STOMATA PHENOTYPE PROTOCOL, BUERKI LAB, BOISE STATE UNIVERSITY

INTRODUCTION – UPDATED NOVEMBER 2023

Investigating phenotypes of leaves is essential to understanding the physiology of plants, allowing for the analysis of adaptive qualities and fitness. Although many protocols and equipment exist to study plant stomata, these are attuned to model or crop species and fail to properly gather data for our target species *Artemisia tridentata*.

This protocol was developed by the Buerki Lab to study *A. tridentata* seedling leaves. It can be applied to more mature individuals as well as other plants with similar success. No protocol, to our knowledge, has been made to specifically study sagebrush stomatal density. We based our workflow on stomatal assay protocols done before using adhesives to separate the leaf epidermis layer (Allsman et al., 2019; Eisele et al., 2016; Wu et al., 2009).

Research on various plant species has shown that epidermis characteristics can vary within species, between the leaves on a plant, and even among specific areas on a single leaf (da Silva et al., 2016). Differences in stomata density has also been observed between ecotypes of *Phragmites communis* as well as heterogeneity in patterning along the blades of tobacco leaves (Pazourek, 1973; Slavik, 1963). Accordingly, this would necessitate multiple observations of stomata along one leaf and on both surfaces of the leaf for an appropriate amount of data to test hypotheses about sagebrush stomata patterning characteristics. Observations on sagebrush leaves should avoid bias in this sampling with spaced imaging frames down the length of a leaf avoiding overlap (Kubinova, 1994). The lack of physical separation of this sampling would normally assume them to be non-independent experimental units for data analysis. In fact, many researchers average multiple observations on the same leaf. However, this methodology prioritizes a systematic positioning of observations- three observations per surface ordered linearly from the leaf tip to petiole. With this standardization, we can test for the independence of these positions along the leaves. A generalized linear model on our data concluded that position had no statistically significant effect on stomata density, allowing for each image, even those physically on the same leaf, to be treated as independent experimental units, allowing for increased power in testing hypotheses (Colegrave & Ruxton, 2018). This approach to stomata density data should be conserved for all leaves with similarly heterogenous stomata patterning and amphistomatous leaves.

Due to limitations imposed by our study species, personnel, time frame, and budget, we adapted previous protocol on stomatal density efforts with theory on stereological methods of measurement to create this workflow to efficiently produce reliable and robust stomata density and size data for *A. tridentata*.

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MATERIALS

- Forceps.
- 2 Large petri dishes.
- 1ml pipette and tip.
- Container with distilled water.
- Microscope slide.
- Spatula.
- Gorilla superglue.
- Transparent tape.
- Cardboard.
- Light microscope with Motic camera.

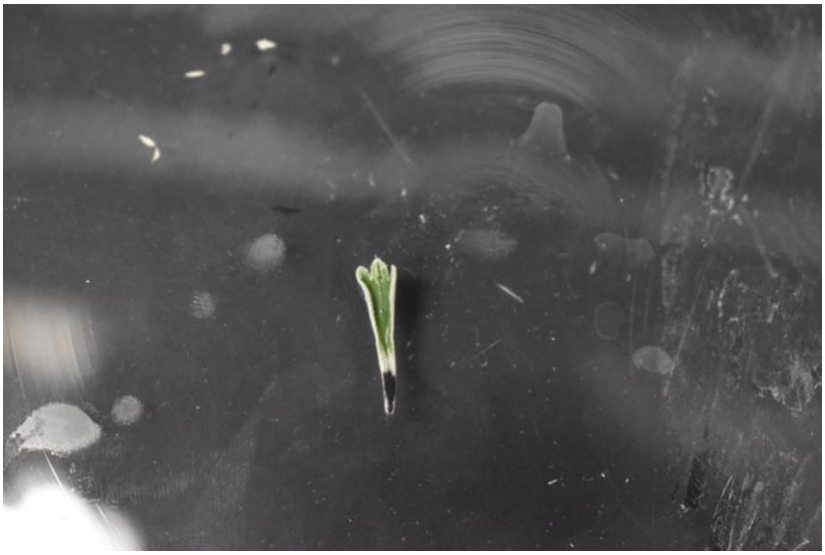
WORKFLOW
LEAF PREPARATION

This procedure should take 30-50 minutes per plant (= 2 leaves).
Stomata assays take place between 9 am and 12 pm when sagebrush seedlings should be performing photosynthesis and therefore have open stomata.

I. Go to the GH and harvest leaves (4 from the top ⅓ part of the main stem if possible) for up to 6 seedlings.
    a. The full length of the leaf should be taken. Abaxial surface of leaves are marked with sharpie at the petiole end.
    b. Store leaves in labeled zip bags. The rest of the protocol takes place in the lab.

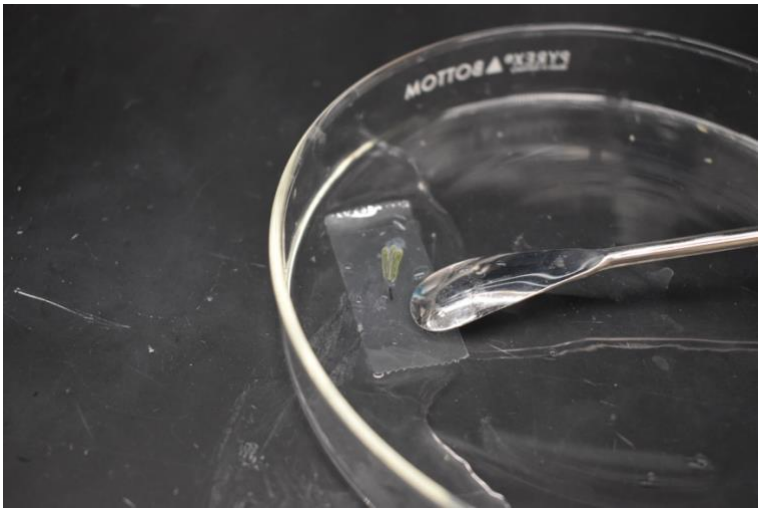
II. Select 2 leaves and prepare slides for each leaf as follows:
    1. Place the leaf on the correct side (Top or Bottom).
        a. We always start with T and then B.
    2. Measure leaf length from petiole to tip and leaf width at 2/3 length from petiole.
    3. Physically remove trichomes with the side of the spatula starting at the petiole and moving onwards in a gentle scraping motion.

   a. Be cautious not to rip off or tear the blade. For more fragile leaves, gentle removal using duct tape or by rubbing with fingers.



4. Transfer leaf onto cardboard and label side of cardboard with Seedling ID and T/B (writing on the bottom left margin at 90 degrees).
   a. Apply a small drop of superglue on part of the leaf without trichomes and spread to cover the leaf with a thin even layer. Cut tape and apply it onto the leaf (from the petiole onwards).
   b. Push the superglue to homogenize it.
   c. Apply pressure with your thumb for 30 seconds.
5. Use forceps to gently remove the tape starting near the petiole. A perfect preparation will have very little mesophyll (= green tissue). Presence of peeled epidermis appears as an opaque leaf on the tape.
6. Move the preparation to a petri dish with the side of the leaf exposed. Take the pipette and spray water onto the preparation.
   a. Repeat this 5-10 times.

b. When this is done, bath the preparation straight into the water in the petri dish, continuously undulating the tape to remove bubbles.



7. Take a slide, put a small drop of water in the center, and transfer the preparation onto it (with the non-sticky part of the tape up).
    a. A cover slip is optional- recommended if tape does not remain flat to prevent uneven viewing surface.

LEAF IMAGING
MOTICAM SETUP

1. Insert electrical plug of Moticam camera into electrical socket, ensure the Moticam LED lights are flashing/on.
2. Open the ‘Moticam Image X 3.0’ software on the Lab MacBook Air.
3. Open the WiFi menu of the MacBook Air and connect to Moticam device.
4. Open the Moticam Live Imaging software by navigating to File > Image Capture; alternatively use the keyboard command ‘CMD + M’.
5. Use ‘Connect’ command on the right side of the live imaging software to receive live feed of Moticam unit.
6. Focus light microscope using macro focus knob.
7. Adjust magnification measurements calibration menu on the top most bar with two dropdown menus. Ensure BOTH dropdown menus are of the same objective as the current objective being used on the microscope stage.
8. Capture image on Live Imaging software using the Image Capture button on the right side of the software menu.
9. Switch to ‘Moticam Image X 3.0’ software window to ensure image was captured appropriately.

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IMAGE CAPTURE

Take 3 images for each leaf surface/slide with the microscope at 10x magnification. Make sure to set a grid with 200x200 μm resolution. Before taking images use 4x magnification to fully inspect preparation and identify the best region for image capture. Images should ideally capture an area close to the leaf end and not at the petiole end, with care not to devote large areas of the image with the very outer edge of the leaf or the veins. Note the time when you imaged the preparation (the software names images with magnification, date and time).
After each 10x image, change magnification to 40x and capture two images with at least two stomata appropriately focused for measuring dimensions and aperture within the confines of the captured 10x image. Grid settings should be removed, replaced with a scale bar of 20 μm.
Export all images (9 per leaf surface: three at 10x and six at 40x) and save them on the shared Google Drive in an individual folder named as follows: GH_Seedling_ID (e.g. "GH_0987"). DO NOT RENAME IMAGES, this is key for the next part.

DATA PROCESSING

When: 1-4pm.

MATERIALS/SOFTWARE

A computer with R and the magick package as well as Fiji installed.
R project (Stomata_project) and associated R script and user defined functions developed for this project. These files are available for download on the Google Drive.

WORKFLOW

Package and workflow used RAW images of 1280x720 resolution using Motic Image Capture software.

For each seedling, prepare images using stomataImProcesseR. Images used to score are in a subfolder entitled "Images_to_score/". Each cell will be labeled and it will be its unique ID for scoring (see DB2).
Generate DB1 and DB2 with stomataDBMakeR. These .csv files will be in the root directory.
Manually score # stomata and trichomes per cell and enter data in DB2. This is done by inspecting images with an Image viewer (you can zoom in to better discriminate stomata and trichomes within each cell). Note: 4x have far less stomata and trichomes than 2x and it is highly possible that few cells will be scoreless.

Update DB1 with data from DB2 using stomataDB1UpdateR1.
Create DB3 for stomata measurements using stomataMeasureR.
Score stomata and guard cells traits in DB3 using Fiji.
Update DB1 with data from DB3 using stomataDB1UpdateR2.
Process leaf scans. TBD.