MEASURING ARBUSCULAR MYCORRHIZAL FUNGAL COLONIZATION OF ROOTS

(NON-TECHNICAL PROTOCOL)

(Giovannetti and Mosse, 1980; Vierheilig et al., 1998)

Introduction

Arbuscular mycorrhizal fungi are endomycorrhizal fungi where the fungal hyphae penetrate the root cell walls and grows within and between root cells and up to cell membrane. To be able to measure mycorrhizal presence in the roots, the root cells need have all their cellular contents (such a nuclei, organelles, and cytosol) removed or roots are cleared. The roots are cleared with an alkaline, lye solution which turns the solution brown. The fungal body parts (i.e. hyphae, arbuscules, spores and vesicles) are stained ink to make them visible inside the clear roots. Note that the root stele may be slightly stained as well.

When sampling roots to detect and/or measure the amount of mycorrhizal colonization, it is important to select finer, more fibrous roots. Older roots or those from plants with taproots or other coarse roots, may have some mycorrhizae, but it usually is sparse, and consists only of hyphae that often is most visible outside the roots. For plants with a fibrous root system, then a random sampling suffices. Darkly pigmented roots should be avoided -- to use them means going through an additional bleaching step (not covered here).

Materials

Colander
Forceps/tweezers
Fume mask*
Gloves, latex or nitrile*
Glycerol/Glycerin
Grid paper with grids of a known size (optional)**
Ink*
Lye/drain cleaner*
Marker, permanent
Microscope, dissecting or compound (optional)**
Microscope slides (optional)**
Oven
Paper bags
Petri plates (with grid, optional)**
Probe/needle
Scale or balance, kitchen or food scale may be used
Stove and sauce pan (optional)*
Vials, bottles, Mason jars or any container large enough to hold roots, preferably glass with a lid
Vinegar

Water, Distilled water preferred but tap water is fine

* Safety:
1. Wear gloves and a fume mask (if possible) and use a well-ventilated area (particularly if using a powdered lye or drain cleaner) when making the alkaline solution in Step 8 or make the solution under a hood. The dust from powder may be harmful to your lungs as well as the fumes when the powder or liquid is combined with the water. To reduce fumes when adding the drain cleaner to water, seal with a lid immediately. Also note heat will be generated when making this solution so you need to make this in a glass Mason or media jar.
2. Gloves should be worn when working with the stain.
3. Optional – If staining needs to be done in one day, you may heat the alkaline solution used in steps 8-9 to a rolling boil in a saucepan on the stovetop but this is not recommended because of safety issues. If you do heat the alkaline solution, you need to use glass containers with lids for the roots, a fume mask, well-ventilated area, and gloves as well as thermal gloves or oven mitts. Caution must be used when pouring the hot alkaline solution (use thermal gloves or oven mitts with your hands in plastic gloves as a protective layer) over the roots and place the lid on immediately after adding the alkaline solution. Incubate with the hot alkaline solution for 5 minutes rather than 24 hours. After completing steps 10-12, heat the ink:vinegar:glycerol:water stain to a rolling boil. Add to the roots and incubate for 5 minutes rather than 24 hours (step 13) and then continue with the remaining steps. Please wear a fume mask and gloves and work in well-ventilated areas if doing the rapid-staining process and working with boiling solutions.

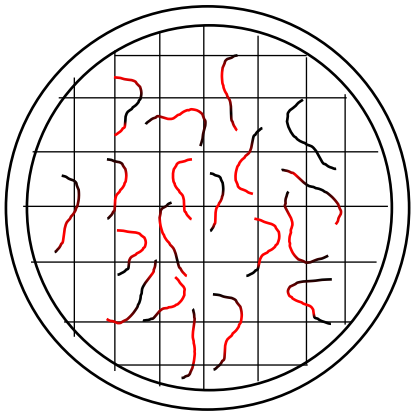
Methods

1) Wash roots with tap water or gently with a sprayer in the colander or sieve to remove soil.
2) Pat roots with paper towels to remove excess water.
3) Cut the roots into about 1 inch or 2.5 cm pieces and mix well.
4) Weigh roots. Immediately take a subsample (at least 0.2 ounce or 0.5 grams) to dry and the remaining roots or a subsample (at least 0.2 ounces or 0.5 grams) for staining. Record the weights of both subsamples as well as the total weight of the roots.
5) Weigh a paper bag and record the bag weight on the bag. Place the root sample to dry in the paper bag and dry at 110-220 ^{o}F in an oven. Weigh after completely dry and calculate the percentage of water in the subsample and use this to calculate the total dry weight of the root sample. See calculations below.
6) Take the remaining damp roots (or at least a 0.2-ounce or 0.5-gram sample) and place them in vials or jars for staining.
7) Roots may be stored in a refrigerator with distilled water for a week before staining. If they need to be stored for a longer period, place the roots in a 1:1:1 vinegar:glycerol:water solution in a refrigerator or a 2:1 vinegar:water solution.
8) Make a 10% alkaline solution (10 parts lye or drain cleaner in 90 parts water).

9) Carefully remove solution or water from the roots from step 7 (using a colander or sieve if needed to collect the roots and transfer them back into the container with a forceps or tweezers) or take the roots in the containers from step 6. Add the 10% alkaline solution. Leave at room temperature for 24 hours.*
10) Carefully remove the 10% alkaline solution and rinse with water 5 times (using a colander or sieve if needed to collect the roots and transfer them back into the container with a forceps or tweezers). Collect the alkaline solution and the rinse water together to be neutralized with the vinegar from step 12.
11) Add vinegar and incubate for 5 minutes.
12) Make the ink stain solution by combining the ink, vinegar, glycerol, and water in a 1:1:1:1 solution.
13) Remove vinegar (using a colander or sieve if needed to collect the roots and transfer them back into the container with a forceps or tweezers) and add it to the alkaline solution and water mix. Add ink stain. Incubate 24 hours at room temperature.*
14) Remove stain (using a colander or sieve if needed to collect the roots and transfer them back into the container with a forceps or tweezers) and destain with distilled water and vinegar in a 3:1 solution. Incubate for 5 minutes. Remove water:vinegar destaining solution (using a colander or sieve if needed to collect the roots and transfer them back into the container with a forceps or tweezers) and add water. Observe under the microscope or store in water in a refrigerator. If you are storing for more than a week, add a 1:1:1 vinegar:glycerol:water solution in a refrigerator or a 2:1 vinegar:water solution in an air-tight container. Monitor for fungal contamination and discard if fungal growth seen. If roots become to destained, repeat steps 11-12.
15) The roots may be observed using either a dissecting microscope at a lower magnification (7.5-20X) or with a compound microscope at 100-200X. The optional (**) methods for these two types of observations are described below:

** Optional microscopic observation of roots:

If using a dissecting microscope to observe the roots at 4-20X magnification, spread the roots out in a single layer with the tweezers and needle in a Petri dish with a grid at the bottom or with gridded paper (0.5 inch or 1 cm spacing in the grid) under the Petri dish. If there are more roots than can fit into the Petri dish, you may repeat this process with another sample of roots but note that you don't need to do more than one Petri dish



per sample. Observe the roots under the microscope and record every time a root crosses a horizontal or vertical line in the grid. If the root contains stained fungal hyphae, spores, arbuscules or other mycorrhizal structures (see figures below), record that separately. At a minimum, you need to have 50 times that roots cross a line. Divide the number of times the root contains mycorrhizal structures by the total number of roots that cross the lines and multiple by 100 which gives the percentage colonization (see calculation below).

Diagram illustrating how roots are spread out in a Petri dish for observation with a dissecting microscope. If a root fragment intersects with a line, it is counted. If that root is stained (i.e. red) indicating mycorrhizal fungi, it is counted as colonized.

If using a compound microscope, spread a sample of roots out on a microscope slide, add a drop or two of water and cover with a coverslip. Observe at 40-200X (objectives are 4X to 20X) using an eyepiece with a grid. Record every time a root crosses a horizontal or vertical line in the grid. If the root contains stained fungal hyphae, spores, arbuscules or other mycorrhizal structures (see figures below), record that separately. If you want, you may record what mycorrhizal structures are observed. At a minimum, you need to have 50 times that roots cross a line. Divide the number of times the root contains mycorrhizal structures by the total number of roots that cross the lines and multiple by 100 which gives the percentage colonization (see calculation below).

Calculations:

1. Calculating root dry weight for the entire root mass collected:

   Dry weight of total roots = wet weight of total roots mass x [1 – ((wet weight of root subsample put in paper bag – dry weight of root subsample in bag) / wet weight of root subsample put in paper bag)]

   Example: wet weight of total root mass = 15 ounces = 425 grams
   wet weight of root subsample = 5 ounces = 142 grams
   dry weight of root subsamples = 0.5 ounce = 14 grams

   Dry weight of total roots = 15 X [1-((5-0.5)/5)] =1.5 ounces

2. Calculating percentage colonization of roots by mycorrhizal fungi:

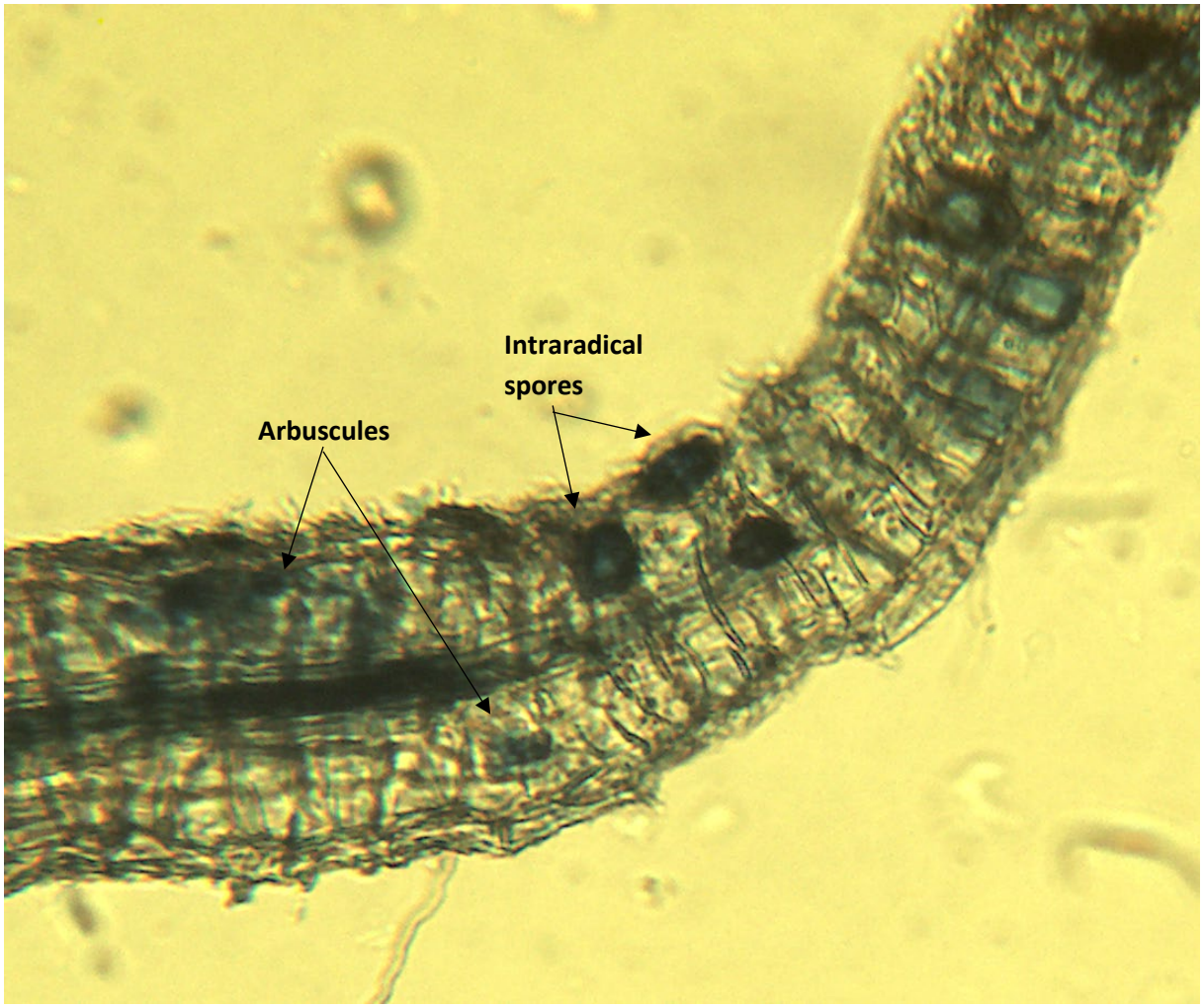
   Percentage colonization = (number of times a root that crosses a vertical line contains mycorrhizal structures/number of times a root crosses a vertical line) x 100

   Example: number of crosses with mycorrhizal structures = 60

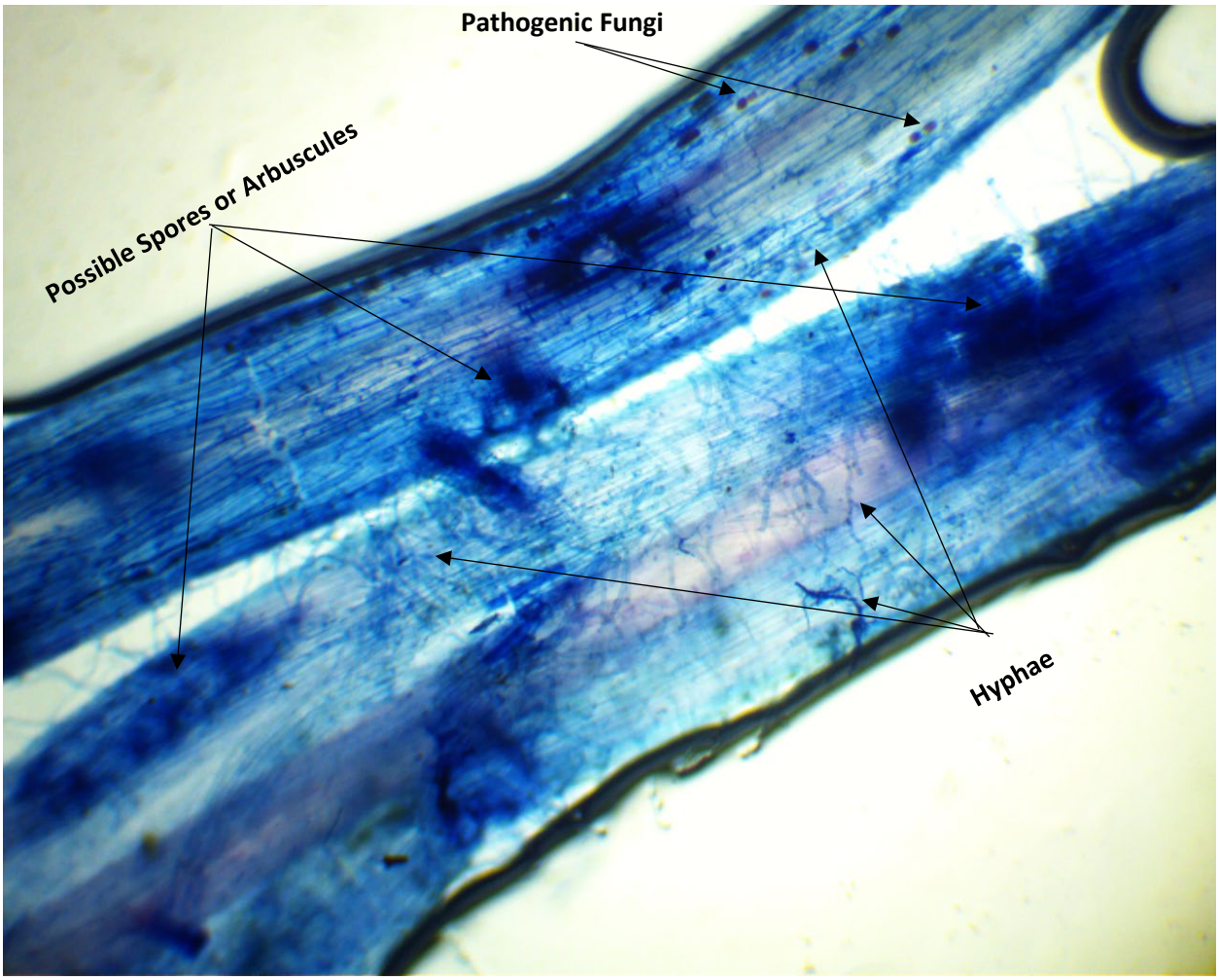
total number of root crosses = 100

Percentage colonization = (60/100) x 100 = 60%

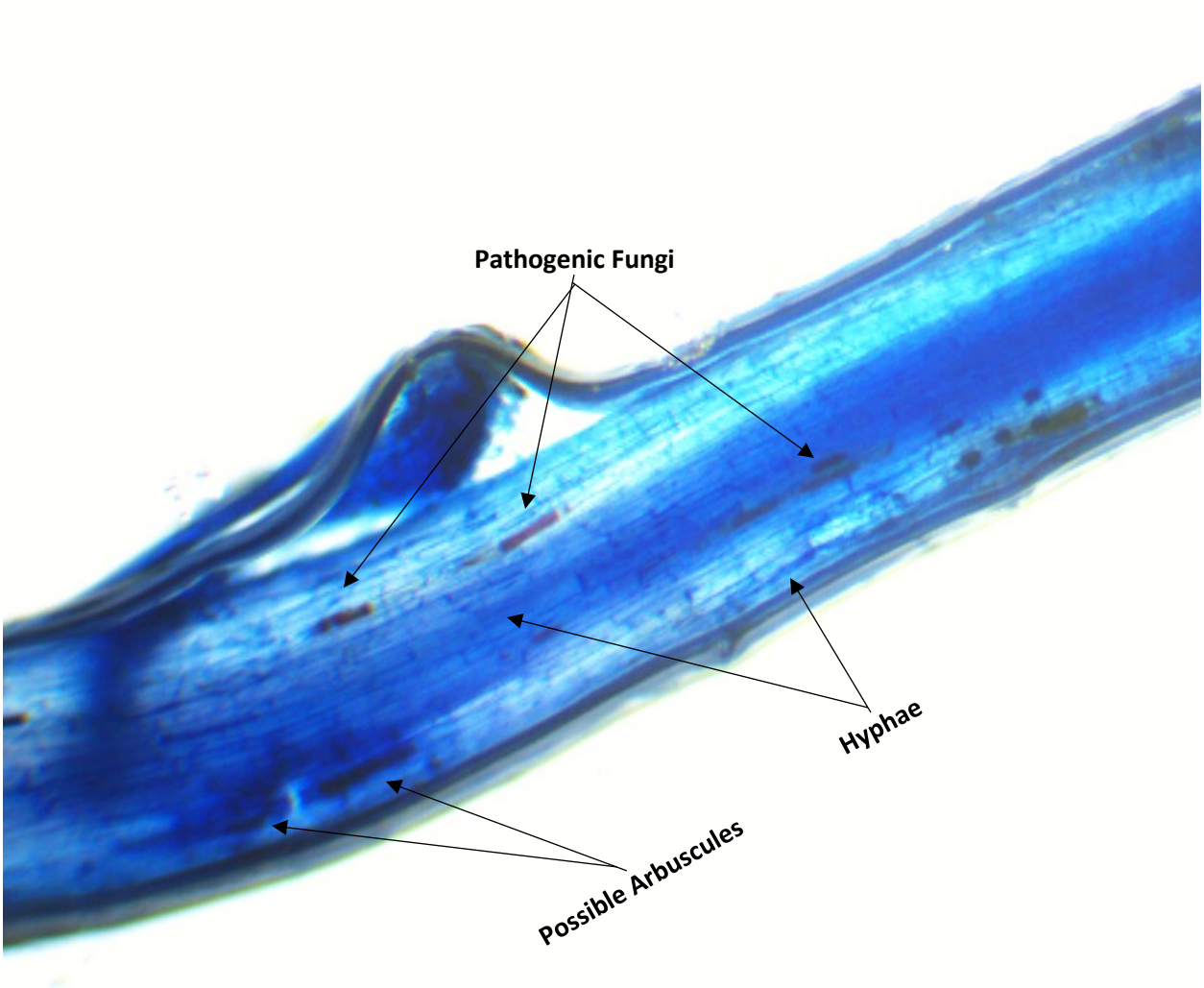
Pictures:



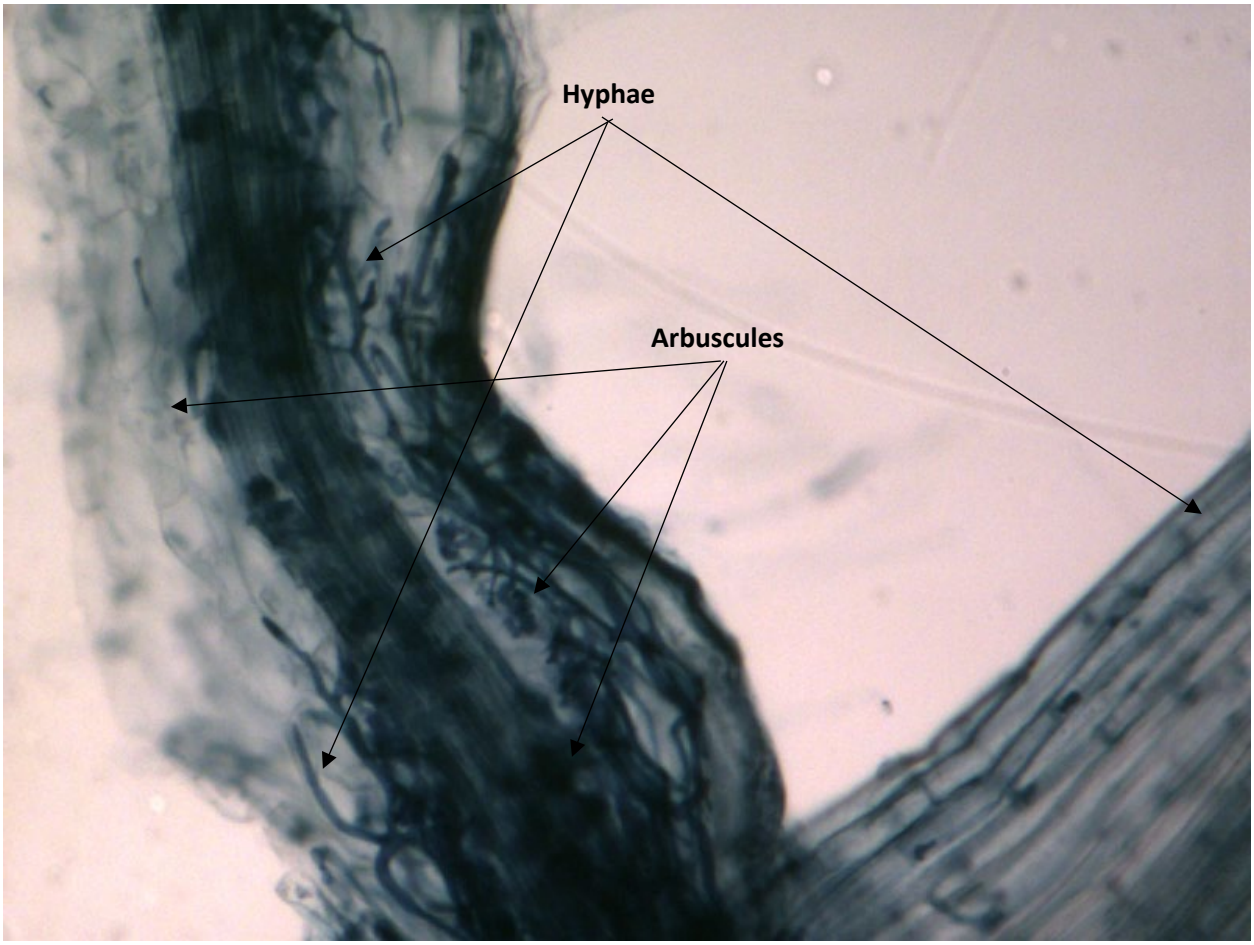
Mycorrhizal spores inside the roots (i.e. intraradical spores) are easily visible while there are indications of arbuscules and hyphae in root cells. Magnification 10X with a dissecting microscope.



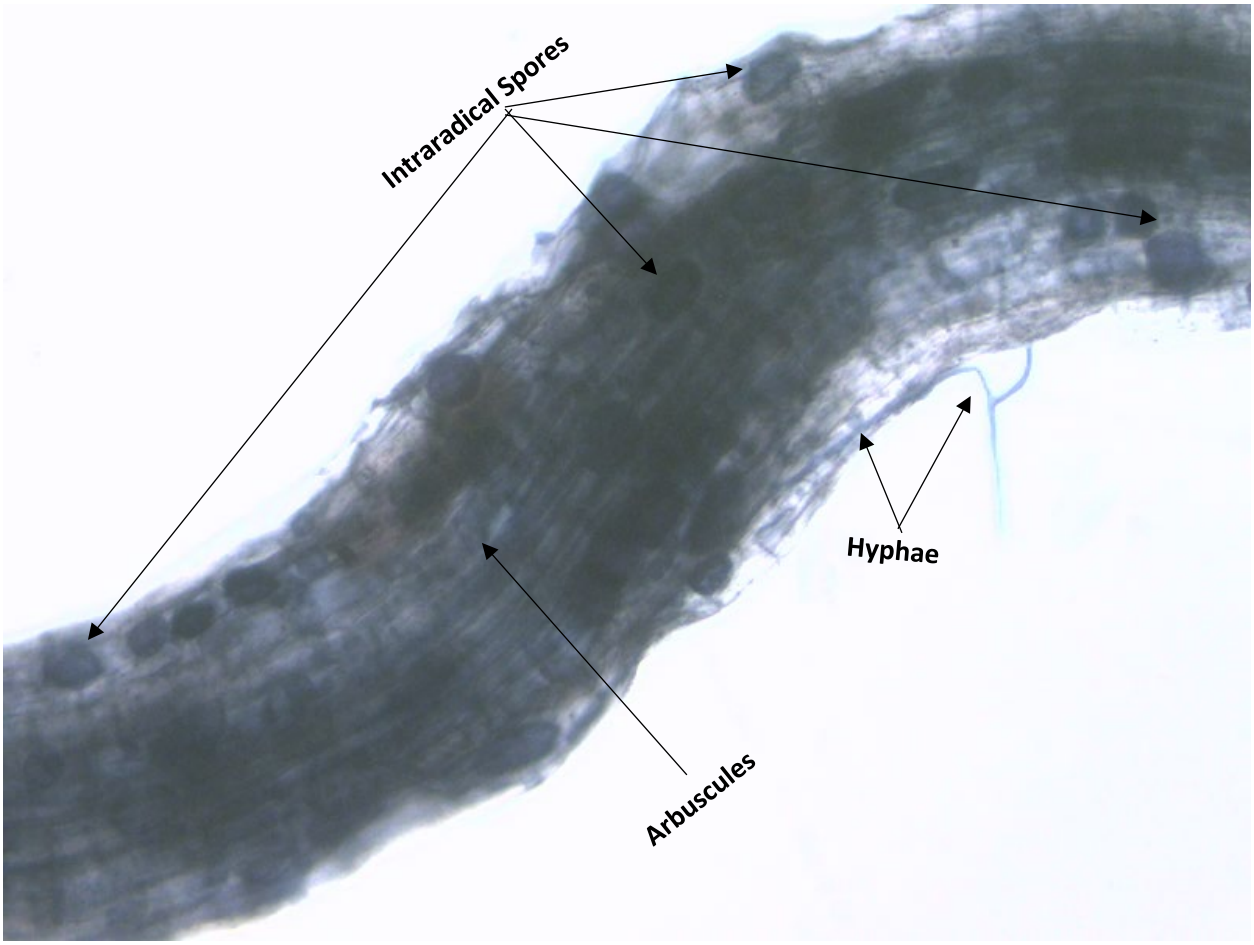
Roots well colonized by mycorrhizal fungi with some hyphal threads visible and indications of arbuscules and spores. Other fungi, probably pathogenic fungi, are also present in the top root. Magnification 40X with a 4X objective.



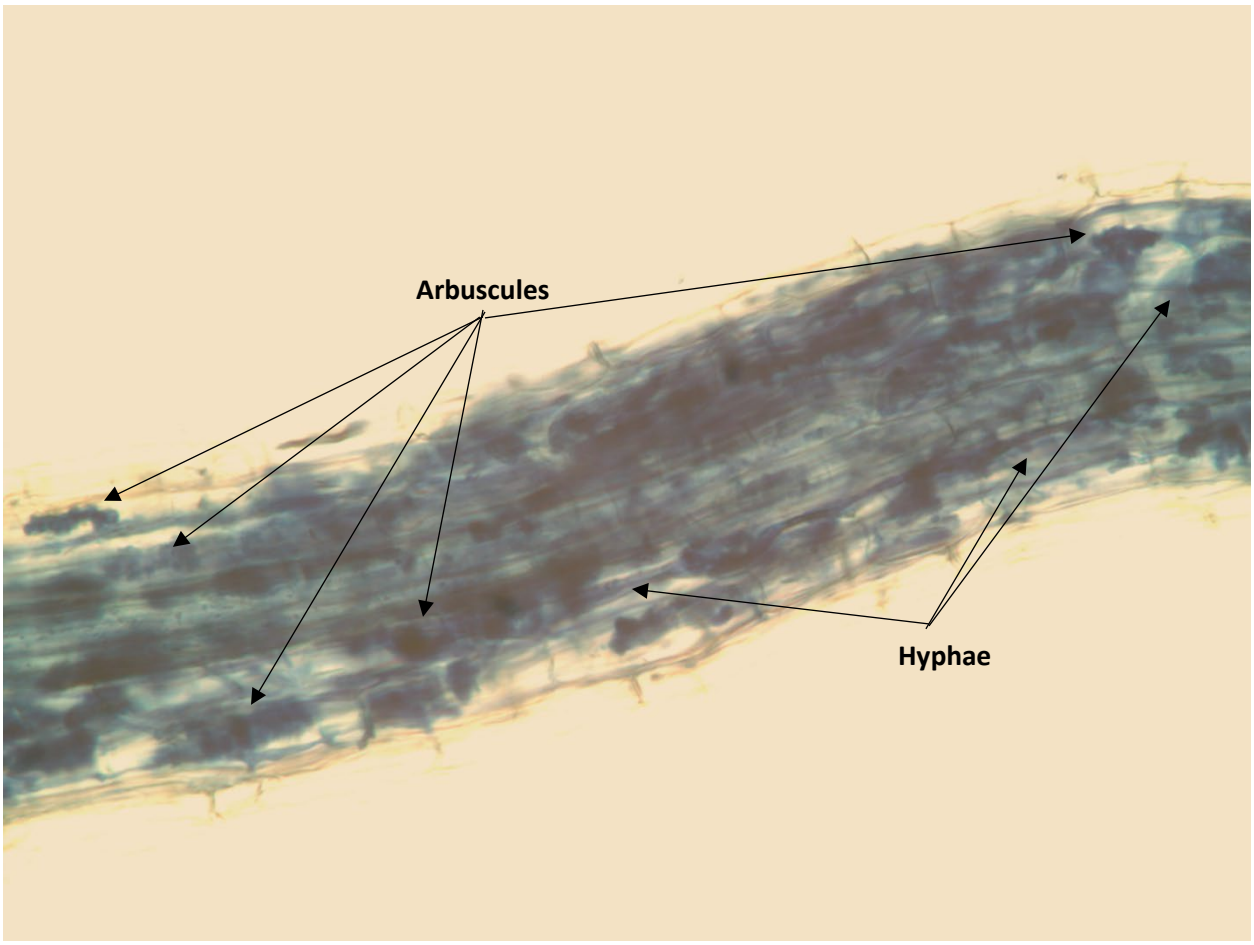
Root about half colonized by mycorrhizal fungi with some hyphal threads visible and indications of arbuscules and spores. Other fungi, likely pathogens are also present in these roots as brown rather than blue structures in root cells. Magnification 40X with a 4X objective.



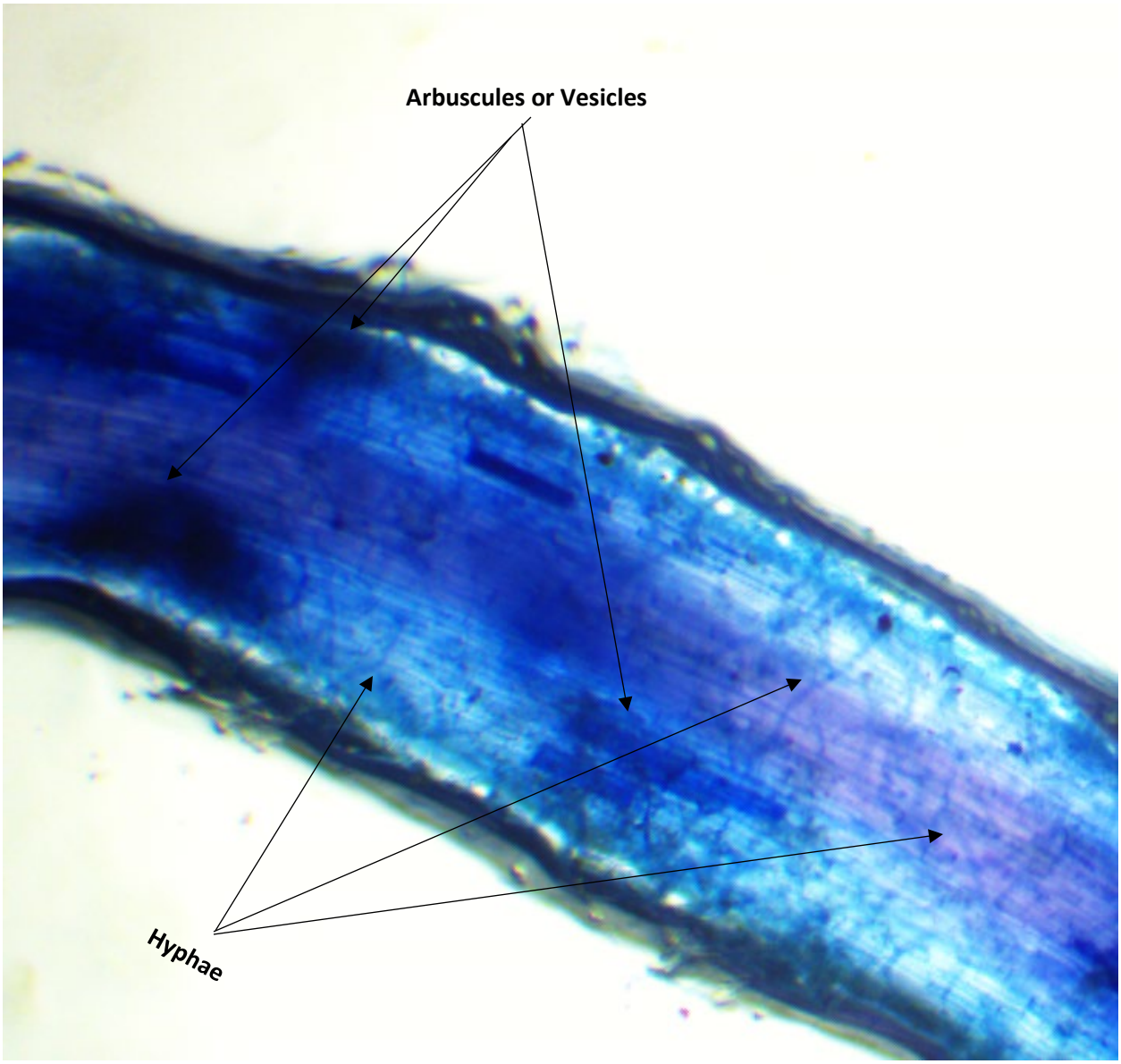
Hyphal threads and shrub-like arbuscules are easily visible in these root cells. Magnification 63X with the 6.3X objective using a compound microscope.



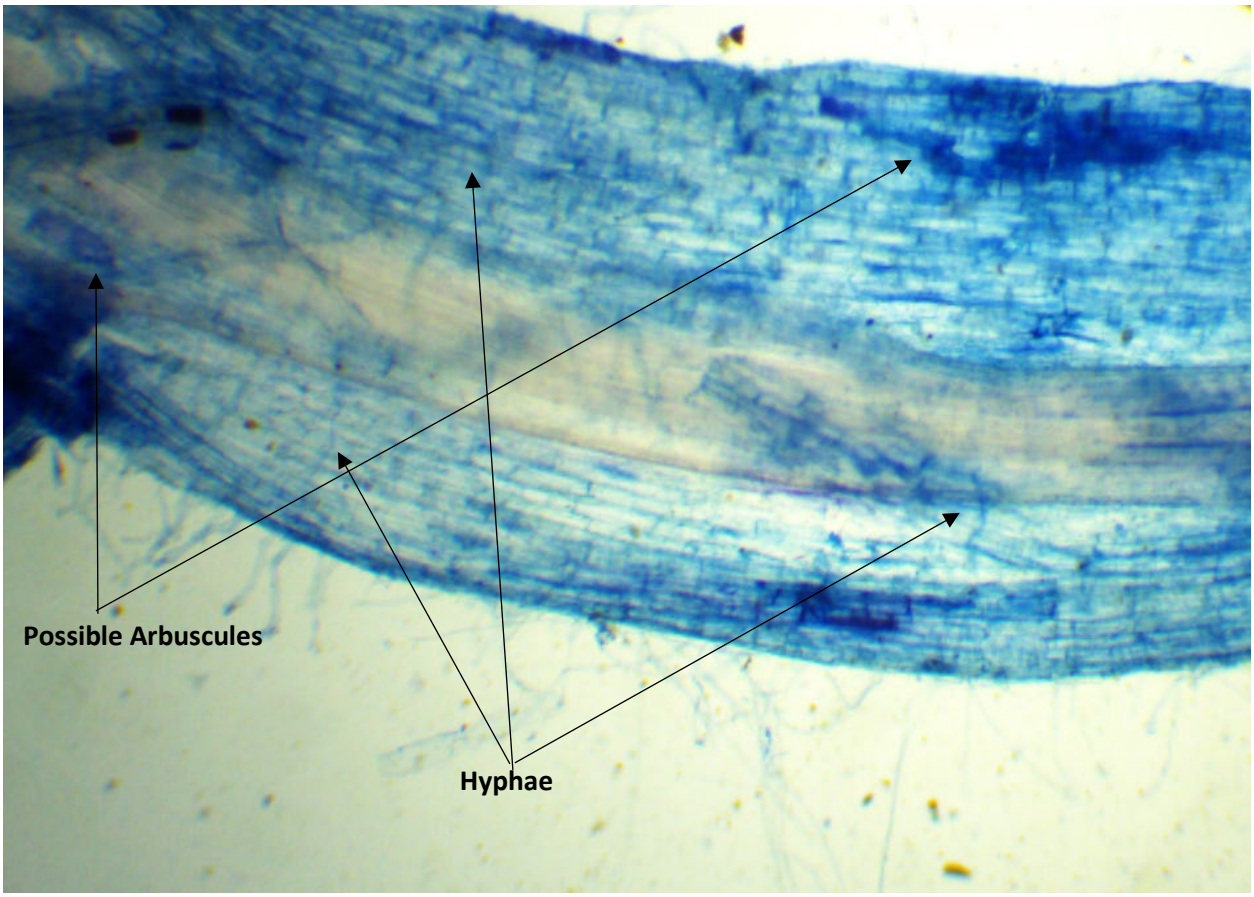
Heavily colonized roots with visible intraradical spores (i.e. spores inside roots) and indications of arbuscules and hyphae in roots. Magnification 100X with the 10X objective using a compound microscope.



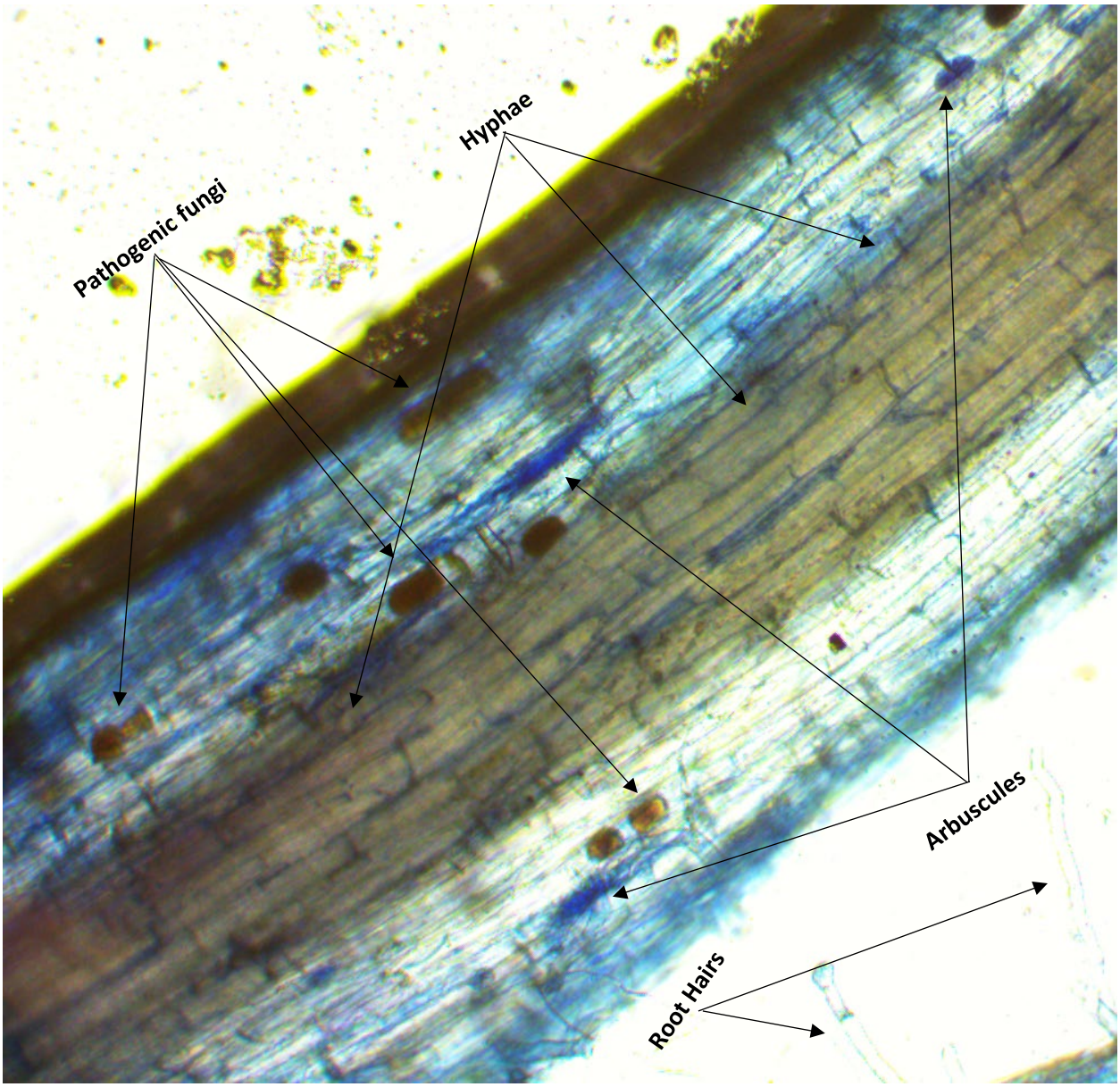
Heavily colonized root with hyphae and highly branched arbuscules filling the root cells. Magnification 100X with the 10X objective using a compound microscope.



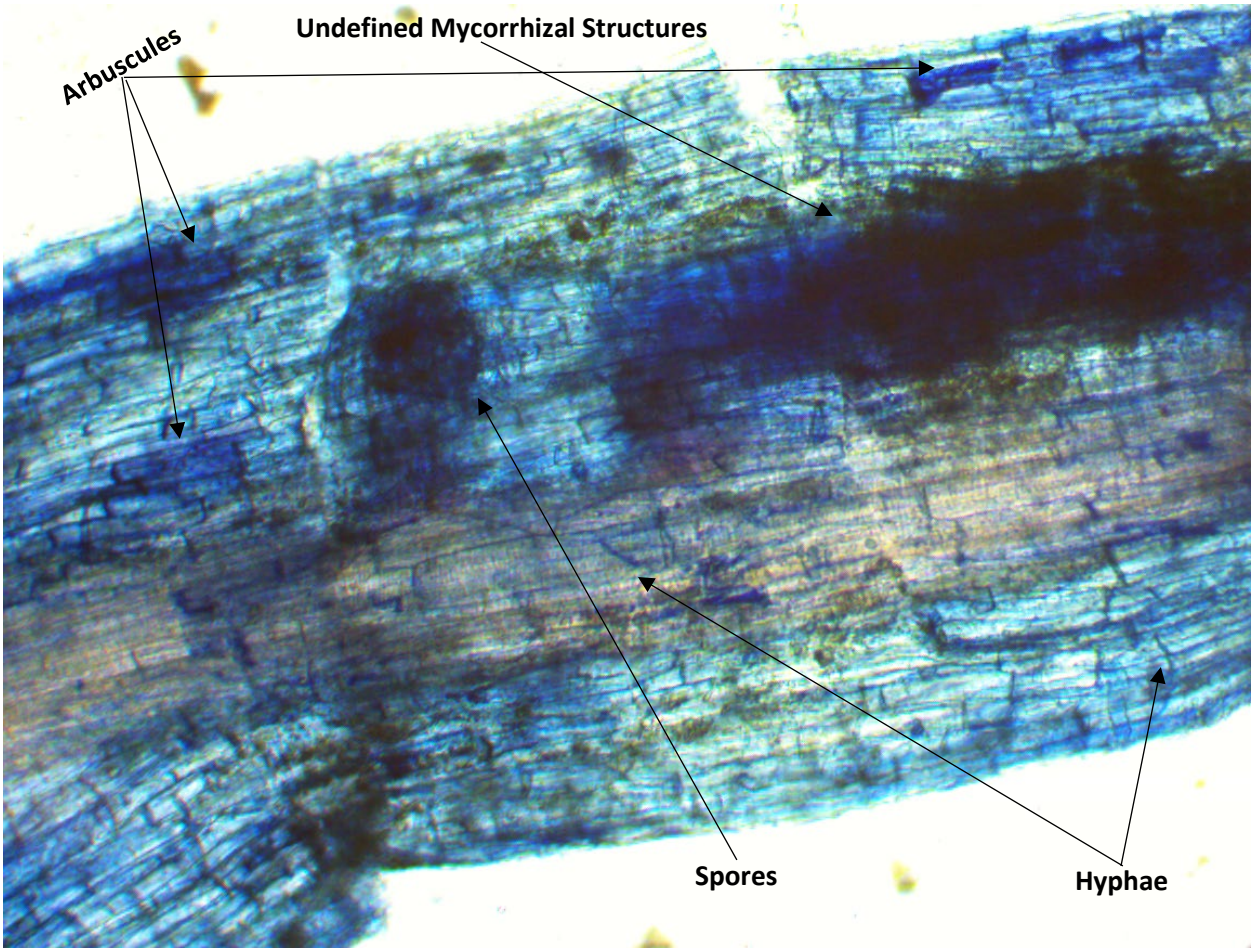
Root well colonized by mycorrhizal fungi with some hyphal threads visible and indications of arbuscules and spores. Magnification 100X with a 10X objective.



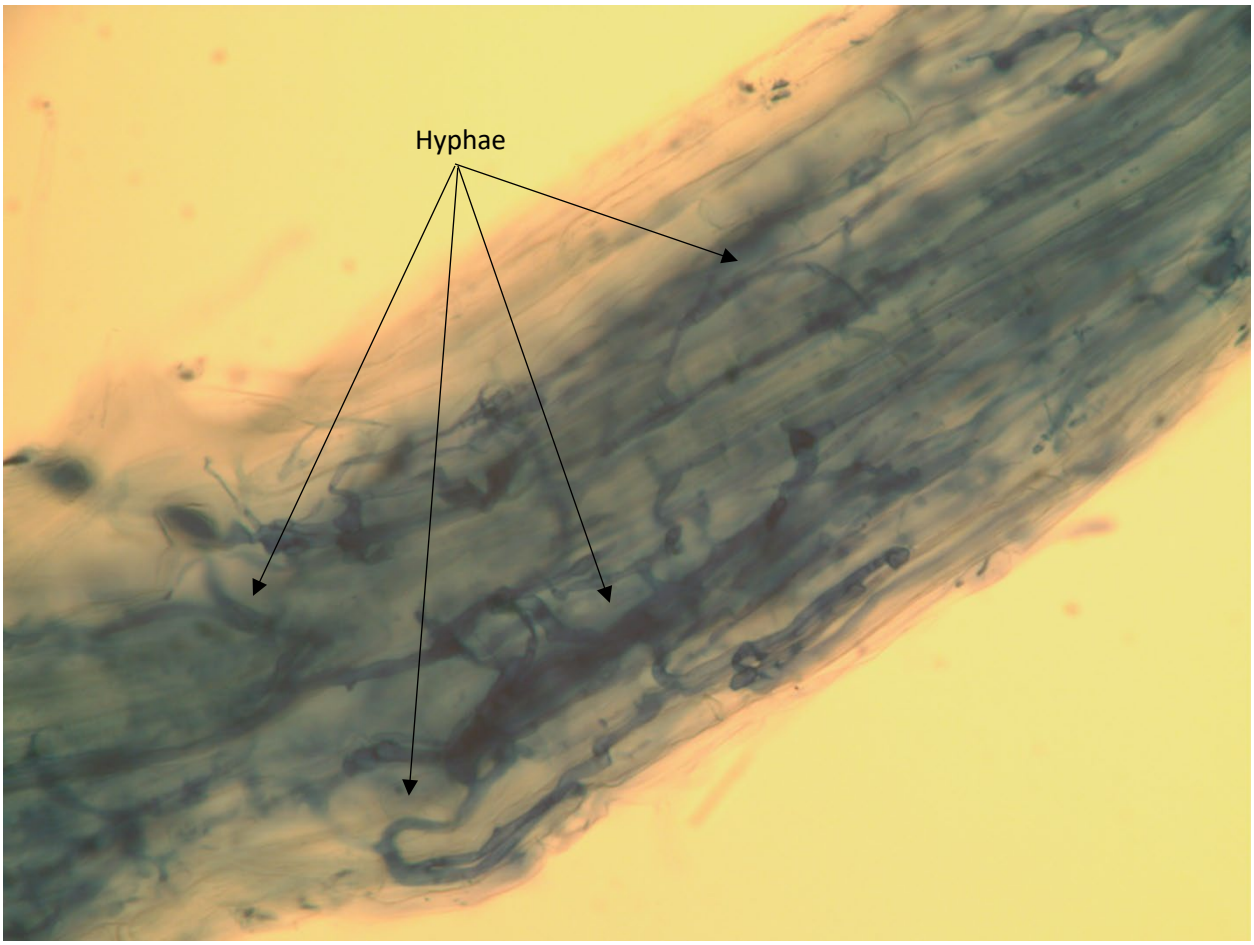
Root highly colonized by mycorrhizal fungi with some hyphal threads visible and indications of arbuscules and spores. Magnification 100X with a 10X objective.



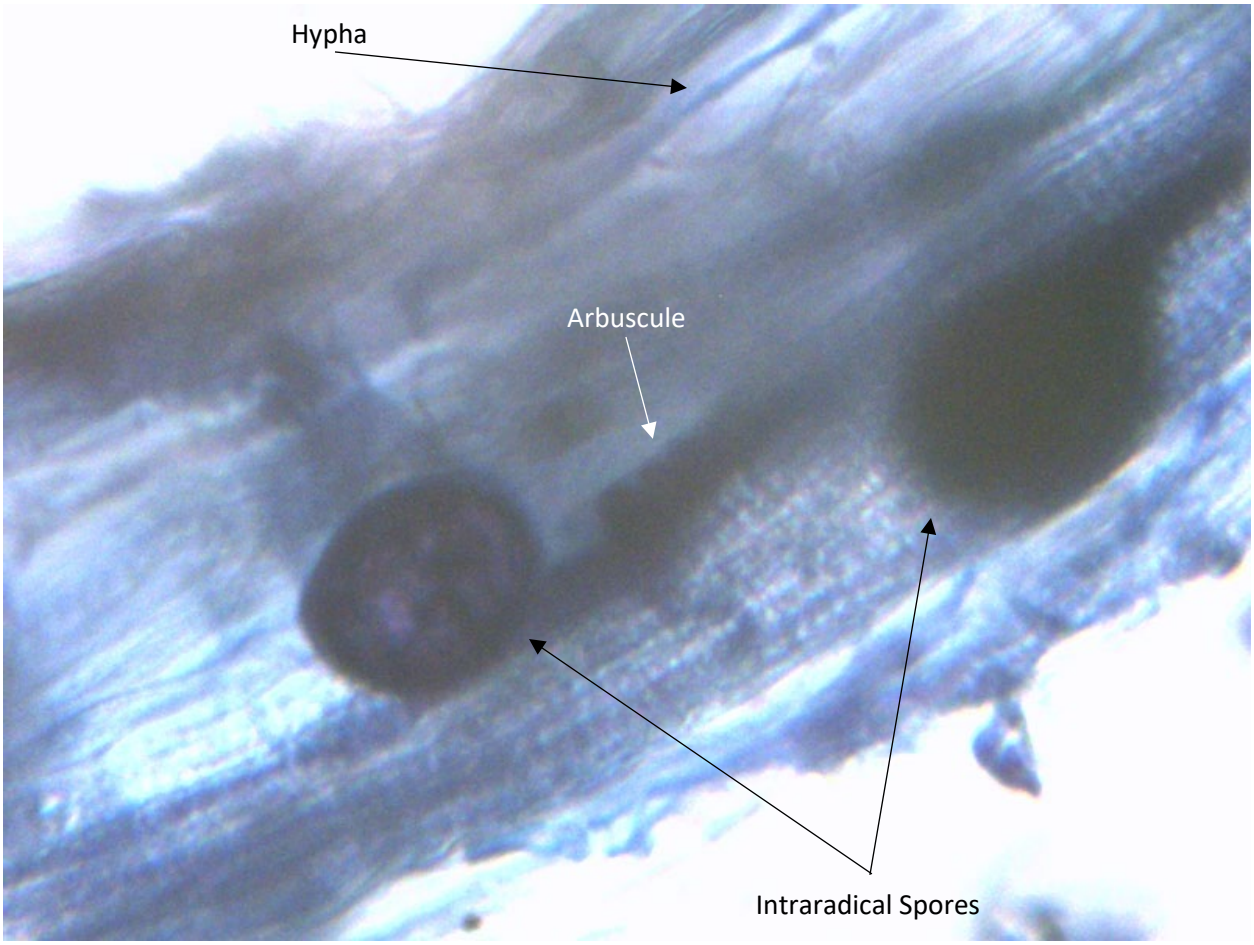
Root colonized by mycorrhizal fungi with some hyphal threads visible and indications of arbuscules. Other fungi, probably pathogenic fungi, are present as brown structures. Magnification 100X with a 10X objective.



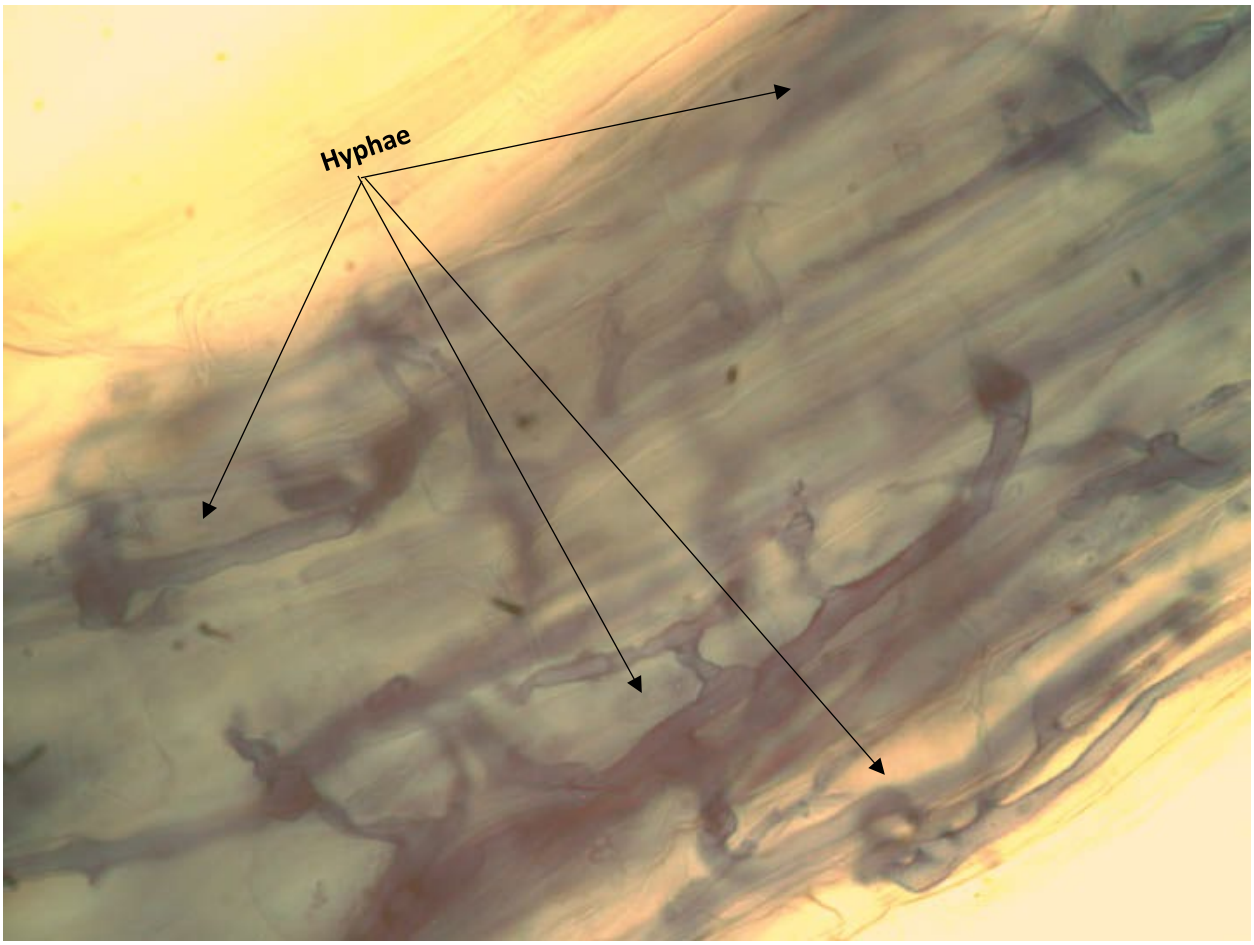
Root highly colonized by mycorrhizal fungi with some hyphal threads visible and indications of arbuscules and spores. Magnification 100X with a 10X objective.



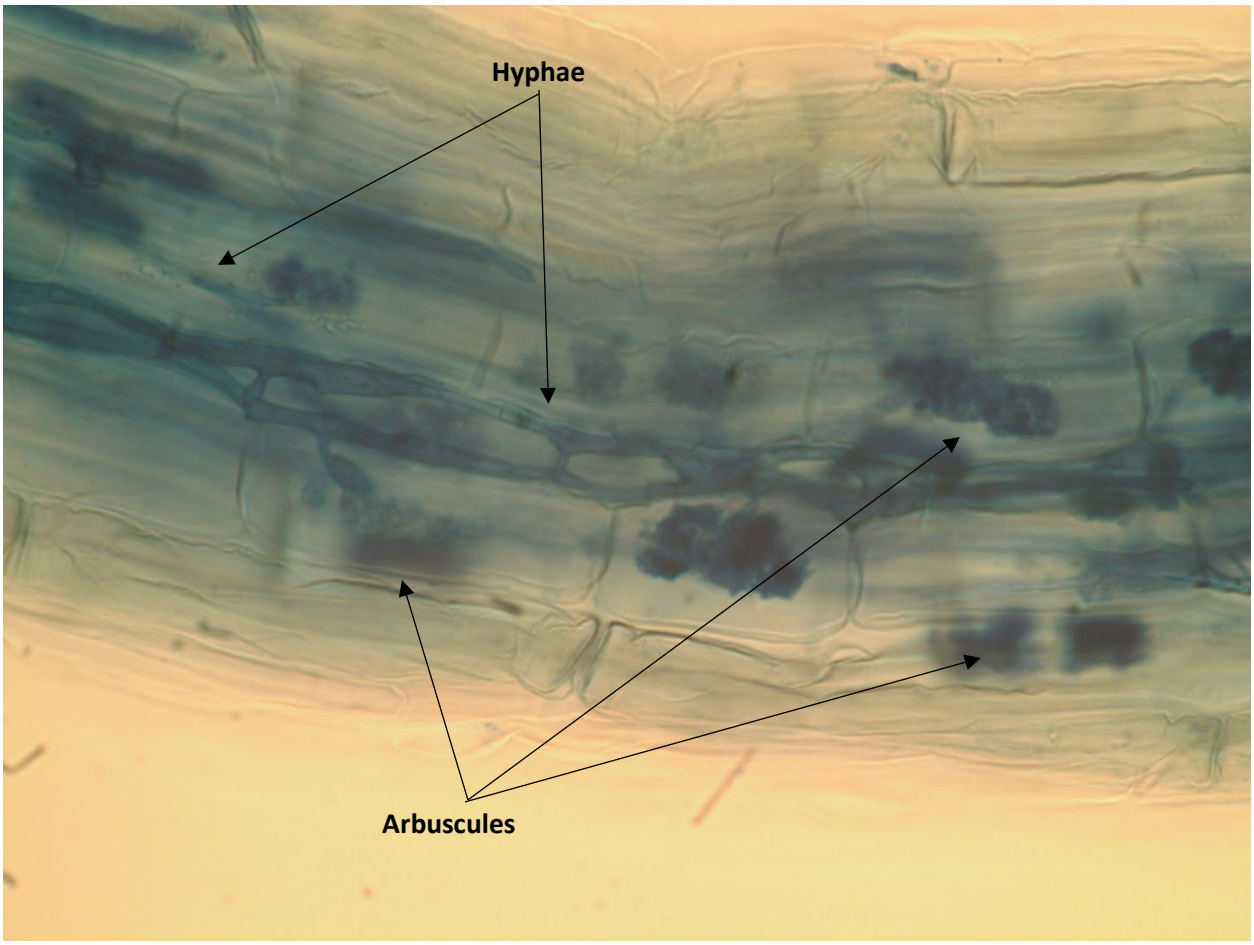
Hyphal threads are easily visible in these root cells. Magnification 125X with the 12.5X objective using a compound microscope.



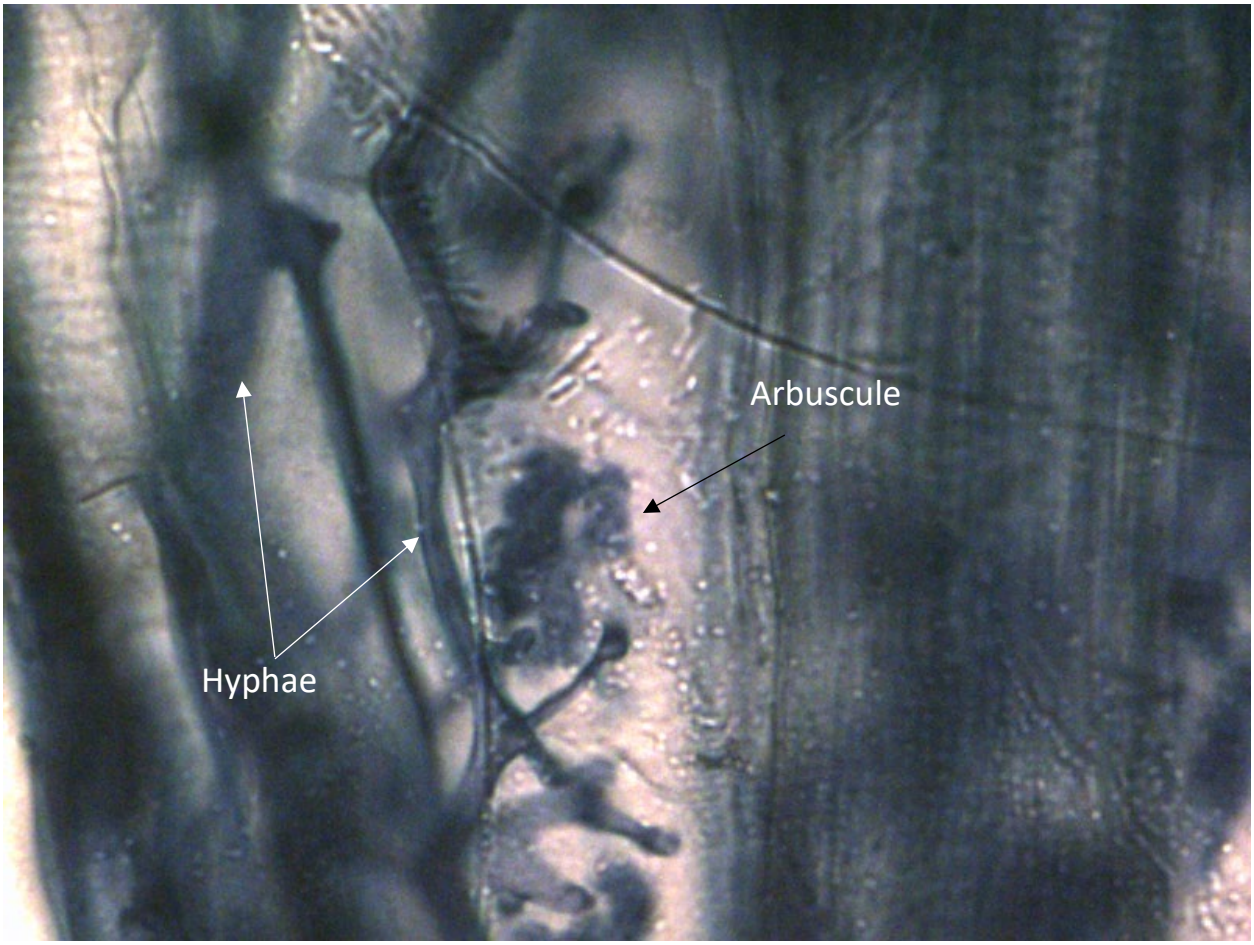
Two mycorrhizal spores present in a root (intraradical spores) with indications of arbuscules and hyphal threads. Magnification 125X with a 12.5X objective.



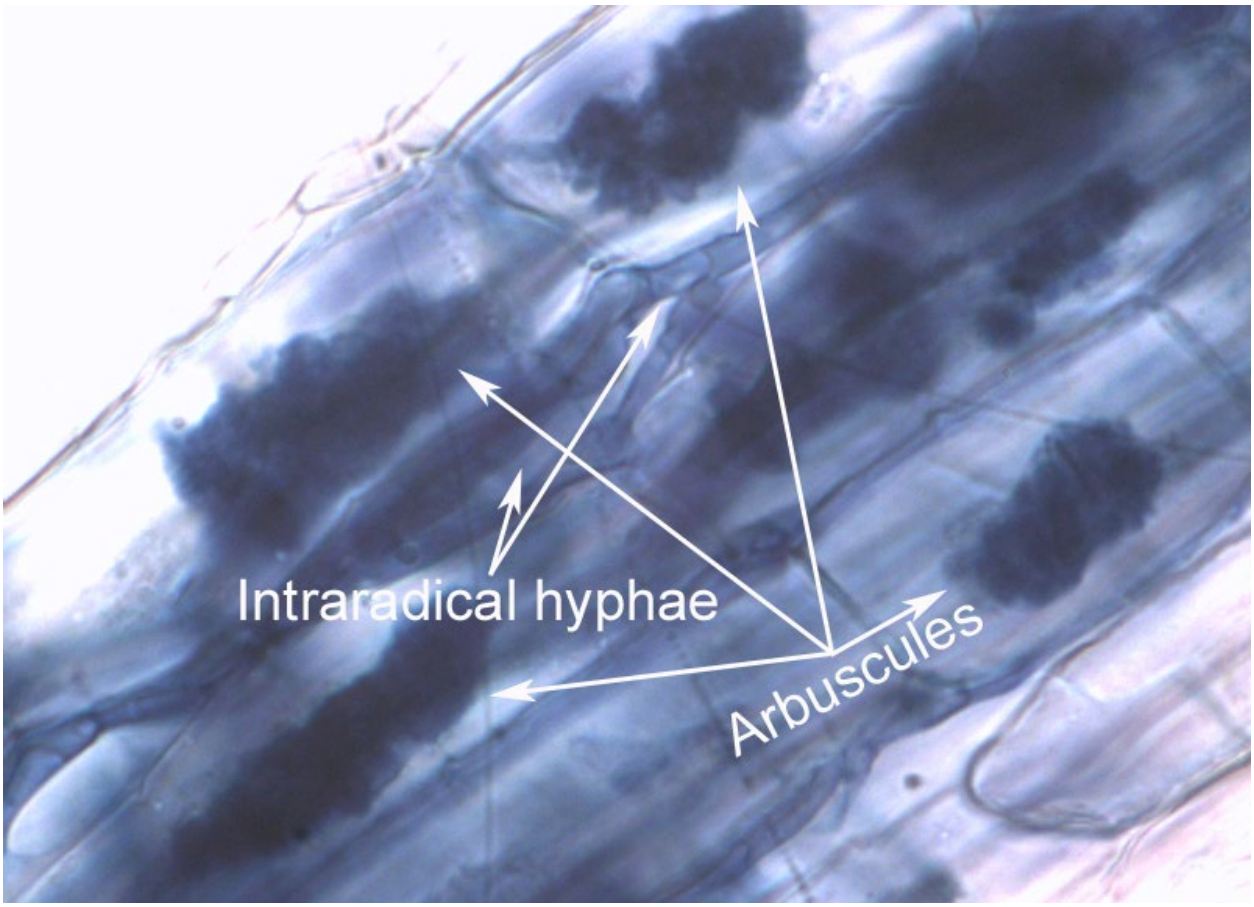
Hyphal threads are easily visible in these root cells. Magnification 250X with the 25X objective using a compound microscope.



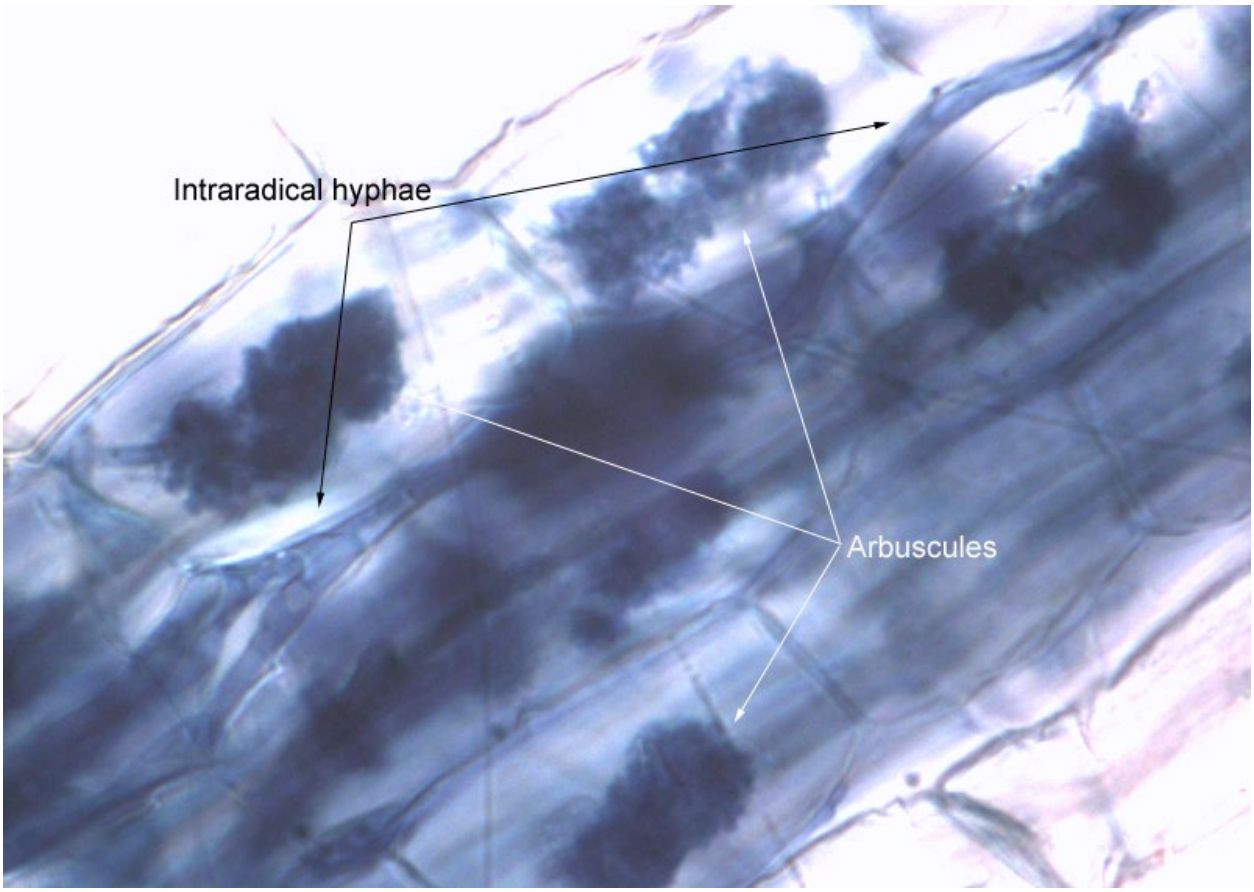
Hyphal threads and shrub-like arbuscules are easily visible in these root cells. Magnification 250X with the 25X objective using a compound microscope.



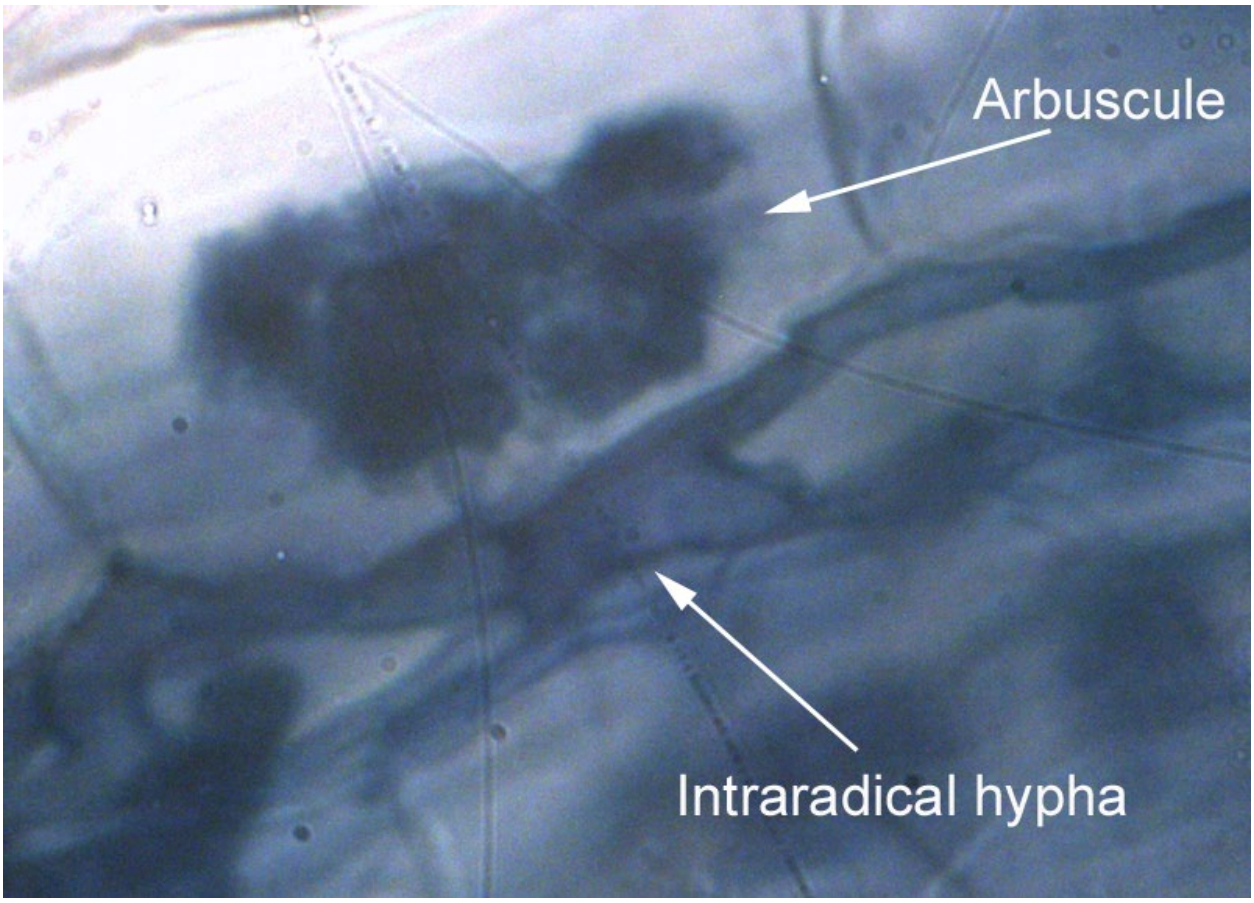
Hyphal threads and shrub-like arbuscules are easily visible in these root cells. Magnification 250X with the 25X objective using a compound microscope.



Root well colonized by mycorrhizal fungi with visible hyphal threads and arbuscules. Magnification 250X with a 25X objective.



Root well colonized by mycorrhizal fungi with visible hyphal threads and arbuscules. Magnification 250X with a 25X objective.



Root well colonized by mycorrhizal fungi with visible hyphal threads and arbuscules. Magnification 500X with a 50X objective.

REFERENCES

Giovannetti, M., and Mosse, B. (1980). An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New Phytol.* **84**, 489-500.

Vierheilig, H., Coughlan, A. P., Wyss, U., and Piche, Y. (1998). Ink and vinegar, a simple staining technique for arbuscular-mycorrhizal fungi. *Applied and Environmental Microbiology* **64**, 5004-5007.

https://invam.wvu.edu/methods/mycorrhizae/staining-roots

https://mycorrhizas.info/method.html#examine