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Chapter

EFFECTIVE AND FLEXIBLE METHODS FOR VISUALIZING AND QUANTIFYING ENDORHIZAL FUNGI

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Abstract: Fungi associated with plant roots are gaining prominence as to their importance for plant survival in a diversity of terrestrial ecosystems. Assessing the importance of interaction types depends in part on quantifying interaction prevalence, particularly in plants harvested from natural ecosystems. In turn, this depends on a sensitive method for fungal visualization, and a reliable method for quantifying potentially multiple endorhizal morphotypes. Recent developments in these areas are discussed.

K**ey words**: confocal fluorescence; endophyte; fungus; mycorrhiza; quantification; root;

1. INTRODUCTION

Plant roots are associated with a diversity of endorhizal and rhizosphere fungi whose interactions vary from endophytic to pathogenic. Few of these interactions produce macroscopic phenotypes, apart from ectomycorrhizae, which are associated with morphological changes in colonized roots (Brundrett *et al.,* 1996, Smith and Read, 1997). However, this does not mean the others are unimportant! Arbuscular mycorrhizal (AM) interactions are found in about 80 % of terrestrial plant families and under experimental conditions have been shown to contribute to plant survival and competitiveness (Smith and Read, 1997). In addition, roots harbour a diversity of endophytic fungi that now are shown to be widely distributed and have roles including stress tolerance (*e. g.,* Márquez *et al.,* 2007, Rodriguez *et al.,* 2008). In order to fully understand the nature of plant-fungal interactions and their relative importance to the plant, it is essential that fungi be clearly visualized and accurately quantified.

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Microscopic visualization methods for endorhizal fungi have recently been reviewed by Peterson *et al.* (2004) and Vierheilig *et al.* (2005). Traditional mycorrhizal visualization used stains with transmitted light microscopy. Classic fungal stains like cotton blue and lactofuchsin, provide relatively lower contrast in transmitted light, or can fade when on storage. For certain experimental studies, roots can be infected with a fungal strain that has been transformed with a fluorescent protein construct (*e. g.* GFP), so that fungal growth and behaviour can be followed in living roots. However this depends on using previously identified and genetically tractable species transformed with a fluorescent construct (still relatively uncommon for some species, and not available for AM fungi) rather than assessing roots harvested from natural environments.

Most roots are several to many cell layers thick and may have pigmented surface layers, whereas most fungi are disseminated mycelia of hyaline hyphae. Only the dark septate endophytes can readily be identified without staining (Jumpponen and Trappe, 1998). For transmitted light microscopy of mycorrhizal fungi, cleared roots are typically stained with chlorazole black E (CBE) (Brundrett *et al.,* 1996), trypan blue (Phillips and Hayman, 1970), or lactofuchsin (Carmichael, 1955). The latter sometimes have insufficient contrast for high magnification transmitted light microscopy.

Quantification of AM fungi described Brundrett *et al.* (1996) used CBE-stained roots examined with a dissecting microscope. This provides an overview of fungal colonization, but assessment depends in part on skill and experience, so results can be difficult to reproduce quantitatively between users. A method described by McGonigle *et al.* (1990) used 200 X magnification to examine roots at defined intersections. With this method, the user's attention was directed to a precise region, examined at relatively high resolution. Reproducibility of the microscopic intersect method even with different users was good, suggesting that it was relatively objective. However, as described, this method was limited to quantifying AM fungi, and did not distinguish between AM morphotypes, nor between levels of AM colonization.

Our studies of AM associates in roots of plants collected from sites in the Canadian High Arctic, where they had been thought to be rare or absent, required that we develop a sensitive method for fungal visualization, a secure method for specimen preservation, and a reliable method for quantifying potentially multiple endorhizal morphotypes. Endorhizal visualization methods using fluorescence microscopy (Allen *et al.,* 2006) were later extended to studying roots with multiple morphotypes and from herbarium specimens (Ormsby *et al.,* 2007). Specimen preservation and endorhizal quantification methods described in the latter paper have led to a

set of robust and flexible methods that will be presented in detail, below.

2. METHODS

2.1 Choice of stain

Trypan blue has been used as a fluorescent stain for fungal foliar pathogens (Wei *et al.,* 1997), although the preparation method appears to be laborious. The structure of trypan blue and CBE is similar (Figure 1) suggesting that CBE might be useful as a fluorescent stain for mycorrhizal fungi. CBE fluorescence imaging was not superior to transmitted light microscopy, since CBE solutions develop a fine particulate background. Acid fuchsin, the dye component of lactofuchsin, which is used as a fungal stain (Carmichael, 1955), has a chemical structure suggesting it might be fluorescent (Figure 1). Merryweather and Fitter (1991) used lactofuchsin for fluorescence imaging, but this method had not been pursued by the authors or others. We independently considered lactofuchsin as a fluorescent stain for fungal cells within plant roots, particularly using confocal epifluorescence microscopy. Use of other fluorescent stains including aniline blue are reviewed in Peterson *et al.* (2004) and Vierheilig *et al.* (2005).

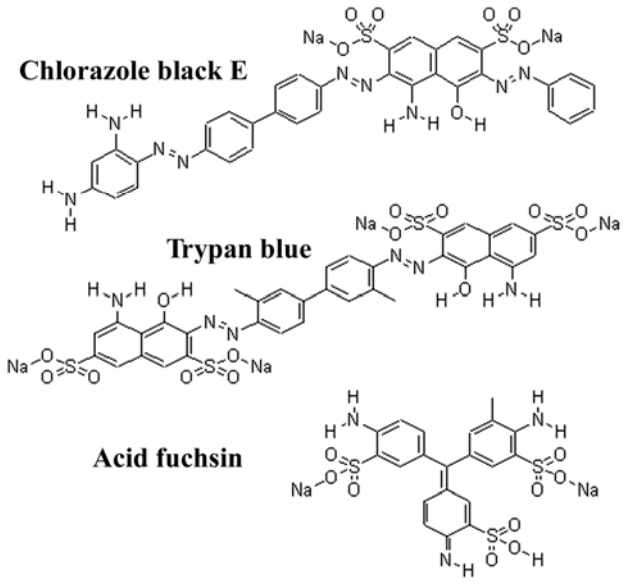

Figure 1. Chemical structures of chlorazole black E (CBE), trypan blue, and lactofuchsin.

2.2 Sample preparation for lactofuchsin staining

Plant roots from different species may grow intertwined. It is critical that the above-ground and root samples are from the same plant. In addition, roots like those of peony (*Peonia chinensis*; Figure 2) have a complicated architecture, and are highly pigmented. For these roots, the bulk soil was gently removed by hand (Figure 2A) and the remaining soil removed by soaking and then rinsing in tap water (Figure 2B). The soil balls in Figure 2A contained abundant fine branches.

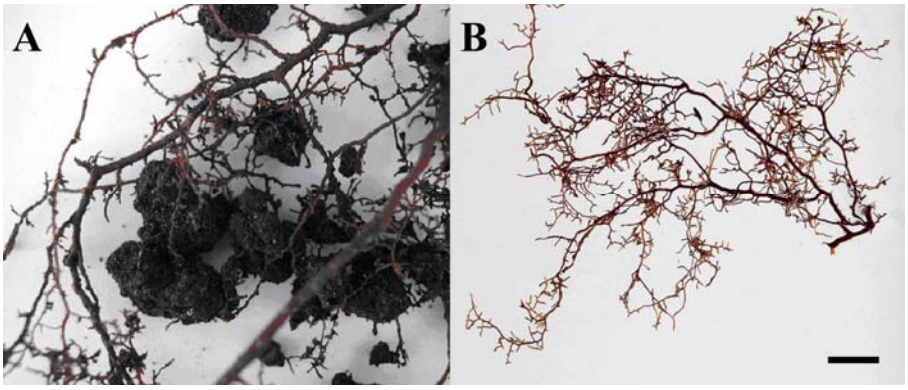

Figure 2. Roots of peony (*Peonia chinensis*) that had been growing in undisturbed garden soil. Bar in B = 1 cm.

2.2.1 Fixing

Washed roots were fixed in 3.7 % formaldehyde containing 0.5 % ethanol, buffered to pH 7 in 50 mM Na-K phosphate. Fixation with 3.7 % formaldehyde alone, and with 70 % or with 95 % ethanol was also tested. All gave similar results for staining. However, it may be advantageous to fix in 100% ethanol or isopropyl alcohol, if subsamples might be used for molecular DNA studies.

As described in Ormsby *et al.* (2007), it is possible to adapt the clearing and staining technique for roots sampled from herbarium specimens. These are rehydrated by autoclaving in 10 % KOH (see below). Even after rehydration, herbarium root samples were sometimes more brittle than chemically fixed roots. All other techniques are the same as for chemically fixed roots. Herbarium specimens can also be used for molecular DNA analyses, and serve as a voucher for identification.

2.2.2 Sampling

For quantitative studies, roots should be cut into segments typically 1-2 cm long, suspended in a large volume of water, and then randomly sub-sampled. For some studies there is a limited amount of root material available, in which case it is preferable to examine the entire length.

2.2.3 Clearing

Root samples are typically autoclaved for 20 min in 10 % KOH, to clear the cytoplasm, using wide glass vials that are topped with a large glass marble to prevent evaporation. Aluminum foil is degraded by hot 10% KOH vapours. Delicate roots survive this treatment, but clearing stems and leaves may require only 95 ºC for 20 min at atmospheric pressure. Cleared samples should be rinsed twice in room-temperature 70% ethanol to remove the KOH, which is more effective than water alone.

2.2.4 Bleaching

Pigmented roots must be bleached before staining. Roots can be bleached in freshly prepared peroxide solution (1 : 1 : 8 – 28% ammonium hydroxide : 30% hydrogen peroxide : distilled water) or in commercial sodium hypochlorite bleach diluted in distilled water to about 1.75 % $NaClO_3$. The latter is effective for bleaching, but these specimens may have higher plant wall fluorescence. Roots are incubated in room-temperature bleaching solution (with occasional swirling, particularly for the peroxide bleach) until pale. Typically this requires 15-30 min. Bleached roots are rinsed twice in distilled water prior to staining. Following staining in lactofuchsin, the vascular cylinder is likely to fluoresce, which can be useful for orientation.

2.2.5 Staining

Roots are stained in lactofuchsin (Carmichael, 1955), 0.1% acid fuchsin in 85 % lactic acid. Staining is for 0.5 - 3 h at 47 ºC to 68 ºC, which can be optimized for different root types. Stained roots are rinsed twice in room-temperature destaining solution (DLAG), which is 1:1:1 distilled water: 85% lactic acid: glycerol) to remove surface dye, and then destained in DLAG at 47 ºC to 68 ºC, typically for 3 h to overnight. Deeply-stained roots will likely have high background fluorescence and poor imaging characteristics. Faintly-stained roots may be still be useful since the fluorescence yield of lactofuchsin is much higher than the visual contrast.

Understained (or overly destained) roots can be re-stained, for longer or at a higher temperature.

2.2.6 Mounting

We mount roots in PVAG solution (recipe below), and cover them with a coverslip. This provides security and permanence, compared to mounting in DLAG, and these slides are easier to clean if immersion optics are needed. Where possible, it is most convenient to mount the roots parallel to the long axis of the slide. Bubbles trapped during coverslipping are all but impossible to remove, but seldom interfere with image acquisition. The PVAG solution is polymerized overnight at 40 ºC. Following PVAG polymerization, the edges of the coverslip, which can chip or lift, should be sealed with nail polish for protection. Slides stored in the dark at 4 ºC are stable for at least two years. PVAG does not fluoresce at the excitation wavelengths used for lactofuchsin staining.

PVAG solution, modified from Brundrett *et al.* (1996), contains 4 g polyvinyl alcohol powder [we use 98 % polymerized, but various grades are available, and many appear to work]: 50 mL distilled water : 20 mL glycerol. This is warmed to 60 ºC, covered, with constant stirring until dissolved, typically 3 h to overnight. Eventually, PVAG solution will solidify at room temperature and become opalescent, but it can be re-melted one or more times with gentle heat and stirring.

2.3 Imaging

Lactofuchsin-stained roots can be examined with transmitted light, or with widefield or confocal epifluorescence illumination. Lactofuchsin has a wide range of excitation wavelengths, spanning at least 405 nm – 534 nm (blue to green) available with most epifluorescence systems. We typically use an FITC filterset (widefield) or 534 nm excitation (confocal). The emission range is also broad. For confocal imaging, we typically use an LP585 filter. Under these conditions, the actual colour of lactofuchsin fluorescence is orange-red. We choose to present images that are false-coloured yellow, in order to increase image contrast.

As with other types of fluorescence imaging, there are tradeoffs to consider. Widefield epifluorescence illuminates the entire field, and both the depth of focus and the lateral resolution are related to the numerical aperture of the objective. Fortunately, lactofuchsin is a very stable fluorochrome, so photobleaching is seldom a problem. Confocal epifluorescence optics provide exquisite control of lateral and depth resolution, but higher resolution images will have reduced depth of focus, which can be

problematic for fine endophytes (FEs). Shallow depth of focus can be offset by collecting multiple focal levels (z-stacks) at the cost of additional time for image collection. Typically we use confocal optics for high resolution imaging, and widefield optics for quantitation.

Examples of endorhizal fungal imaging using lactofuchsin staining and confocal epifluorescence microscopy are shown in Figures 4 – 6.

Figure 4 shows transmitted light (A) and confocal fluorescence (B) images of a lactofuchsin-stained *Taraxacum officinale* (common dandelion) root, collected from garden soil in Saskatoon SK, 52 ºN. These images were collected simultaneously. The greater depth of focus with transmitted light microscopy provides a sense of continuity for the Paris-type coils (P), but details of arbuscule (Ar) structure are more evident with confocal fluorescence because out-of-focus glare is reduced. Multiple focal depth (z-stack) confocal imaging shows that the hyphae which form coils and arbuscules are continuous.

Figure 5 shows FE hyphae in roots of a *T. phymatocarpum* plant collected from highly mineral tundra soils overlying shallow permafrost, on Axel Heiberg Island in the Canadian High Arctic, 80 ºN. The FE network shown in (A) are typical of near root-surface morphology, whereas those in (B and C) is typical of networks deeper in the root. This FE colony appears to be relatively newly established, since it has yet to form arbuscules, which typically form near the vascular cylinder (see Figure 5, Allen *et al.,* 2006). Due to their narrow width (1 – 1.5 μm) FE hyphal networks are difficult to study at high spatial resolution using transmitted light microscopy. FE hyphae are highly abundant in plants from High Arctic sites (Ormsby *et al.,* 2007) and so are likely to be ecologically important.

Figure 6 shows FE hyphae, arbuscules, and vesicles of a *T. phymatocarpum* root. This plant was collected near the specimen shown in Figure 5, stained with lactofuchsin, and imaged with confocal fluorescence. This relatively high resolution image shows the detail that can be acquired with confocal microscopy.

Figure 7 shows lactofuchsin-stained fungi in a peony root sample collected from garden soil in Saskatoon, SK. These roots have not been studied previously for their fungal associates. The macroscopic appearance of these roots suggested they would be ectomycorrhiza; however, no evidence of a mantle or Hartig net system was found. Morphologies consistent with ectomycorrhizae are readily recognized with lactofuchsin fluorescence (not shown). Peony roots are challenging to work with because of their architecture and pigmentation. In this case, the adjacent field-of-view procedure shown in Figure 3 is useful for keeping attention focused during quantitation.

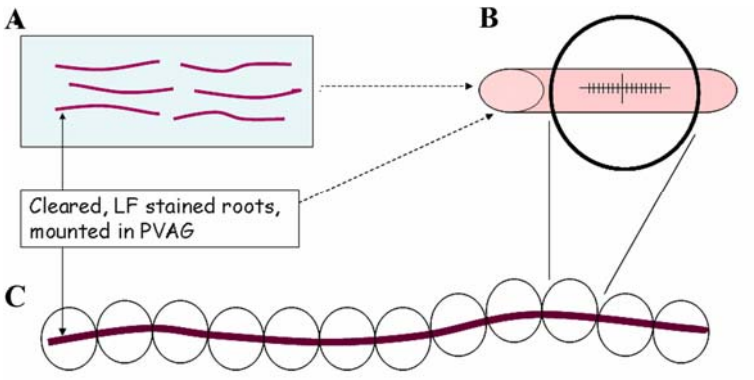

Figure 3. The scanning procedure for the Multiple Quantitation Method. Roots (A) are visualized using at least 200 X magnification (B). Fungal structures that intersect the vertical line on the graticule are considered, throughout the entire focal depth of the root. (C) Intersections are evenly spaced by moving the stage by one field of view each time.

3. THE MULTIPLE QUANTITATION METHOD

Quantitation is essential to assess the relative physiological importance of particular interactions. Molecular DNA techniques can be excellent for identification, with caveats as described in Shepherd *et al.* (2007), but are not well-suited to quantitation. Our multiple quantitation method (MQM), built on that of McGonigle *et al.* (1990) and described in Ormsby *et al.* (2007), accommodates samples that have abundant endorhizal fungi besides AM, and that vary in colonization abundance (Table 1).

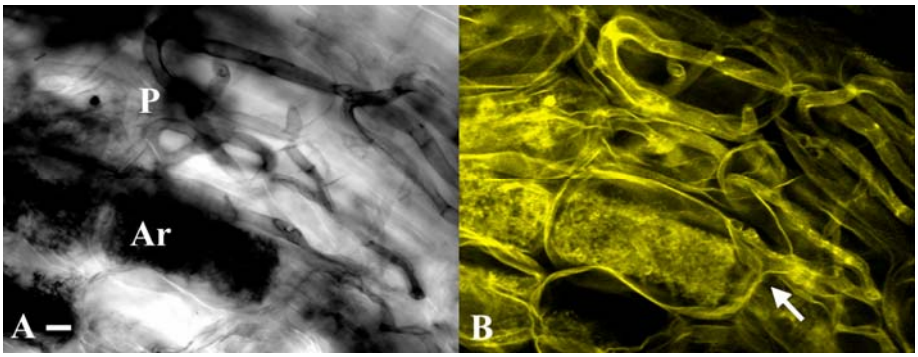

Figure 4 Arbuscular mycorrhizal colonization in *Taraxacum officinale* (common dandelion). This specimen was stained with lactofuchsin and imaged with transmitted light (A) and confocal epifluorescence optics (B). Arbuscule (Ar). *Paris*-type intracellular hyphal coil (P). The arrow indicates continuity between an intercellular hypha and the arbuscule. Bar in A = 10 μm, for both.

As indicated in Figure 3, we recommend (where possible, but see Figure. 2) that root samples be arranged parallel to the long axis of the microscope slide, so they can be examined systematically (Figure 3A). Statistical studies by McGonigle *et al.* (1990) show that quantitation estimates by different users examining the same sample converged by about 150 intersections. There is no guarantee that different portions of a large root system will be colonized to the same extent, so increasing the number of intersections leads to a diminishing return for effort. Nevertheless, for large root samples, assessing colonization at 150 intersections may leave some portions unexamined. If so, the remaining material should be scanned for rare types of interaction.

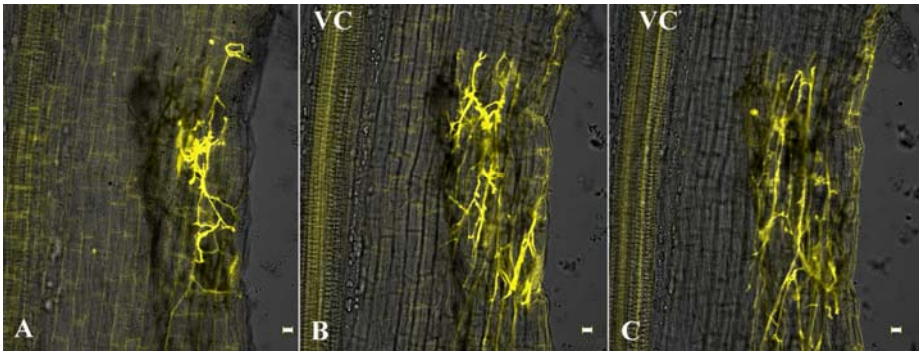

Figure 5. Fine endophyte (FE) hyphae in a *Taraxacum phymatocarpum* root. This lactofuchsin-stained sample was imaged with confocal epifluorescence merged with transmitted light to provide spatial context. Root cortical cell walls and the vascular cylinder (VC) have faint fluorescence. Optical sections were A) 8 µm, B) 21 µm, and C) 32 µm beneath the root surface. Bars = 10 µm.

We start at one corner of the slide, position the field of view and the orientation of the eyepiece graticule so that the intersection line is perpendicular to the root axis at that point (Figure 3B). The entire depth of the root should be examined (adjusting the fine focus as needed) and the data recorded. Then the stage should be moved laterally by one field of view, and the scoring process repeated. This systematic approach reduces the very real temptation to be attracted to the colonized parts of the root, which can lead to overestimation. It can also help when dealing with complicated root architectures as shown in Figure 2B.

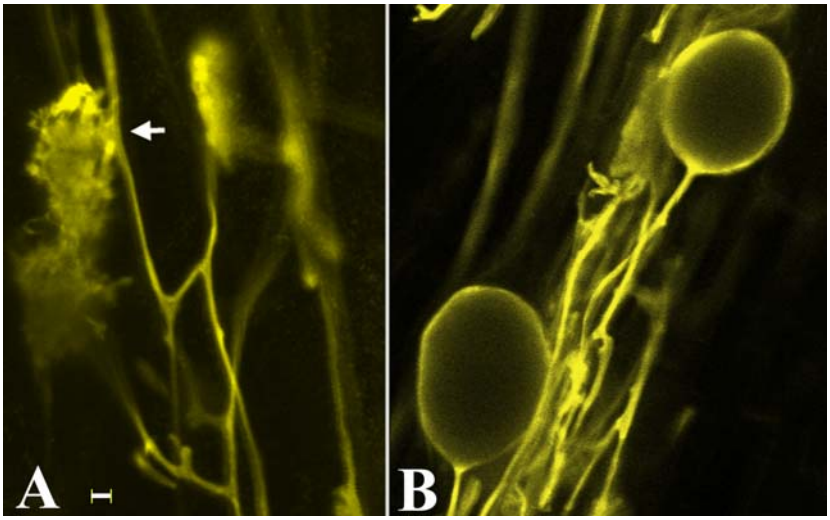

Figure 6. Fine endophytes (FEs) in a *Taraxacum phymatocarpum* root stained with lactofuchsin and imaged with confocal epifluorescence. FEs produce (A) arbuscules, and (B) vesicles. The arrow in A shows continuity between an FE hypha and an arbuscule. Bar in A = 2 μm, for both.

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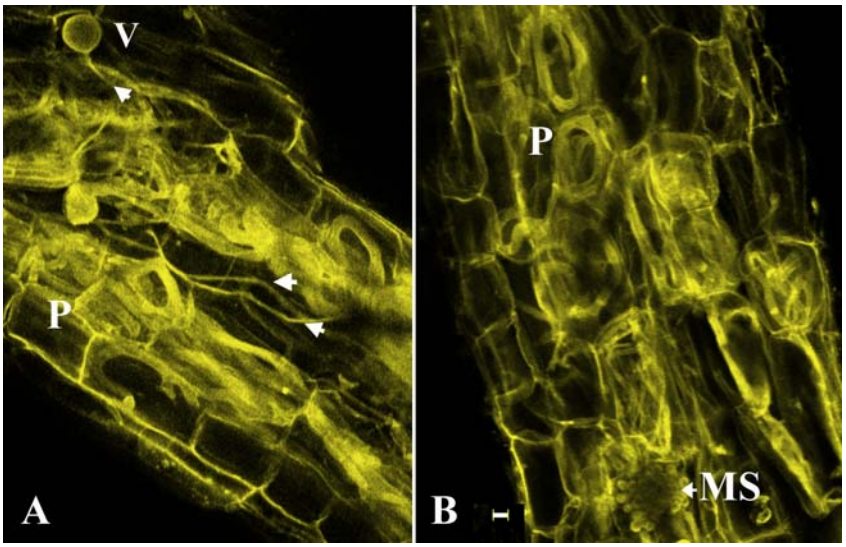

Figure 7. Endophytic fungi in peony roots visualized with lactofuchsin staining and confocal epifluorescence optics, including arbuscular mycorrhizal (AM) fungi and fine endophytes (FEs). AM fungi formed *Paris*-type (P) intracellular coils. FE hyphae (arrowheads in A) produced vesicles (V), and arbuscules (not shown). A putative microsclerotium (MS) suggests there may also be septate endophytes. Bar in B = 10 μm for both.

Table 1. Fungal endorhizal colonization[a] for Asteraceae from Axel Heiberg Island, 80ºN, assessed using lactofuchsin epifluorescence, and the multiple quantitation method.

Species (# of plants)	**AMA**	**AMV**	**AMH-L**	**AMH-M**	**AMH-H**	**AMH-tot**
Arnica alpina (10	0.7±0.5	1.3±1.3	0±0	1.1±0.5	7.9±5.2	9.0±5.2
Erigeron compositus (5)	0±0	0±0	0.6±0.6	0.8±0.8	2.0±1.3	3.4±2.2
E. eriocephalus (8)	7.5±3.0	0.5±0.3	0.4±0.3	7.9±2.4	29.7±7.5	38.0±7.6
Taraxacum hyparcticum (6)	15.8±4.6	9.0±4.0	1.0±0.6	10.3±3.7	19.2±4.8	30.5±5.5
T. phymatocarpum (13)	13.5±4.3	11.4±3.5	1.8±0.6	6.7±1.1	39.8±7.6	48.3±7.7
All species	9.1±1.8	5.2±1.4	0.8±0.2	5.6±0.9	25.5±3.7	31.9±4.1

Species (# of plants)	**FEA**	**FEV**	**FEH**	**SEH**	**Total fungal Colonization**
Arnica alpina (10)	27.8±10.1	10.4±3.6	50.5±13.6	27.4±10.5	82.7±6.3
Erigeron compositus (5)	6.4±3.6	4.6±3.0	44.2±17.9	27.6±14.1	59.0±16.9
E. eriocephalus (8)	0.6±0.4	0.2±0.2	10.2±4.3	50.7±6.4	76.1±6.0
Taraxacum hyparcticum (6)	21.0±4.2	28.3±3.2	76.8±10.4	26.5±10.2	88.3±5.2
T. phymatocarpum (13)	5.6±3.4	2.8±1.4	16.5±8.4	42.1±8.6	78.3±7.3
All species	11.6±2.9	7.5±1.7	33.8±5.7	36.7±4.4	80.0±3.6

a Summary data of endorhizal colonization is quantified for endorhizal fungi, and expressed as percent abundance ± standard error of the mean. These data were originally presented in Ormsby *et al.,* (2007). Arbuscular mycorrhizae (AM) were categorized as arbuscules (AMA), vesicles (AMV) and hyphae (AMH). Root samples from each plant were assessed at about 100 intersections per sample. AM hyphae varied in abundance, so were subdivided into abundance classes: low (1 hypha per intersection), medium (2 - 5 hyphae per intersection) and high (> 6 hyphae per intersection). Fine endophyte (FE) fungi were categorized as arbuscules (FEA), vesicles (FEV) and hyphae (FEH). Septate endophyte hyphae (SEH) were found in the same roots. Total colonization was the number of intersections assessed, minus intersections lacking fungal structures.

With the MQM technique, each type of fungal interaction is scored separately, and new categories can be added if necessary. In samples that contain multiple types of fungal endophyte (*e.g.* Figures 4, 7) scoring can be facilitated by considering the morphology of the hyphae to either side of the intersection (as they are likely to be continuous), but quantification data should only be collected at the intersection. In addition to scoring fungal interactions, the absence of interaction is also important. Intersections that do not contain fungi should be scored as a separate category, so that overall colonization can be calculated. Otherwise, as in Allen *et al.* (2006) summing abundances could be misleading.

In Ormsby *et al.* (2007) we were faced with the challenge that some intersections had only one AM hypha, whereas in others they were very abundant. We defined additional categories (in this case low, medium and high hyphal abundance) to describe this variation (Table 1). Roots of Arctic

Asteraceae had few AM hyphal coils; the data in Table 1 are almost exclusively for *Arum*-type intercellular hyphae. Thus it was perhaps surprising that there were relatively few arbuscules (these plants were collected in early July 2004, at the height of the Arctic summer), however, Ryan *et al.* (2003) have shown that intercellular AM hyphae may also participate in mineral nutrient transfer.

4 DISCUSSION

Discrimination and quantitation of endorhizal fungal structures is an important correlative technique for molecular and physiological studies, because it is likely to be directly related to the significance of an interaction to the symbiotic partners. The methods described in this chapter include the most sensitive staining and imaging currently available. Fluorescence microscopy has long been the method of choice for cell biology, because luminous objects are highly contrasted with a black background, which increases detection and resolution of fine structures. In this application, high detectability and spatial resolution are particularly important for examining details of arbuscule structure and FE hyphal networks.

To date, for samples harvested from field sites where there is often little control of the fungal root symbiont(s), lactofuchsin staining viewed with epifluorescence provides a convenient combination of relative simplicity of preparation and imaging quality.

The choice of confocal *vs* widefield epifluorescence imaging depends on the need, and naturally on availability of equipment. Confocal epifluorescence optics are often superior for documentation, whereas widefield optics are more efficient for quantitation. Modern widefield epifluorescence microscopes are typically equipped with high-sensitivity imaging systems, so there can be considerable flexibility.

We have found endorhizal quantitation using the MQM method to be reproducible as well as flexible. MQM is well suited for plant roots harvested from natural locations where the endorhizal fungi are not necessarily well described. Furthermore, MQM can describe the relative contributions of different endorhizal interactions, which must intrinsically be related to their importance to the plant's physiology.

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