PROTOCOL

PRESERVATION, HAND SECTIONING AND STAINING FOR QUANTIFICATION OF ROOT ANATOMICAL TRAITS

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Understanding plant adaptation to edaphic factors requires knowledge of plant traits and responses of the root system. Root anatomy is a good indicator of the strategies and limitations of plants to its environment, yet, it is underutilized in plant breeding programs. The following was written to serve as a guide for the identification and quantification of root anatomical traits.

Tissues

A basic knowledge of plant root anatomy is required:

1. **Rhizodermis/epidermis:** The outermost layer of cells of the root. Entry point of nutrients and water.
   Root hairs: Extensions of root epidermal cells where most of the nutrient and water are up taken.
2. **Hypodermis:** Layer of cells just below the epidermis. It may comprise one or several layers of cells.
   Exodermis: Hypodermis with cells showing Casparian bands**.** Cells with Casparian bands may also develop a suberin lamellae. The exodermis is believed to influence on aposplastic water and solute movement, radial oxygen loss, and may represent a physical barrier to pathogens.
   Casparian bands: A band of suberin and lignin in cells of the exodermis or endodermis.
   Suberin lamellae: A layer of suberin deposited within the cell wall of exodermis and endodermis.
3. **Sclerenchyma ring:** Following the hypodermis, roots of some species may have a ring of sclerenchyma cells. It may comprise more than one layers of sclerenchyma cells. The sclerenchyma ring is mainly comprised by lignified cells and acts principally as support tissue and may be involved in apoplastic water and solute movement, and radial oxygen loss.

Collectively, rhizodermis,hypodermis and sclerenchyma (if present) are referred as Outer Part of the Root (OPR).

4. **Cortex:** Cell layers between the OPR and stele.
5. **Endodermis:** Innermost layer of the cortex with cells showing Casparian bands in cell walls. Cells with Casparian bands may also develop a suberin lamellae. The endodermis is believed to influence on aposplastic water and solute movement
6. **Stele:** Zone internal to the endodermis and containing vascular tissues (xylem and pholem), perycle and sometimes a pith.
7. **Xylem:** Lignified cell walls involved in water and mineral transport.
8. **Phloem:** Tissue involved in photosynthates transport.
9. **Pericycle:** Layer of cells between phloem and endodermis where lateral roots are developed

Sampling of roots

Selection of roots for anatomical studies may represent a hard task, as roots are often grown in soil media. If plants are grown in soil media, to help remove rhizosphere soil from roots, the entire root system of each plant is placed in a container with few drops of low foaming detergent (e.g. tween-20) for 10-15 minutes and rinsed again with tap water to clean up loosened soil. For anatomical studies, only growing roots should be considered. These roots

can be easily recognized as they have a soil-free white elongation zone next to the root cap (McCully 1995 cit. by Neergard et al, 2000). For anatomical studies, roots should be immediately processed or preserved. In our case, roots kept in 50% ethanol and stored at 4ºC up to two months are effectively fixed, preserved and used for posterior anatomical studies. We avoid the use of formalin based fixatives (e.g. FAA) as Formaldehyde may be carcinogenic to humans. http://www.reagent.co.uk/uploads/msds/FORMALDEHYDE%2037-40%20percent%20W%20V%20LRG.pdf

Sectioning of roots

Conventional histological methods involve the time-consuming processes of fixation, embedding and microtome sectioning. On the other hand, free-hand cross sectioning provides an adequate, rapid and inexpensive method for the observation of plant´s anatomy and often results in high quality images (Lux et al, 2005). Moreover, resin embedding may not allow the penetration of dyes including those for suberin and lignin detection (Zelko et al, 2012). Sectioning of roots by this method can be effectively done in either fresh material or fixed roots with ethanol.
Two supporting mediums can be used for efficient free hand sectioning: 1. styrofoam pieces and 2. Agarose blocks.

Sectionioning using Stryrofoam pieces as a support medium.

Thin cross sections can be done with the help of styrofoam pieces. Styrofoam used for packing is ideal for this purpose (Figure 1). We have found that using styrofoam to provide mechanical support whilst sectioning represents the cheapest, still, very effective way to examine root anatomy in grasses such as *Brachiaria*.

1. Hold the root with the styrofoam and with a wet sharp razor blade cut sections along the root (Figure 2). Consecutive and numerous cross sections along the root should be rapidly done. Depending on individual skills very thin cross sections can be obtained (<0.5mm). However, most of the time, the limitation for extraction of information from cross sections arises from the angle in which cross sections were taken; **Avoid oblique sectioning of the root** as possible (Figure). More important is the angle, which should be perpendicular to the root axis. Styrofoam pieces can also be used for sectioning of leaves, leaf sheaths, and stalks (Figure a, b, c).
2. With a fine and wet brush remove cross sections from the blade and put them into a Petri dish with water. Roots and cross sections obtained must be wet or in water at all times to avoid root dehydation and collapase (Figure).
3. Under a dissecting microscope, select thin and uniform sections to view under the microscope or further processing of the sections.

Sectioning using Agarose (or Agar) blocks as a support medium.

Sometimes, roots can be too thinned or too weak as to resist the pass or a razor edge (even if its new and sharp) and entire cross sections are almost impossible to obtain. We have found that particularly truth when cross sectioning roots of various legumes (e.g. *Cannavalia*

brasiliensis, Arachis pintoi, Stylosanthes guianensis, Phaseolues vulgaris), and parts of cross sections obtained using Styrofoam as a support medium are almost unvariably lost (Figure). To avoid this, roots can be embedded in agarose (or agar) and then sectioned (Figure) as in sectioning using styrofaom. Agar (5-10%) may be used to lower costs (agar is at least 3.5times cheaper than agarose) but with less satisfying results.

1. Construct a mold where roots and agarose (or agar) solution near solidification can be poured. We have used "home-made" customized blocks of aluminum paper of a similar size to that of Styrofoam pieces (approximately 1.5cm width x 2cm length).
2. Prepare a 3% Agarose solution (w/v) by pouring agarose in boiling water or by short bursts of heat in a microwave. Swirl continuously to avoid lumps. Let the solution cool down and before it solidifies (solidification of agarose occurs at 45ºC), pour the solution (5mm layer) onto the aluminum blocks. Let further cool down (as you can touch the block without burning yourself), put the root section as straight as possible on top of agarose layer. Pour another layer of agarose that fully covers the root (another 5mm of agarose).
3. Let the agarose to harden and after that, take out the agarose form the mold and place it into water to avoid drying.
4. Perform consecutive and numerous cross sections along the root as when using Styrofoam.
5. Place sections in a petri dish with water.
6. Under a dissecting microscope, remove agarose (if possible) with a dissecting needle and select thin and uniform sections to view under the microscope or further processing of the sections

Clearing

We have found that 50% ethanol is generally a good clearing agent. However, roots for whole mount sections or cross sections can be improved by the use of other clearing agents such as KOH or saturated lactic acid (for further information see Lux et al, 2005).

Staining of root sections

We are interested in the detection of suberin and lignin in root tissues as they are involved in responses of roots to their environment. Suberin is deposited in cells of the exodermis and endodermis and represents a barrier to apoplastic movement, pathogens and radial oxygen loss (for more information see:

Sudan Red 7B for suberin detection

Sudan Red 7B is the most effective, non-fluorescent, stain for Lipids (Brundrett et al, 1991).

1. Prepare solution 1:1 of Polyethylene glycol 400 (PEG-400) and Glycerol (90%).
2. Dissolve 0.01g Sudan Red 7B in 50mL PEG- Glycerol solution.
3. Heat solution at 90ºC for 1h.

Note: we have prepared solutions of Sudan Red 7B in PEG-6000-Glycerol, and Sudan Red 7B in Glycerol, with relatively acceptable results.

4. Cross sections are then mounted on a slide and directly into the staining solution for 2 hours. Framing with nail polish is effective to avoid loss of cross sections from the slides.
5. After 1 hour, dye is rinsed with water and then washed with 1% (w/v) sodium dodecylsulphate (SDS) (Soukup et al, 2002). SDS minimizes the precipitation of dye on the sections (Soukup et al, 2002).
6. After washing, mount the cross sections on a clean and oil free slide. Degrease with ethanol or methanol. Mount sections on a drop of glycerol (>30%).
7. Sections are ready for examination under bright field microscopy. Suberized walls and Casparian strips will stain red (Figures)

Phloroglucinol-HCL for lipid staining

To confirm lignin presence, phloroglucinol in HCl is used (as in Gunawardena et al, 2007)

1. Mix 0.1 g phloroglucinol, 16 mL of concentrated HCl (12 N), and 84 mL 95% ethanol.
2. Incubate sections in solution in a hot plate/petri dish for 60s.
3. Rinse sections with water to remove excess of solution. Mount sections on a drop of glycerol (>30%)
4. Sections are ready for examination under bright field microscopy. Lignified walls will stain red (Figure).

Measurement of root traits in cross sections using digital images.

Rapid advances on digital imaging has made more affordable the recording of images from a microscope. The recording of digital images allows posterior visualization of root anatomical traits of interest. Several image software such as Adobe´s Photoshop and Open ImageJ are user friendly and allows the manipulation and rapid quantification of areas within a digital image. At CIAT we use ImageJ which is a potent open source image software for the routine quantification of traits known to be related to waterlogging tolerance (e.g. aerenchyma and stele areas) in root cross sections.

Quantification of areas in a digital image using ImageJ software

ImageJ is public domain software downloadable at http://rsb.info.nih.gov/ij/. It runs in Windows, Mac and Lynux operational systems and is frequently updated.
Here we briefly describe the process of measurement of actual and proportional areas for the determination of root diameter and root aerenchyma proportion. Using the same principle, other features can be also quantified (e.g. areas of stele, metaxylem vessels, root hairs, etc)
Any measurements of length in an image are based on the comparison of the object under study with another of known dimensions. Such task is simply filled with the use of a standard

stage micrometer. An image of the stage micrometer at known magnification must be recorded. This image is then used as a reference for the determination of the actual dimensions of the images taken of the cross section of interest. This technique is very reproducible with an accuracy of approximately 1µm. A detailed description of calibration of images using a stage micrometer or micrometer slide can be found in the following link: http://skepticwonder.fieldofscience.com/2009/11/quick-imagej-tutorial-scalebar.html

Measurement of root diameter in a cross section

1. Install ImageJ
2. Opening an Image from a file:
   Select **File** from the main menu and then **Open.** Choose the image of interest
3. Set the scale of your image:
   As described before, measurement of actual dimensions must be based on a standardized object of known dimensions.
   a. Draw a line of known dimensions
   b. Go to **Analyze** and then **Set Scale**. A new window will show with the length of the line in pixels. Type in know distance, the length of the line with the appropriate unit.
4. Select the area using one of the selection tools available ()
5. Go to **Analyze** and then **Measure**
6. The actual size of the image will be displayed

Measurement of proportional areas

The measurement of proportional areas is even a more strait forward process as you are comparing at least two areas within one image.

1. Repeat steps 2 and 3 previously described
2. Select the area of interest using one of the selection tools available
3. Go to **Analyze** and then **Measure.** The number of pixels within your selection will appear
4. Select the second area you want to measure with one of the selection tools available. The number of pixels of your selection will appear.
5. Divide the number of pixels in the second selection with the number of pixels of your first selection.

Quantification of number of cells showing Caspari Bands and Suberin lamellae

High quality pictures of root cross sections stained with Sudan Red 7B allows posterior analysis of cells showing Caspari bands and Suberin lamellae. For the quantification of cells in the exodermis showing caspari bands and suberin lamellae we have used the methodogy as described by Peterson and Enstone.

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