#### Plant Growth and Development, 15p

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#### LAB2: Growth & Defense trade-off in *Nicotiana benthamiana* and Turnip Mosaic Virus interaction

Schedule:

2024-11-25 Day 1 9:15-12:00 Infection of seedlings

2024-12-10 Day 15 09:15-12:00 Symptom scoring and samples harvesting

2024-12-11 Day16 09:15-16:45 RNA extraction and cDNA synthesis

2024-12-12 Day17 09:15-14:00 qPCR

2024-12-13 Day18 09:15-12:00 Discussion of qPCR results

**Day 15: Samples harvesting, scoring of viral symptoms, and first discussion**

It is an ongoing debate in the plant-pathology field, especially in plant virology, whether and how disease symptoms and pathogen accumulation correlate. Is the disease phenotype truly dependent on pathogen accumulation? Today, we will examine your infected *Nicotiana* plants and score the TuMV-induced disease by measuring the fluorescence intensity of the GFP protein co-expressed in each Agrobacterium construct previously inoculated. **Please bring your laptop for this exercise and ensure you have installed** [**imagej software Fiji version**](https://imagej.net/software/fiji/downloads)

**Material**

* *Nicotiana* infected plants, 15 dpi
* Laptop with Imagej software
* Gloves
* Goggles
* UV reflective ruler
* UV torch
* Photographic camara and tripod
* Black cloth
* 2 mL Eppendorf and Eppendorf tube stand
* Eppendorf pestles
* 1ml pipettes + tips
* Hole puncher plant
* RNeasy kit extraction buffer RLT buffer with β-mercaptoethanol (10uL:1mL RLT)
* Liquid nitrogen liN2.
* Zippered bags of Eppendorf tube stand size

**What do you think (please prepare these questions for an in-class discussion):**

* Does the TuMV accumulation correlate with the symptoms you are observing in your plants?
* What are reproducible, easy-to-score, non-invasive readouts for viral disease?
* Are viral infections comparable to bacterial/fungal infections? What are differences/ similarities?
* What does a pathogen need from its host?

**Photography of GFP expression to address symptoms severity**

Before processing the plant tissue for RNA extraction, we will take take photographs of the leaves expressing GFP proteins for each of the four conditions: GFP only, TuMV “ΔHCPro” expressing free GFP (ΔHCG), TuMV “6K2-GFP” (6K2G) and both ΔHCG X 6K2G. We will use a UV reflective ruler and a black cloth as background, as shown in the setup:

A close up of a leaf

Description automatically generated

**OBS!** When working with UV light, especially in laboratory settings, there are significant safety concerns to be addressed. UV radiation can cause severe damage to the eyes, including corneal burns, which is why it is critical to wear proper protective goggles that block UV rays whenever UV light is in use. Similarly, exposure to UV radiation can also harm the skin, so ensuring minimal skin exposure is necessary.

**OBS!** Do not forget to wear gloves to protect your skin and prevent cross-contamination. These precautions—goggles for UV protection and gloves for handling infected tissue— are necessary measures to ensure both personal safety and biosafety compliance in the laboratory.

**Sample harvesting:**

1. Once detected the regions of GFP expression signal, proceed to collect the tissue. Depending on the size of the infected tissue, use either the whole leaf or the smallest hole puncher. To collect a single biological sample, we will collect four different leaf disks from different locations on one leaf of each group, as shown in the picture below:

A green leaf with white circles and numbers

Description automatically generated

1. Label four 2mL Eppendorfs for each treatment and place them in your liquid nitrogen tank.

**OBS!** Direct skin or eye contact with liN2 can cause severe damage, including cryogenic burns, frostbite and eye damage. Wear goggles whenever you work with liN2!

1. Place the infected leaves disk (per treatment) in the previously cooled 2mL Eppendorf and add liquid nitrogen liN2.
2. On a tube-by-tube base, grind the plants to fine powder (be careful not to let the material thaw!) using the Eppendorf pestle. Before continuing to the next tube, proceed to the next step
3. Go to the fume hood where the RLT Buffer is located, and add 450uL in your tube. Vortex and place your sample on a tube stand at room temperature (RT) in the fume hood.
4. Once you have processed each of the four tubes, place the entire tube stand into a zippered bag and store this bag in the refrigerator. This is the material for RNA extraction on the following day.

**GFP Fluorescence Image Analysis to Assess Symptoms Scoring**

1. Once you have taken UV pictures of the GFP-expressing tissues, save them in the same folder and open them in the Fiji version of ImageJ software.
2. Open the images

A screenshot of a computer

Description automatically generated

1. **Adding scale-bar:** First, use the straight tool to draw a line approximately 1 cm in length, as shown:

**A screenshot of a computer

Description automatically generated**

1. Set the scale by going to the “Analyze” tab and clicking on “Set Scale”. Type “1” in the “Know distance” and write “cm” as unit of length.
2. Add the scale bar by going to the “Analyze” tab and then clicking on “Tools”. Finally click on “Scale Bar”. In the new menu, select “Lower Right” as “Location”.

A screenshot of a computer

Description automatically generated

1. Duplicate your image by clicking on the “Image” tab and then selecting “Duplicate”.
2. To avoid fluorescence quantification at the pixel resolution level, select the duplicate image, go to the "Process" tab, search for the "Filter" option, and select "Gaussian Blur." Type "2.
3. **Filtering Green channel:** With the lower-resolution image, go to the "Image" tab, select the "Color" option, and then click on "Split." You will get three new windows—select the one labeled "(green)," as shown: A screenshot of a computer

   Description automatically generated
4. Re-add the scale bar following the previous four instruction but selecting only the green window.
5. Using the Rectangle tool, select only the leaf region. Then go to the “Image” tab and click on “crop” option. Duplicate the image without selection marks, and save the image by going to the “Image” tab and clicking on “Save as” in JPEG format.

A screenshot of a computer

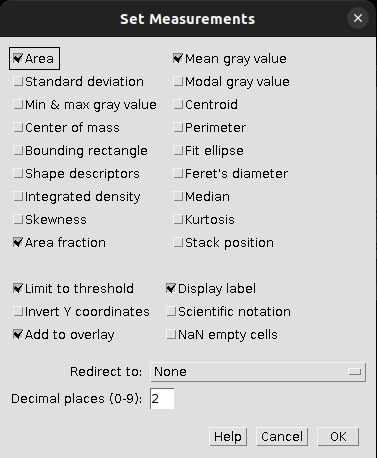
Description automatically generated

1. **Fluorescence quantification**: We are now ready to quantify the fluorescence intensity, go to the "Image" tab, click on "Adjust," and search for "Threshold." Make sure "Don’t reset range" is selected. Then search for “MaxEntropy” option and move the upper bar to filter out the “non-green” tissue, then click on “Set”. All pixels with an intensity below 59 will be filtered out:

A screenshot of a computer

Description automatically generated

1. Now go to the “Analyze” tab and click on the “Set Measurements” option. In the new dialog box, click on the same boxes as the example ahead:



1. Once you click on “OK”, then select the image with the red threshold and click into the “Analyze” tab, and click on Measure, a new window will appear called “Results”. Copy the “Area”, and “Mean” intensity per each leaf measured, we will use these values as a proxy of the GFP are (cm2) and mean GFP fluorescence intensity.

**Compare your infections with the other groups:**

* Do you see differences in the same viral strains?
* Who is winning in our competition test, based your decision on mean intensity values

**Further Reading**

**Nicaise, V. (2014).** Crop immunity against viruses: outcomes and future challenges. *Frontiers in plant science*, *5*, 660.

**Bashandy H. *et al.* (2015).** Within leaf variation is the largest source of variation in agroinfiltration of Nicotiana benthamiana. Plant Methods, 11, 47.

**Fitzpatrick M.** **(2014). The Open Lab Book: Measuring cell fluorescence using ImageJ.** https://theolb.readthedocs.io/en/latest/imaging/measuring-cell-fluorescence-using-imagej.html