laser scanning confocal microscopy [1]. This procedure was used to determine the surface area and volume of arbuscules, as well as hyphal coils that formed inside of *Allium porrum* and *Lilium* spp. respectively [1]. An unidentified Glomus spp. was inoculated onto the roots of *Allium porrum* and *Scutellospora calospora* was inoculated onto the roots of *Lilium* spp [1]. Sulforhodamine G and phloxine B were used to differentiate fungal tissue from plant tissues [1].

Kumar and colleagues used LSCM to visualize the relationship between AMF and roots of three Mangrove species; *Acanthus ilicifolius* L., *Ceriops tagal* (Perr.) Robins, *Excoecaria agallocha* L. [4]. Kumar and colleagues used Trypan blue, a diazo dye synthesized from a derivative of toluidine blue, to act as a fluorochrome in fluorescence microscopy [4]. Kumar and colleagues state that trypan blue has been used to visualize mycorrhizal fungus at low magnification but had never been used as a fluorochrome in fluorescent microscopy [4]. Kumar., *et al*. explains that LSCM is an excellent tool for visualizing AMF in plant roots because fluorescent structures can be imaged with high resolution without mechanical sectioning [4].

Differential Interference Microscopy (DIC) is a wide field light microscopy technique that uses the properties of polarized light, and special optics to capture an image that has a three-dimensional appearance. Blaszkowski and colleagues used DIC to identify 2 new species of AMF from costal dunes in northern Poland [5]. *Glomus achrum* sp. nov. and *Glomus bistratum* sp. nov. are described in this manuscript, where there spore morphology, as well as 18s small subunit rDNA is used for phylogenetic analysis [5]. Spore samples were mounted on glass slides with either lactic acid, polyvinyl alcohol – lactic acid – glycerol (PVLG), or a mixture of PVLG and Melzer's reagent (1:1 v/v). Some spore samples were stained with 0.1% Trypan blue, although the procedures were not men-

tioned in the materials and methods section of that manuscript [5]. Blaszkowski and colleagues published a follow-up manuscript in 2010 describing another new species of AMF, *Glomus indicum*, and used the same procedures as mentioned above [14].

Transmission electron microscopy (TEM) is used to study materials, metals, polymers, and biological specimens. In the biological realm, TEM's are often used to image the ultrastructure within cells, by producing a two-dimensional image that is captured by an analog or digital camera. The use of TEM is advantageous to image cross sections of cells and is used specifically to image the internal structures of cells. During bright field TEM, a primary electron beam passes through the negative space of a sample, while an aperture blocks secondary elastic scattered electrons that are produced by a nuclear interactions of the primary electron beam and the sample material. This creates a dark image formed on the light background, and a detector is used to collect the signal. During dark field TEM, an aperture blocks the primary electrons, and the secondary elastically scattered electrons are collected by the detector, creating a dark background, and a light colored image.

One of the advantages of TEM is that they can resolve an image up to 0.04 nm. They can also be used for immunolabeling experiments, as well as histochemical studies. TEM's are usually used to image the internal contents of cells, including membranes, organelles, and viruses. TEM's are advantageous because of their versatility to image both biological, as well as materials samples. One of the disadvantages of TEM's is that they are ineffective to image samples that are greater than 100+nm thick, with an optimal thickness of roughly 60-80nm. This limits the sample to an object that can be ultra-thin sectioned. Another disadvantage to TEM, is that biological samples produce a low signal to noise ratio, unless they are stained with heavy metals. Heavy metals are often toxic to humans and pose a potential health risk the applicator.

TEM of plants and fungi use glutaraldehyde for a primary fixation, and then usually use osmium tetroxide for a secondary fixation, and uranyl acetate as a tertiary fixation. Plant tissues are imbedded in epoxy resin, usually Spurs, or white resin. After polymerization of the resin blocks, ultra-thin (50 - 100 nm) sections are made using an ultramicrotome, and collected on TEM grids. Sometimes a quaternary stain, such as lead citrate is used post-sectioning to provide better resolution of membranes. All of these fixatives act as stains that could provide resolution between fungal tissue and plant tissues. All of the fixatives used for electron microscopy techniques have health hazards associated with them, which means they need to be used with extreme caution.

Stain	Microscopic Technique	Advantages and Disadvantages	Reference
Acid Fuchsin	LM BF/ LSCM	Used in tandem with freeze sectioning techniques to provide 2D and 3D micrographs of arbuscules , fades quickly	Dickson and Kolesik [1], Bundrett., *et al.* [2], Schaffer., *et al.* [3]
Chlorazol Black E	LM BF	Highest Contrast for Light Microscopy, known animal carcinogen and possible human carcinogen	Kumar., *et al.* [4], Grace and Stribley [8]
Trypan blue	LM BF/LM DIC/LSCM	Common stain, Confirmed animal carcinogen and possible human carcinogen	Kumar., *et al.* [4], Blaszkowski., *et al.* [5]
Phloxine B	LSCM	Can be used to stain through LR-White Resin, rapid staining technique	Melville., *et al.* [13]
Eosin B	LSCM	Can be used to stain through LR-White Resin, rapid staining technique	Melville., *et al.* [13]
Rose Bengal	LSCM	Can be used to stain through LR-White Resin, rapid staining technique	Melville., *et al.* [13]
4,5,6,7-tetrachlorofluoroscein	LSCM	Can be used to stain through LR-White Resin, rapid staining technique	Melville., *et al.* [13]
Sulforhodamine G	LSCM	Can be used to stain through LR-White Resin, most effective staining technique in melville., *et al.* 1998	Melville., *et al.* [13]
SYBR Green	LSCM	Fluorescent DNA binding probe, not good for long term studies, fades	Lee., *et al.* [15]
DAPI	LSCM	Fluorescent DNA binding probe, performs better than SYBER Green, but autofluorescence is stronger	Lee., *et al.* [15]
PATAg	TEM	Localizes β-1,4 and 1,6 polysaccharides	Cordier., *et al.* [16]

Toluidine Blue	LSCM	Used for post embeding, staining of fungal hyphae	Cordier., *et al*. [16]
Wheat-germ agglutinin	LSCM	Histochemical used as a fluorescent label	Hause., *et al*. [17]
Green Fluorescent Protein	LSCM	Very specific labeling, used for fungal transformations and molecular physiological studies	Genre., *et al*. [12], Genre., *et al*. [11], Kobae., *et al*. [18]
DsRED	LSCM	Reporter for molecular studies, had less autofluorescence than GFP	Helber., *et al*. [19]
Ink and Acetic Acid	LM BF	Low toxicity, Nontoxic, Shaeffer Black ink highest contrast	Vierheilig., *et al*. [6]
Sudan Black	LM BF	Differential stain for total lipids	Nemec., *et al*. [7]
Sudan IV	LM BF	Neutral lipid stain	Grace and Stribley [8], Mosse 1970
Nile blue	LM BF	Differentiate between neutral lipid and phospholipid	Nemec., *et al*. [7]
Feulgen	*	Stain for carbohydrates, stain for mitotic activity	Nemec., *et al*. [7], Trotta., *et al*. 1996
Fast green	*	Basic protein stain	Grace and Stribley [8]
Azure B	*	Successfully stain for RNA	Grace and Stribley [8]
Fast blue	LM BF	Many added steps for staining procedure	Tisserant., *et al*. [10]
Nitro Blue Tetrazolium Chloride (NBT)	LM BF	Used with Acid Fuchsin as a counter stain, fades quickly, asses immediately	Schaffer., *et al*. [2]
Mithramycin A	LSCM	DNA specific fluorescent stain	Becard., *et al*. [20]

Table 1: Commonly used stains that differentiate Arbuscular Mycorrhizal fungus tissue from host plant tissue for various microscopic techniques.

LM BF: Light Microscopy Bright Field; LSCM: Laser Scanning Confocal Microscopy, *: Microscopic Technique not stated in literature.

Concussion

Nearly all of herbaceous plants form relationships with Arbuscular Mycorrhizal Fungi, and they are an important member of the agricultural ecosystem. The host plant receives nutrients, water, and protection from pathogenic invaders, where the fungus receives a niche in which to survive. This review provides a comprehensive collection of stains used to image Arbuscular Mycorrhizal Fungi in host tissues, which will allow researchers quickly determine a stain the is appropriate for a specific application. This is the most complete collection of stains for Arbuscular Mycorrhizal Fungi to date [21].

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