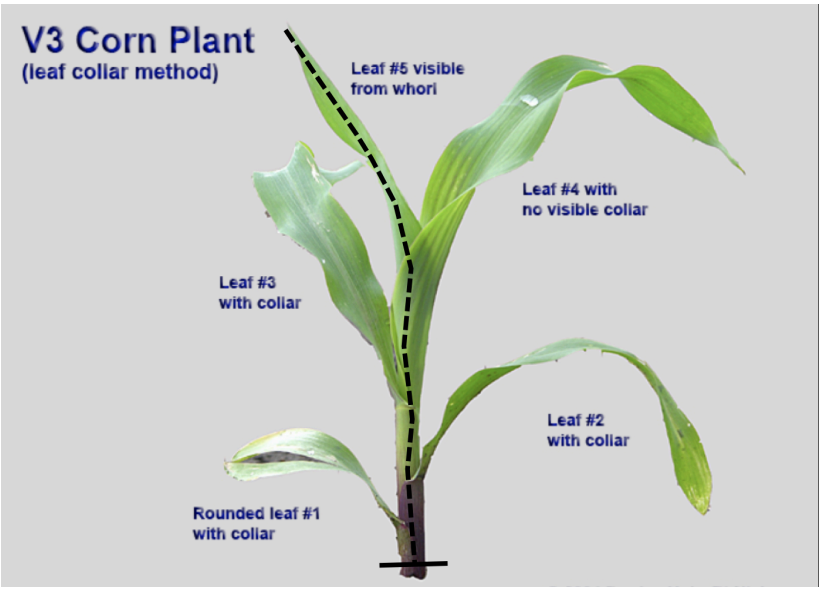
Shoot Phenotyping Protocol



This figure shows the axis up to leaf #5, but **you should measure leaf #4.**

Keeping track of your plants

It is very important that the stem portion of each plant can be connected back to the root portion of the plant. Therefore, each plant should receive an identification number. When you separate the stem and shoot portion, remember to label carefully so that you know which root system belongs with which shoot system downstream.

4th Leaf measurements

You will need to observe your growing seedlings **everyday** to assess when leaf #4 blade tip is visible in the whorl. Once the tip of leaf #4 is above the leaf whorl, take a sharpie and make a mark at the base of leaf sheath #1, as close to the bottom of the shoot as possible but above the soil line. This is the "zero" mark for taking **daily** leaf length measurements. Starting from the first day leaf #4 is visible, measure from the sharpie mark at the shoot base to the tip of leaf #4, doing this at the same time each day. You will need to gently straighten the leaf so it is relatively linear. Take care to NOT pull off the leaf tip. If you do, record the length of the torn off tip and make sure to add that measurement to all subsequent length measurements. Each day, at the same time, measure the length of leaf #4 from the "zero" mark to the tip of the blade making sure to keep these data with the correct plant number. Leaf #4 will keep growing for about 2 weeks and the growth will gradually slow. When leaf #4 length has not changed for 3 consecutive days, it has reached its mature length and you can now proceed to termination of the plant, end-point leaf measurements, and root processing.

Telling when leaf #4 has stopped growing isn't trivial. The best way to do this is to continuously graph (leaf length vs time) your data as you measure. You will see when the leaf plateau and will be able to stop the growth at the right time by watching the growth curve carefully.

Termination of plant

When leaf #4 has stopped growing for 3 days, cut the base of the shoot at the sharpie mark (as low on the 1st leaf as possible). Holding the base of the plant upwards with the leaves hanging downwards, carefully peel off each leaf at the sheath base, starting with the outermost leaf #1, and taking care to not damage it. For end-point measurements, dissect out the 4th, 3rd and 2nd (if still healthy) leaves (including the sheath) and number them accordingly. For each leaf (#'s 2 to 4), measure the sheath length (from base of sheath to auricle/ligule). Cut sheath from blade at the auricle/ligule. Measure the length of the leaf blade from the auricle/ligule to the tip of the blade. At 50% of the blade length, measure leaf blade width. If you cannot scan the leaf blade the same day, place the leaves into a plastic bag, blow in some air and seal the bag until the next day. For longer storage, keep the leaves in the fridge.

Wash the root system according to the Root Phenotyping Protocol. Keep the root system cold until you are ready to wash them. Do not store longer than a couple of days. For longer storage, freeze the root system after washing.

Setting up the leaves for scanning

- Cut the blade of the leaves (Leaf 4) into portions that would fit onto the scanner surface.
- Lay the leaves so that all portions of the **blade** are flat with the adaxial (flat/smooth) side against the scanner (only scan living leaves).
- **Add a ruler.**
- Make sure everything is flat and close the scanner lid with the white background inserted.

Note: You may set up the scanner so that multiple leaves can be scanned at once. However, make sure you also insert a label associated with each plant. Each leaf should have a ruler next to it.

Scanning the leaves

- Open the Epson Scan 2 software. Note that the following parameters may be different depending on if you are using a Mac or PC to control the scanner.
- Set up the scanner parameters as below.
- **Scan one leaf at a time.** In other words, each image should contain only one leaf and one ruler.

Scanner parameters
Scanner: EPSON Perfection V800/V850 (USB)
Scan Settings: Select saved settings or create one for future use
Mode: Photo Mode
Document Source: Scanner Glass
Document Type: Reflective
Image Type: 24-bit Color
Resolution: 300 dpi
Scanning Quality: Standard
Image Format: JPEG
File Name: Genotype-Plant#-leaf#.jpg
Folder: Shoot Phenotype

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ImageJ software to measure leaf area

Install the open access ImageJ software to your computer: https://imagej.nih.gov/ij/download.html

1. Open your image to be analyzed in **ImageJ**

2. Set measurement scale. **You must set a new scale every time you change a scale (ruler), scanning parameters (especially resolution), and reopening ImageJ!**
    - Using the "Straight" line tool, draw a line over a 50 mm section of the ruler
      Go to Analyze > Set Scale
    - In Set Scale window enter 50 into the 'Known Distance' box
    - Change the 'Unit of length' box to mm
    - Check 'Global' (this will set the scale for all your images for this working session)

3. Segment the image (phenotype speak for getting rid of everything you don't want)
    - Convert scanned image into black and white
        - Process > Binary > Make Binary
    - Find the edges of all objects
        - Process > Find Edges
    - June 21, 2024: Tai switched these steps
4. Calculate surface area
    - Use the Rectangle tool, select the leaf blade (or all parts of a blade) that you want to measure.
    - Analyze > Analyze Particles
        - Enter 50 as the minimum particle size (it will not include anything that is smaller than 50 mm^2, thus effectively getting rid of all small particles)
        - Show: Outlines (this will show you what it actually measured so make sure for each image that it measured what you think it measured).
        - Check 'Display Results'
        - Check 'Include holes' (your leaf blade is now considered a hole according to ImageJ).
        - Click 'OK'.

    Your results will be displayed in a table (the table is appended each time you take a measurement unless you check 'clear results', which you DO NOT want to do). It will also show you the outline of the object that it just measured. **The "Area" is the measurement that you want!**

Saving data

Save your data after the processing of each image into your Shoot Phenotype folder. Do this often because there is a chance that you accidentally delete all your data or that the program crashes (This happens often)!

Click on the "Results" box
File > Save As
Save your data in csv format, making sure you label the appropriate data.

Combine final data

Once you have processed all of your images, combine the data into a single spreadsheet so that you can process your raw data in R.