HAWKES LAB – COLD STAINING FUNGI in ROOTS

Rationale

This procedure stains the fungal structures inside roots for visualization, clearing away the majority of root structures that might interfere. When working with a new plant species, be sure to optimize the amount of time in each step of the process. Tougher roots will take longer to clear and stain (e.g., grasses), while softer roots will clear and stain faster (e.g., leek roots).

Materials, Equipment, Reagents

20% KOH
1% HCl
Destain, 14:1:1 lactic acid: glycerol: water
Stain, 0.01-0.05% acid fuchsin, aniline blue, or chlorazol black
Forceps, narrow
Razor blades and cutting board
Microscope slides, 75x25x1mm with one end frosted
Coverglasses, 60x24mm or 50x22mm
Test tubes, with holes near bottom to allow flow of solutions, numbered at top with tape (we usually use 12x75 or 12x100 culture tubes)
Test tube racks, chemical resistant
Staining containers, chemical resistant and large enough to hold test tube racks

Method

1. Make the KOH, HCl, stain, and destain solutions in boxes with large enough volumes to immerse test tube racks to a depth that completely covers root samples.
2. Wash root sample and cut into pieces ~2-3 cm long. If roots are very fine, cut them longer to avoid losing them through the holes at the bottom of the tube.
3. Transfer roots to a numbered staining tube; record the number for your sample. Do not overstuff tubes, leave space for solutions to move in and out of the tube.
4. Load tubes into rack and move entire rack between the solutions as indicated below.
5. Soak roots in 20% KOH for 1-3 days, until most internal root structures are cleared but fungal structures remain visible.
6. Drain and wash thoroughly in water.
7. Acidify overnight in 1% HCl.
8. Stain in 0.01-0.05% acid fuchsin, aniline blue, or chlorazol black for 1-3 days, until fungal structures are stained.
9. Destain overnight or longer as necessary to clear root tissues but not fungal structures.
10. Store roots in destain in the dark.

Microscopy – Magnified Intersection Method

1. Set up the microscope (ask for training if this is your first time!). Acid fuchsin is best viewed under fluorescence (excitation is 540λ, emission is 630 λ), but can also be viewed under a compound light microscope. For other stains (e.g., aniline blue, chlorazol black, ink), use light microscopy. Insert a cross-hair reticle in the eyepiece.
2. Mount roots in fresh destain on a microscope slide, with the roots laid out in the long direction of the slide and parallel to one another (Figure 1). Alternatively, roots can be mounted in advance using a semi-permanent to permanent mounting fluid (e.g., PVLG, Permount, ProLong, Flouro-gel).
3. Place your slide on the microscope and focus at 100X (10X objective + 10X eyepiece), then increase the magnification to 200X (20X objective + 10X eyepiece). Move your first field of view to the uppermost or lowermost root at either the far left or far right of the slide.
4. Move the stage up or down to move between roots. Systematically move the field of view to the left or right as you move across the slideIn each field of view, you will count the number of times that the vertical cross hair touches the following (Figure 2):
    a. Aseptate hyphae
    b. Arbuscules
    c. Vesicles
    d. Non-mycorrhizal fungal structures (septate hyphae, usually brown)
5. Once you reach the last root on the slide moving up or down, move the field of view a small distance to the left or right (depending on the edge of the slide where you started). Be sure to move over a minimum distance of one field of view. Continue the process of moving the slide up/down to score roots. Score a minimum of 100 fields of view per slide, being sure to record the number of total number of fields of view examined.
6. Quantification can be refined by adding more information. For example, hyphal width or position in root cortex, arbuscule shape and position, vesicle shape and wall thickness.
7. Roots can be replaced in their vial of destain (for storage) or discarded (if temporary). Slides can be wiped with ethanol and reused. Dispose of slides in the glass waste bin.

Calculations

$\%RLC_{hyphae}$ = # of intersections with hyphae/ total number of intersections counted
$\%RLC_{septate}$ = # of intersections with saprophytic hyphae/ total number of intersections counted
$\%RLC_{arbuscules}$ = # of intersections with arbuscules / total number of intersections counted
$\%RLC_{vesicles}$ = # of intersections with vesicles / total number of intersections counted

References

Grace C and Stribley DP. 1991. A safer procedure for routine staining of vescicular-arbuscular mycorrhizal fungi. Mycological Research 95: 1160-1162.

Merryweather JW and Fitter AH. 1998. The arbuscular mycorrhizal fungi of *Hyacinthoides non-scripta* II. Seasonal and spatial patterns of fungal populations. New Phytologist 138: 131-142.

McGonigle TP, Miller MH, Evans DG, Fairchild GL, and Swan JA. 1990. A new method which gives an objective measure of colonization by roots of vesicular-arbuscular mycorrhizal fungi. New Phytologist 115: 495-504.

Figure 1. Mount roots on slide as depicted below.

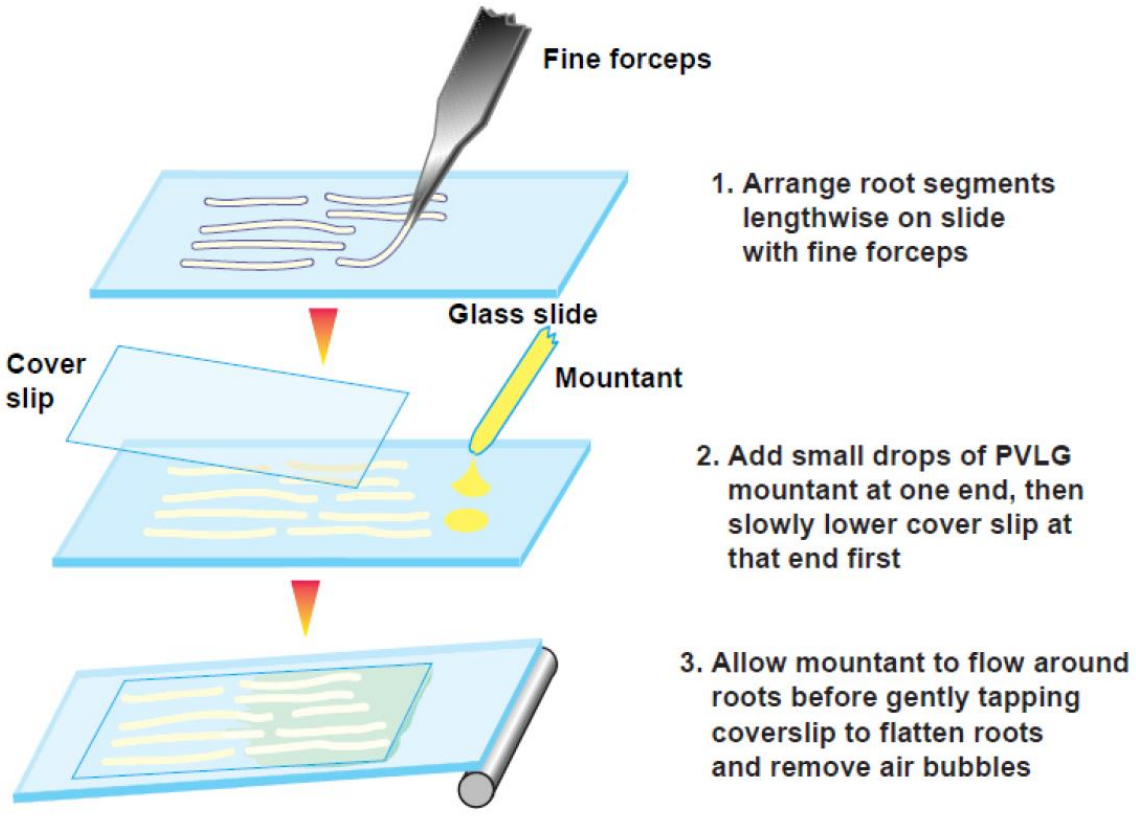

Figure 2. Example fields of view with crosshair intersecting different structures.