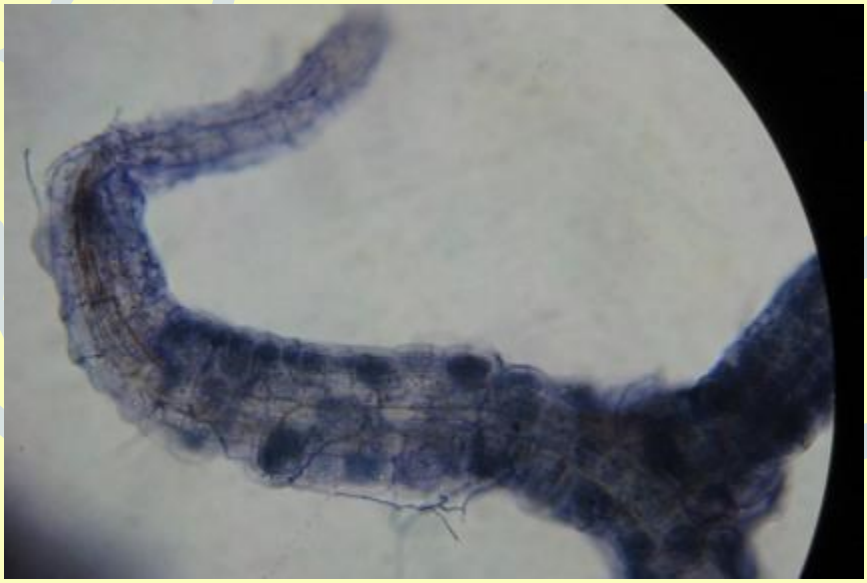

Mycorrhizae Observation Made Simple

A "Farm Lab" Method for Low-cost, Non-toxic Mycorrhizae Observation and Quantification

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The farm-based research enabling the production of this booklet was funded by a SARE Farmer Grant, “Cost/Benefit Analysis of Inoculating Blueberry Bushes with Ericoid Mycorrhizae.” Five hundred blueberry bushes were inoculated with a commercially available Ericoid product, and 500 bushes were left untreated. Watermans Berry Farm worked with University of Vermont (UVM) Extension, UVM Plant and Soil Science and UVM Biology to trial simple, low-cost mycorrhizae observation and colonization quantification methods, the most refined and replicable of which is detailed in the following pages. Full results of the 3-year study can be found in the database at www.sare.org .

Many thanks to Mark Starrett of UVM Plant and Soil Science for advising on all aspects of this project.

Many thanks to Alison Brody and Jon Gonzales of UVM Biology for advising and guidance with Ericoid mycorrhizae clearing, staining and observation methods in a university laboratory setting.

The root ball at the left has not been inoculated by the Ericoid product. The root ball on the right has been inoculated by the Ericoid product, which is simply rubbed onto the outside of the wet root ball so it sticks and covers all visible roots.

The purpose of this booklet is to provide farmers with a method for observing and quantifying mycorrhizae on roots of their crops.

Why is this important?

There is an enormous base of research and evidence to prove that mycorrhizae fungi in soil form symbiotic associations with crop plant roots, resulting in improved nutrient uptake, nutrient use efficiency and a host of other benefits to the plant. Yet those of us who derive our livelihoods from this microbiological, plant root interaction typically only experience it in theory. Farmers are not able to confirm the existence of mycorrhizae in fields or on crop roots without sending samples to sophisticated labs or paying for testing services at high costs.

This booklet walks you through a low-cost method to observe mycorrhizae on plant roots first hand. It is for farmers with basic scientific background and access to a microscope. The procedures can be done on the farm in what we call a "farm lab," a simple, small table space with a 120 VAC outlet in a shed, shop, barn or other building. After using the method, you might be able to confirm whether or not crop roots have mycorrhizae symbiosis, to what degree or what % of cells have been colonized by the beneficial fungi. This is valuable information for soil fertility planning, decisions about fertilizer applications (theoretically the more mycorrhizae symbiosis the less fertilizer needed), or whether to purchase one of the myriad of mycorrhizae products on the market and confirm its effectiveness.

The following method was adapted for blueberry roots, however it can be used for other plants. It might take some refining and tinkering, but that is what "farm lab" mycorrhizae observation is all about. By starting with our method and trying some of the tricks we used, you'll be well on your way to finding the method that works best for you.

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Mycorrhizae Observation Made SImple: A "Farm Lab" Method for Low-cost, Non-toxic Mycorrhizae Observation and Quantification

WHAT YOU WILL NEED

From left to right: Potassium Hydroxide pellets sourced from lab supply depots; Hydrogen peroxide sourced at your local drug store or supermarket; Lactic Acid (optional) sourced from lab supply depots; Pen ink sourced from pen ink suppliers, Glycerol (optional) sourced from lab supply depots; and vinegar sourced at your local supermarket

Safety First: A Note About the Process

Many mycorrhizae observation methods developed over the years involve the use of cancer causing staining reagents, such as Chlorazol Black or Trypan Blue. Our initial goal was to compare these methods with the safer method of using ink and vinegar. Our success with ink and vinegar was so striking that it is not even worth detailing the use of the more toxic staining reagents. Why risk exposure to these chemicals when you can get satisfactory results with a less toxic method!

With that said, pen ink is by no means safe for human consumption, and we learned the hard way how quickly and easily it can stain your hands and skin. Always wear gloves and goggles when handling concentrated ink.

Safety First: A Note About Potassium Hydroxide

We don't know of any way to avoid using Potassium hydroxide (KOH), a strong and very caustic base. It can easily eat a hole through your skin or make you blind. Its vapors burn and extreme care must be exercised at all times when working with potassium hydroxide. Most labs require the handling of KOH under a ventilation hood, but in a farm lab, the best you can do is ensure good ventilation and protect yourself by wearing goggles and gloves and long sleeved clothes. Make sure to source KOH pellets and not KOH solution, which is more likely to spill or be mishandled. KOH pellets are strongly hydroscopic and will absorb humidity from the air and clump, making it more difficult to handle.

KOH can be handled safely. Always wear gloves and safety goggles. Always close containers of KOH right away after using. Always label KOH solutions. KOH comes in child proof containers, so make sure to keep KOH in these containers and store them far out of reach of children.

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Mycorrhizae Observation Basics

Common methods for mycorrhizae observation involve a two-step process:

1. Clearing natural pigments out of the roots using a strong base solution (Potassium Hydroxide)
2. Staining the roots using a stain (ink and vinegar solution). This stains the root cell walls and mycorrhizae fungal arbuscules and hy-

CROCK POT

The "high" setting on this crock pot worked the best for staining and clearing.

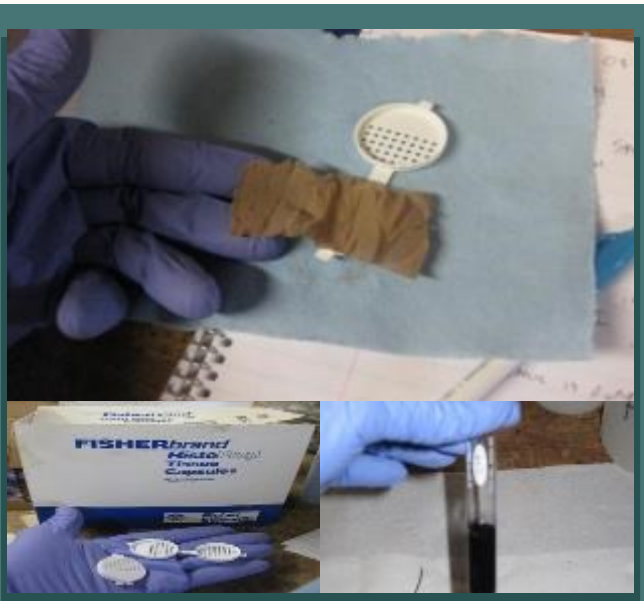
Review of Materials and Reagents Needed to Start

Materials (Not including microscope which many people might let you borrow):

1. Crock Pot. ($25) This serves as a hot water bath into which you place test tubes or other beakers for both the clearing and the staining process.
2. Test tubes. ($0.50 each) These come in various sizes but anything will work.
3. Graduated cylinder. ($10) For measuring precise volumes of liquids when making your KOH and ink/vinegar solutions.
4. Polyethelene bottles. ($5 each). These are very useful for safely storing solutions for later use.

5. Tweezers , needlenose pliers or a dissecting needle (pictured here). ($5) These come in extra handy for handling roots throughout the process.

TOTAL STARTUP COST ESTIMATE FOR MATERALS: $50 (This estimate is for a bare bones approach. If you want to make the process easier, more glassware and test tube racks are useful. On the other hand, you can get creative and work with what you have.



CASSETTES VS TEST TUBES

To handle roots efficiently, you can use a number of strategies that allow the roots to stay in one container as different solutions are added and rinsed through the entire process. One method uses plastic cassettes sourced from medical supply, along with panty hose cut into small pieces as an additional sieve to hold fine roots in the cassette. We preferred the test tube method where we could see the roots at all times. We also felt that roots were less crowded in test tubes, making soaking in each solution more uniform and effective.

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Farm Lab

Welding helmet + lab supplies?

Whoever's lab this is, they probably could have been more tidy. ;)

Materials and Reagents (continued)

Reagents:

1. Potassium Hydroxide, KOH (500 grams of pellets for $30). This might be your most difficult chemical to source, as it tends not to be widely available and found only at lab supply depots. It needs to be handled with caution (see p 4). You might need to open an account at a lab supply company to order this. KOH is used for clearing natural pigment off roots before staining the mycorrhizae.
2. Distilled water (1 gal for $2). Used for washing glassware and for making the KOH solution for clearing.
3. Pen ink (50 ml bottle for $12). This can be any bulk pen ink, but we used Sheaffer brand because other research found it to be one of the most effective and we confirmed its success. Ink is mixed with vinegar to create a staining solution.
4. Vinegar (1 L bottle for $2). White vinegar is used as a solvent for the pen ink to make a staining solution.
5. Hydrogen peroxide, H2O2 (small bottle for $2). Used in our method as a supplementary clearing solution used after the primary KOH clearing step.
6. Lactic Acid and Glycerol (optional). Lactic acid and glycerol and distilled water are mixed at a ration of 1:1:1 to make "lactoglycerol, " which is a great, clear semi-viscous solution you can use for preparing slides. Distilled water can be used for slide prep instead, but they won't last as long before spoiling. Lactic acid and glycerol can be sourced from lab supply depots. They are generally safe chemicals and don't require special handling.

TOTAL STARTUP COST ESTIMATE FOR REAGENTS: $50 (for a bare bones approach)

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Step 1: Root Washing

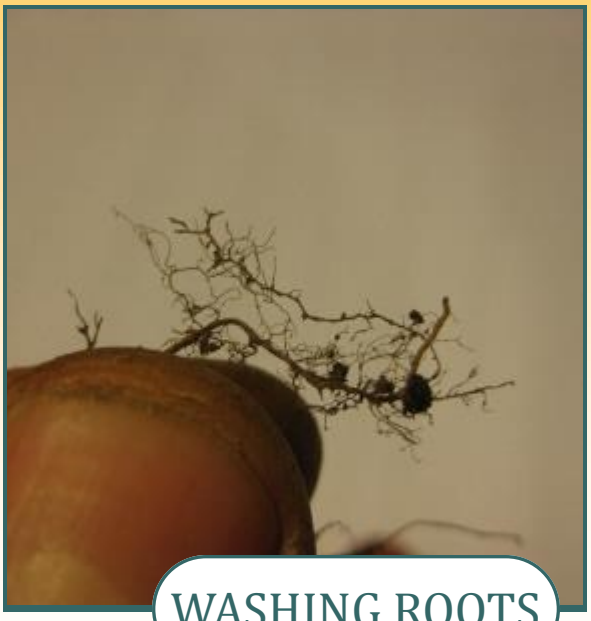
Mycorrhizae are typically found colonizing the finer feeder roots (known as hair roots on blueberry) as opposed to the thicker roots used for nutrient transport.

Roots should be kept in loose field soil in the fridge in unsealed plastic bags before processing.

It is not practical to isolate all feeder roots in a sample, but do your best to ensure every sample contains a good amount of feeder roots that you can observe when it comes time to preparing your slides.

It is easy to wash roots once you have snipped a few and put them into a cup or beaker (pictured below). Rinse them with tap water several times over to get rid of all soil particles. A final rinse with distilled water ensures uniformity in your samples.

It is debatable how long roots can be left in water in a refrigerator before initiating the clearing and staining method. The safest thing to do is time the processing of your roots right before clearing and staining.



A piece of blueberry root showing thicker transport roots and finer hair roots, which are much more likely to contain mycorrhizae

Step by Step Summary

A) Store root samples in refrigerator in loose soil in unsealed bags prior to washing.

B) For what will eventually end up as one slide for viewing under the microscope, pinch 2-3 root networks, each about the size of a dime. Make sure to include feeder roots; these will have mycorrhizae if it is present in that root's rootzone.

C) Put these dime-size pinches in a beaker or cup for rinsing repeatedly with tap water until all soil particles are rinsed.

D) Rinse one last time in distilled water.

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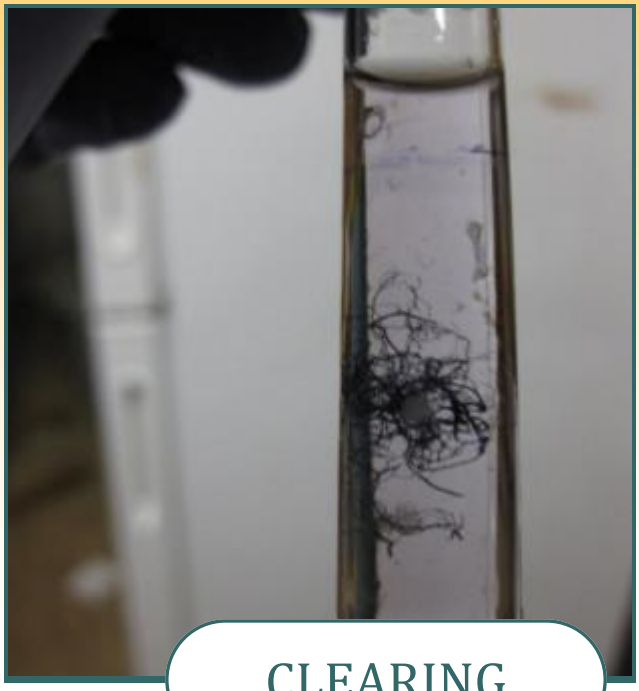

CLEARING

Enough KOH solution is added to immerse the root sample

Step by Step Summary

A) Prepare a 10% weight:volume KOH solution by dissolving 10 grams of KOH pellets in 100 mL of distilled water. (NOTE: SEE SAFETY CONSIDERATIONS ON P 4)

B) Fill test tube with root sample about half full with KOH solution.

C) Cap test tube with a little piece of aluminum foil to prevent KOH vapors from escaping during the hot water bath soak.

D) Put test tube in hot water bath in the crock pot.

E) Soak for 24 hours in KOH solution in hot water bath set on HIGH temperature. (Note, we tested various scenarios including 12, 18, 24 hours, 1,2,3,4 and 5 days on LOW and at room temperature. The optimum scenario: 24 hours on HIGH.

Step 2. Clearing with KOH

In order for you to be able to stain mycorrhizae fungi for clear viewing, you first need to get rid of any natural pigment that might occlude the fungi.

Several factors influence how quickly and thoroughly a KOH solution will clear a root of its natural pigment. The strength of the KOH solution, the time of the root soak, and the temperature at which it soaks can all be adjusted to optimize clearing. Darker colored roots will need more time and/or higher temperatures to clear.

Clearing is usually done in an autoclave under intense temperature and pressure. In a farm lab, however, a crock pot hot water bath works fine, although you need to plan ahead to make sure you have enough time.

We tested various time and temperature scenarios. We also compared a 10% KOH solution to a 15% KOH solution.

Too much time soaking in KOH, too high a temperature or too strong a KOH solution will lead to over-clearing. Evidence of over-clearing is root cells bursted apart like the root has exploded. All mycorrhizae structures will have been disintegrated at this point as well.

Too little time in KOH, too weak a solution or too cold a temperature will lead to under-clearing. Evidence of under-clearing is roots still containing their natural pigment and mycorrhizal structures too difficult to distinguish (after staining) because the stain has mixed with the natural pigment still left in the root.

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Step 3. Water Rinse and Secondary Clearing with Hydrogen Peroxide

After 24 hours in KOH in the water bath the roots should be 90% cleared of their pigment. You can use hydrogen peroxide to clear any stubborn roots with remaining pigment, and to ensure the roots are thoroughly cleared prior to mycorrhizae staining.

Be careful with this step. Hydrogen peroxide is not nearly as caustic as KOH, but if you soak the roots too long in hydrogen peroxide at this stage, because they have already been softened by the KOH they will disintegrate easily.

Ten minutes soak in hydrogen peroxide is adequate, and 15 minutes is the maximum.

You know the KOH has done its job if it has dissolved the root pigment, which then can be rinsed to complete this step.

A batch of cleared samples coming out of the 24 hour water bath. We had the samples in KOH in test tubes, the test tubes in water in the foil covered beaker, and the beaker in a hot water bath in the crock pot.

Step by Step Summary

A) Be careful with samples in KOH fresh out of the hot water bath, for vapors can be caustic. Let them cool a few minutes.

B) Rinse out the KOH at least three times with fresh, cold water. If you are using a test tube, use a small scrap of window screen or other method to act as a sieve to prevent roots from being dumped out of the test tube at each rinse.

C) Fill sample test tube half full with hydrogen peroxide so roots are immersed.

D) Soak 10-15 minutes and then rinse three times with water.

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A sample test tube filled with ink/vinegar solution ready for its 12-hour hot water bath.

Step by Step Summary

A) Prepare an ink/vinegar 5% volume:volume solution by dissolving 5 mL of ink per every 95 mL of vinegar.

B) After samples have been thoroughly rinsed of hydrogen peroxide, fill test tubes about half full with ink/vinegar, making sure samples are immersed.

C) Cover test tubes with tin foil and place in hot water bath on HIGH for 12 hours.

NOTE: We tested various scenarios for stain times and hot water bath temperatures, including Low vs High temp and 8 hours, 12 hours, 24 hours and 2 days. It is generally easy to over stain roots. We found 12 hours on HIGH to be optimum.

Step 4. Staining with Ink and Vinegar

Ink and vinegar can be a low cost, non toxic option for an effective stain solution.

The HUGE lesson we learned working with ink and vinegar is THE SOLUTION MUST BE MIXED FRESH EVERY TIME BEFORE STAINING. We once processed about 50 samples using a solution we mixed about 3 weeks prior. The result was a disaster with the ink precipitating and clumping. It was very difficult to wash out the ink clumps when it came time to preparing our slides for viewing.

Mycorrhizae is not the only thing this ink will stain! Be extra careful to wear gloves and protect all surfaces, materials and yourself from splash. Concentrated ink, especially when dissolved in vinegar does not wash out! We learned this the hard way, coming away from the farm lab some days with interesting new tattoos.

You're almost there. This is a blueberry hair root that has been nicely cleared and stained. Not only are numerous arbuscules visible within cell walls (virtually every cell colonized), but the hair-like hyphae fungal structures in this sample are also impressive.

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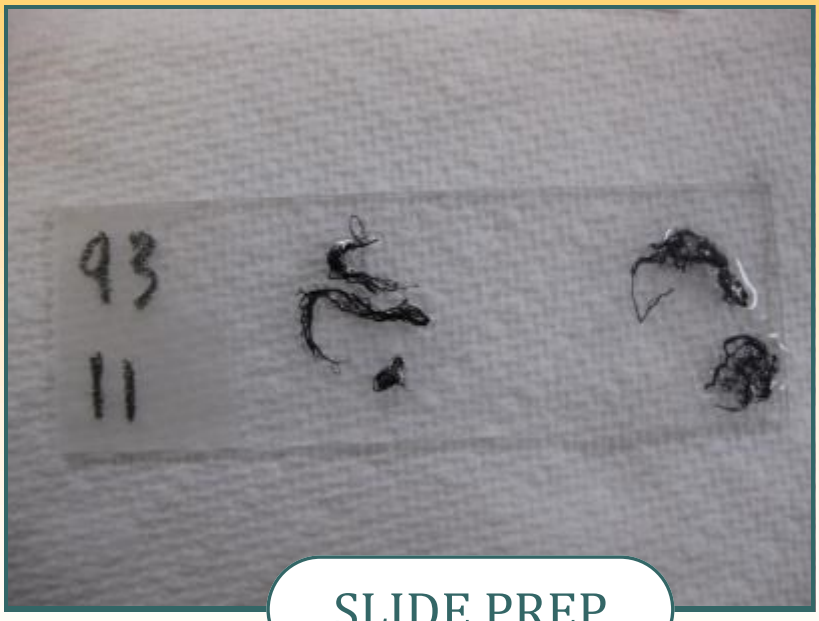
Step 5: Slide Preparation

This step can be a bit tricky. By this time your root sample(s) have gone through a lot, between washing, clearing, staining and many rinses with water in between. They will be delicate.

The other challenge is they will be clumped, simply by virtue of having been in the stain and rinse solutions for some time.

A dissecting needle or any other type of fine pointed needle, e.g. a toothpick, is an ideal tool for handling roots at this stage. You can fetch the roots from within the test tube with the needle and use it to spread the roots out on the slide. The goal there is to give each fine root some space so you can view all roots on the slide instead of one big clump of roots on top of one another.

Pictured here is the same slide as in the picture above, but with the roots spread out. This slide will be perfect for viewing.



SLIDE PREP

Once stain is rinsed off samples, roots can be placed on slides. These roots still need to be spread out on the slide to make viewing of each individual root easier.

Step by Step Summary

A) "De-stain" samples by rinsing thoroughly with distilled water until rinse water is not the least bit dark tinted.

B) Carefully spread root sample on a microscope slide.

C) At this point a drop or two or three of lactoglycerol or distilled water will really help you spread the roots out with a needle, tweezers or toothpick.

D) Spread roots out on slide the best you can so they don't overlap.

E) If you have not added distilled water or lactoglycerol yet, you will need at least a few drops for mounting the cover slip.

F) Mount a cover slip over the sample, pressing firmly and evenly to disperse any excess mounting solution.

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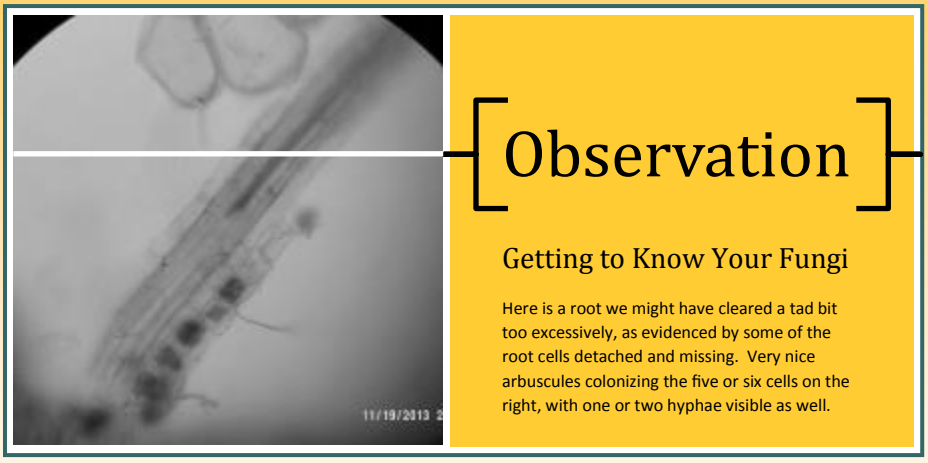


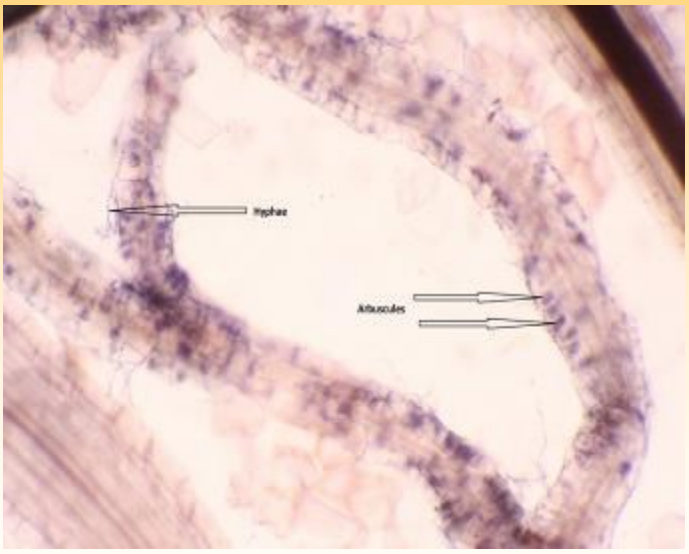
Mycorrhizae structures can vary depending on species of fungus and the type of interaction the fungus has with roots. For our blueberry roots, arbuscules and hyphae are the two primary structures we can see. Hyphae are the hair-like fungal mycelium that grow in great lengths and explore nearby soil and other roots, in essence, both extending the plant’s root network for nutrient acquisition and greatly increasing the chances for further colonization of host plant root cells. Arbuscules are bundles of tightly wound mycelium inside root cells that have resulted from hyphae penetrating root cells from their outside.

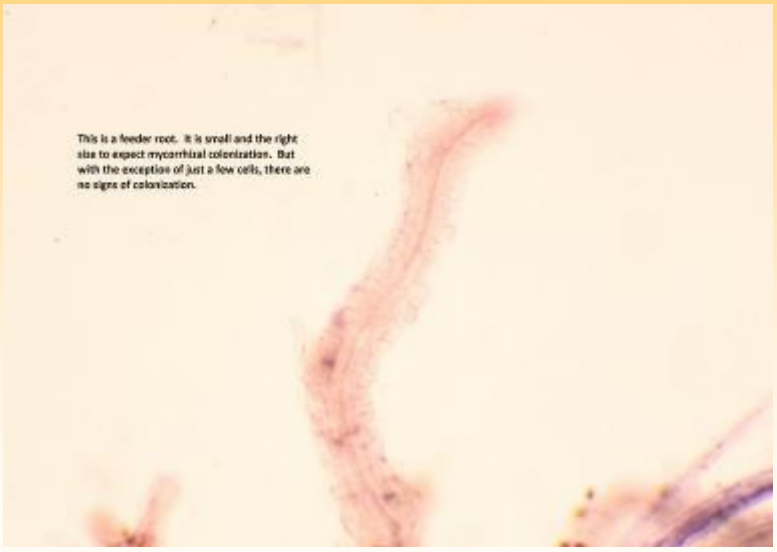
Good mycorrhizae viewing starts with good staining and clearing, but microscope quality also makes a big difference in how clear arbuscules and hyphae will be on the backdrop of distinct cell walls and other cellular structures. But even an old, second hand compound microscope should provide sufficient enough viewing for you to be able to compare colonization from one slide to another. Start on low magnification, hone in on a root, center it in your view and progress to higher magnification.

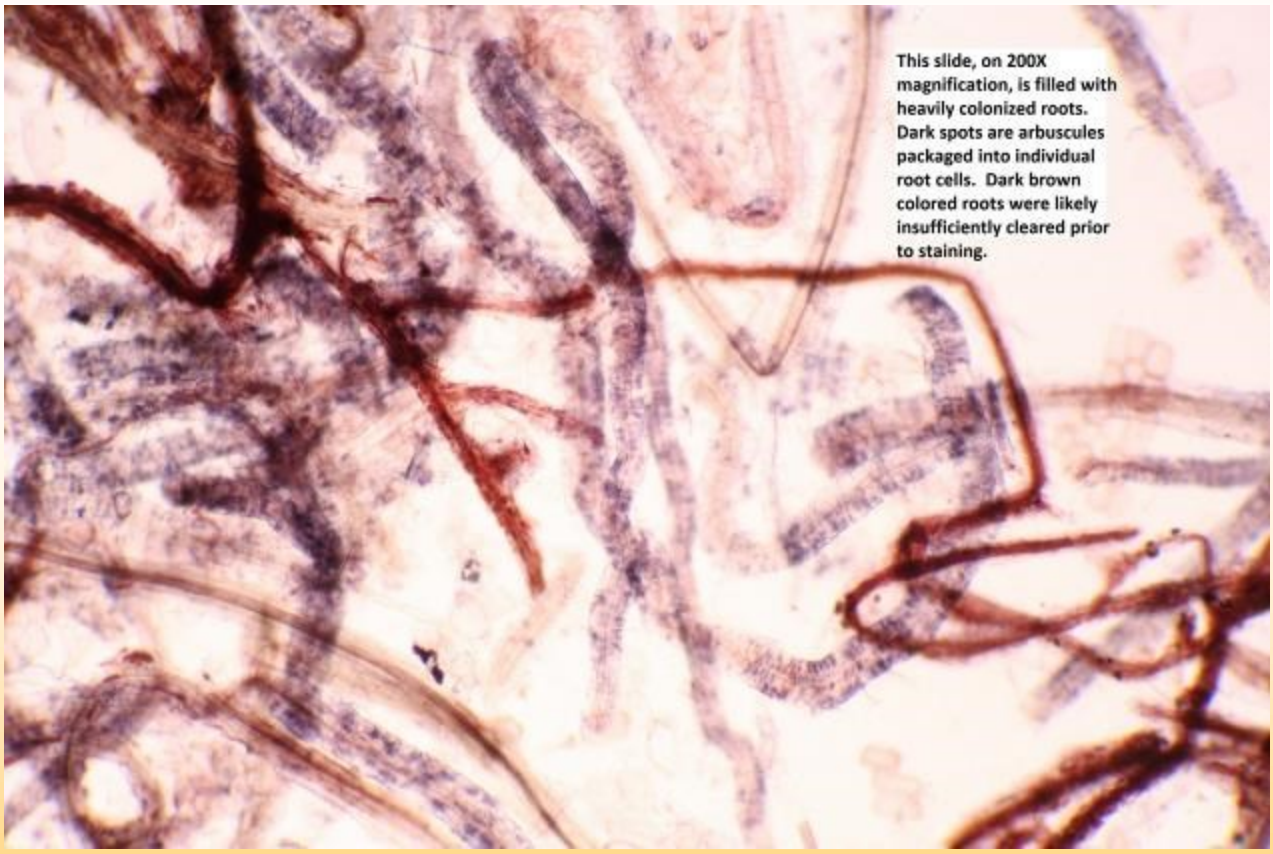
If you want to get a rough idea of yes/no whether or not a sample has mycorrhizae, you don’t need to worry about detailed quantification. It will become obvious after observing just a few slides and surfing around a little bit with the microscope slide toggle whether or not roots are colonized and to what degree. For some slides, all you might see is cell walls with blank, transparent middles, i.e., no mycorrhizae. Other slides might be teeming with fungus, with virtually every cell packed with an arbuscule and hyphae extending out around the roots all over the place. As long as your method of root handling, clearing, staining and slide prep remains the same across your samples, you can make very valid comparisons this way.

There is of course a caveat. After completing our three-year research project we have come to believe that there is tremendous variability among soil conditions that influence mycorrhizae colonization within any given root zone. In other words one root on one side of a rootzone might have access to a different pH, type of organic matter, soil fertility status, etc than another root from the same plant. That plant’s rootzone might have the same degree of variability as another plant a couple feet away in the same row or field. This is all to say that any given sample represents just that: a sample. Take a few samples from different areas of the rootzone to get a more representative picture about what is happening with the whole plant. Even more samples up and down a row or across a field can be viewed to confirm broader suspicions or paint a picture of what is happening on a broader scale.

Researchers have developed many methods over the years for quantifying the degree to which mycorrhizae have colonized roots. There are two common methods. One counts each cell or counts a sample of each root cell in a sample and counts what % of those cells contain arbuscules or have been colonized. This provides data on % of root cells colonized. The other method looks at standard samplings of random intersections on a slide, and the % times colonization is viewed at each intersection is counted. This method provides data on % of root lengths that are colonized. For an excellent overview of these methods as well as much more information on mycorrhizae biology and observation methods, see MYCORRHIZAL ASSOCIATIONS: The Web Resource at www.mycorrhizas.info .







Give the methods a try and tell us what you learn. Good luck! Thanks for reading!

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