EcoFab Ringtrial
Overview and shipment

Participating labs
Lab 1 Joelle Schlapfer, Northen lab, JGI
Lab 2 Andrew Klein, Dangl lab, UNC
Lab 3 Borjana Arsova and Josefine Kant, Watt lab, Forschungszentrum Jülich IBG-2

Timeline

Sept 18:	ship material
Sept 19/20:	material arrives
Sept 25:	start stratification
Sept 29:	check if logger interval changed from 8 s to 4 s (start recording)
Oct 2:	seed out Brachy seeds on plate
Oct 4, 0d:	transfer Brachy to ecofab
Oct 11, 7d:	add medium
Oct 16, 12d:	check medium
Oct 18, 14d:	add medium
Oct 23, 19d:	add medium
Oct 24, 20d:	sample medium, add new
Oct 25, 21d:	sample medium, end of experiment

Material shipped by the Northen lab
To each of the participating labs

- 15 Ecofab devices
- 15 Ecofab boxes
- HOBO data logger (light, temperature)
- 1 L pouch with regular MS
- 1 L pouch with MS no phosphate
- 3 g phytoagar in 15 ml falcon
- 100 ml sterile soil extract, in two 50 ml falcons
- 15 ml sterile 1 M K_2HPO_4 in 15 ml falcon
- 60 *Brachypodium distachyon Bd21* seeds in envelope
- 7 rolls of Micropore tape

Upon receiving the shipped material:
please move MS pouches and soil extract to a fridge for storage
check the material for shipping damage
check the data logger: the red light should be blinking at an interval of 8 sec. The logger is set to start recording on Sept 29, and the interval will switch from 8 sec to 4 sec.

Experimental procedure:

Layout of the experiment:

Brachypodium will be grown in quadruplicates in Ecofabs in three conditions (0.5 MS + P, 0.5 MS – P, soil extract, one blank for each condition). Root morphology and sterility will be monitored during the course of the experiment. After three weeks, root morphology will be imaged, root exudates and tissues collected, and phosphate quantified.
The goal is to test the Ecofabs for reproducibility.

Growth conditions:

- Growth chamber with 16 h light (8:00 – 24:00), 150 µE, 24˚C day and night. We do not control humidity in our chamber, if you can, please set it to 70%.
- Please put the logger into the growth chamber latest on Sept 29, 8:00 PST when it starts logging temperature and light intensity. Recordings will be made on an hourly basis.

Brachypodium seeds stratification, *Sept 25*

Figure 1: left, Brachy seed with husks attached. Right, Brachy seed (top) with husks removed (bottom). Arrow: embryo

1. Dehusk seeds (see figure 1): remove husks from seeds with either fingernails or tweezers. The embryo sits at the base of the seed (arrow), try not to damage it. Prepare 25 seeds (germination rate is about 80%) for the 12 Ecofabs with plants.

2. Sterilize seeds: Immerse dehusked seeds in 70% Ethanol for 30 s, remove supernatant. Immerse seeds in 50% bleach, 0.1% Triton X-100 for 5 min, remove supernatant. Wash 5 times with sterile water.

3. Stratify seeds: after sterilization, immerse seeds in water, and store in fridge at 4˚C for 1 week

- from here on, all the work has to be done in a sterile environment -

Check logger, *Sept 29*

The data logger is configured to start recording on Sept 29, 8:00 PST. The interval of the red light on the logger will change from 8 s to 4 s. The logger will record temperature and light intensity on an hourly basis. The logger will keep recording until the storage is full (about 2 months), or unless the recording is stopped with the base unit connected to the software.

Brachypodium seeds plating, *Oct 2*

1. Pour 0.5 MS + P plates: Dissolve 0.55 g MS+P in 0.25 L water, add 1.75 g phytoagar, adjust pH to 5.7 with KOH. Autoclave, pour 25 ml plates.
2. Prepare MS media: try to find the cleanest bottles in your lab. LCMS is very sensitive, and residual soap or rich medium in bottles will generate high backgrounds. If possible, bake the bottles for 30 min at 200˚C or 400˚F in an oven. Dissolve 2.2 g of MS+P or xx g MS-P in 1 L of water, adjust pH to 5.7 with KOH. Autoclave.
3. Plate Brachy seeds: remove supernatant from stratified seeds, spread seeds on 3-4 plates with a sterilized tweezer. Try to orient them with the embryo towards the bottom (see Figure 1 and 2). Allow a distance of about 1 cm between seeds. Wrap the plates with Micropore tape. Incubate the plates in a growth chamber for two days.

Ecofab sterilization, *Oct 2 or Oct 3*

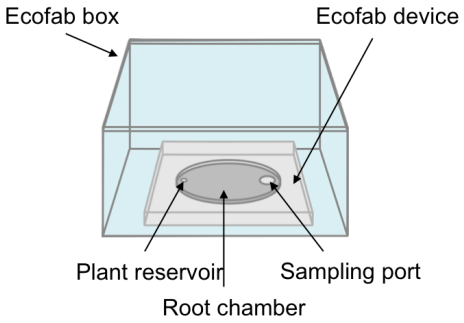

Figure 2: Ecofab device setup. The Ecofab consists of an outer plastic box, and the Ecofab device. The Ecofab device is made from PDMS, bonded to a glass slide. The PDMS layer contains a reservoir, which is filled with growth medium. The seedlings are transferred to the plant reservoir, such that the roots are submerged in the liquid. The sampling port enables changing the growth medium.

1. Rinse: rinse the Ecofab boxes with milli-Q water three times. I rinsed the Ecofab devices already three times with water, and with 100% Methanol to remove contaminants from the production process.
2. Sterilize the boxes and devices: Place all the devices in boxes. Add 70% ethanol until the device is submerged. Close the lid of the box, and gently wet all surfaces of the box with ethanol. Make sure the Ecofab device contains no or few air bubbles. Incubate for 30 min. Pour off 70% ethanol, and repeat the procedure with 100% ethanol, incubation time 5 min. Drain the ethanol, and dry the Ecofabs (takes about 16 hours). If possible, additionally sterilize the Ecofabs by turning on UV light for 1 hour. Continue with the seedling transfer, or wrap the Ecofabs with micropore tape before removing from sterile environment.

Transfer of seedlings to Ecofab, *Oct 4:*
Day 0 of timecourse

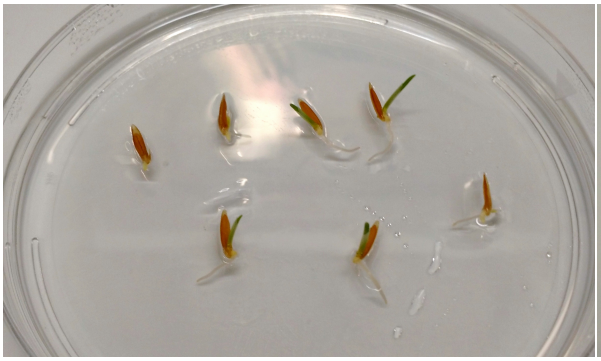

Figure 3-1. Brachypodium seeds have been germinated for two days and are ready for transferring to EcoFABs.

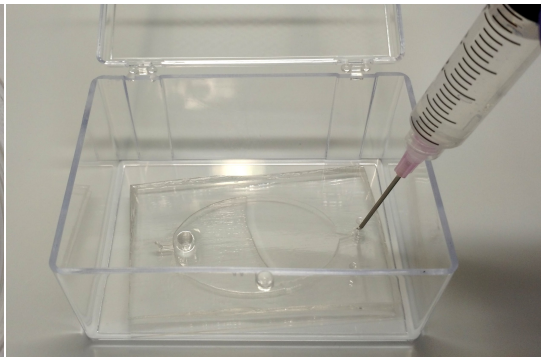

Figure 3-2. Filling the root chamber with growth media.

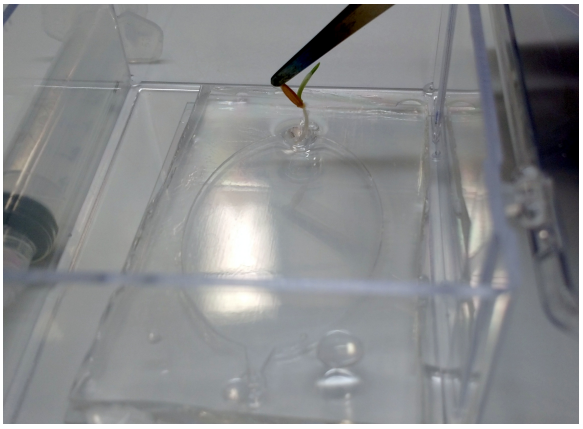

Figure 3-3. Using tweezers to carefully insert root end of seedling into the plant reservoir. Be careful not to damage the root or shoot.

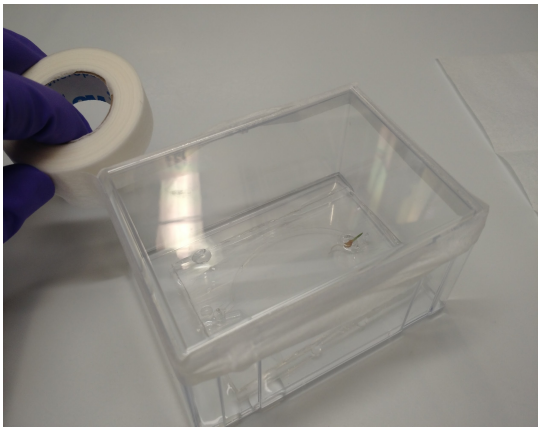

Figure 4-3. Sealing EcoFAB container with Micropore tape, after adding 2-5 mL of water to the bottom of the container (2mL for small EcoFABs, 5 mL for large EcoFABs).

1. Retrieve the Brachy plates from the growth chamber (Figure 3-1).
2. Flush the root chamber: rinse the root chamber three times with the growth media to use. Prepare 5 Ecofabs with 0.5MS+P, 0.5MS-P, and soil extract (1 blank, 4 with plants). The volume of the chamber is approximately 2 ml. Either use a sterile syringe (as shown in Figure 3-2), or a regular pipette with sterilized tips to pipet media through the sampling port. Fill as much medium as possible.
3. Transfer the seedlings: use sterilized tweezers, and grab the seedlings gently by the seed (as shown in Figure 3-3). Gently insert the root into the plant reservoir, such that most of the root is submerged in the growth medium. In total, 12 seedlings are transferred (4 each for 5MS+P, 0.5MS-P, and soil extract).
4. Add 5 ml of sterile water to the box around the Ecofab device, to keep humidity high.
5. Close the box, and wrap with Micropore tape. Transfer carefully to growth chamber.

Add medium to Ecofabs, *Oct 11:*
Day 7 of timecourse

1. Check sterility: plate 50 µl of growth medium in root chamber on an LB plate. Dry the LB plate, then wrap with parafilm, and incubate at room temperature. After 2-3 days, check for colonies.
2. add new growth medium: refill the root growth chamber with new medium. Due to evaporation and plant growth, some medium will have evaporated. Replenish the medium via the plant reservoir or the sampling port with a syringe or pipette, whichever is more convenient for you. Try to remove all air bubbles in the root chamber.
3. Close the Ecofabs with Micropore tape.
4. Image the Ecofabs: take a picture for each of the Ecofabs with a Gelimager (xxx), so that the root morphology is visible. Return the Ecofabs to the growth chamber.

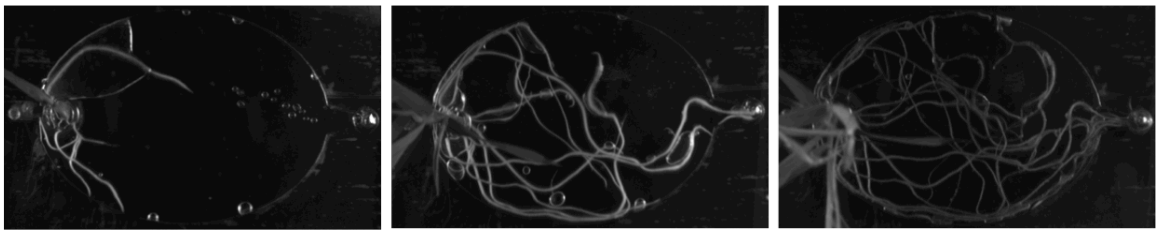

Figure 4: Timecourse for Brachypodium grown in Ecofabs (7 d, 14 d, 21 d old plants, from left to right). Pictures taken with gelimager. Make sure the entire root chamber is on the picture, so I have a reference for the root measurement. Also, save the pictures with a minimum of 300 dpi.

Check medium amount, *Oct 16:*
Day 12 of timecourse

1. Check medium amount: check how much medium is present in the root chambers, refill if necessary.

Add medium to Ecofabs, *Oct 18:*
Day 14 of timecourse

1. Check sterility: see above
2. add new growth medium: see above
3. Close the Ecofabs see above
4. Image the Ecofabs: see above

Add medium to Ecofabs, *Oct 23:*
Day 19 of timecourse

1. add new growth medium: see above
2. Close the Ecofabs see above

Sample medium Ecofabs, *Oct 24:*

Day 20 of timecourse

1. Check sterility: see above
2. Sample exudates: instead of just adding new medium as before, the root exudates will be collected at this time point: tilt the Ecofab such that the liquid in the root chamber can be pipetted out. Transfer as much liquid as possible into a 2 ml eppendorf tube, and write down the approximate volume of the sample. Store the samples at -80˚C.
3. add new growth medium: see above, will be a bit more difficult than before, because the root chamber was completely emptied. As before, refill with as much medium as possible, and try to avoid air bubbles.
4. Image the Ecofabs: see above

Sample Ecofabs, *Oct 25:*

Day 21 of timecourse, end of experiment

1. Sample exudates: same procedure as above. Try to empty the root chamber completely, transfer the exudates into 2 ml tubes and write down the amount sampled. Add a maximum of 1.8 ml exudate to a tube, else the ice will pop open the tube. Use a second tube for the remainder of the sample, if necessary.

From here on, there is no need to work sterile anymore. I suggest handling the Ecofabs one by one, following steps 2-4

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2. Image the root morphology on a glass plate: Carefully pull on the shoot to remove the root system from the root chamber. The root system fits through the plant reservoir, the key is to pull slowly. Arrange the roots on a glass plate with some water, and take a picture of the root morphology with the gelimager.

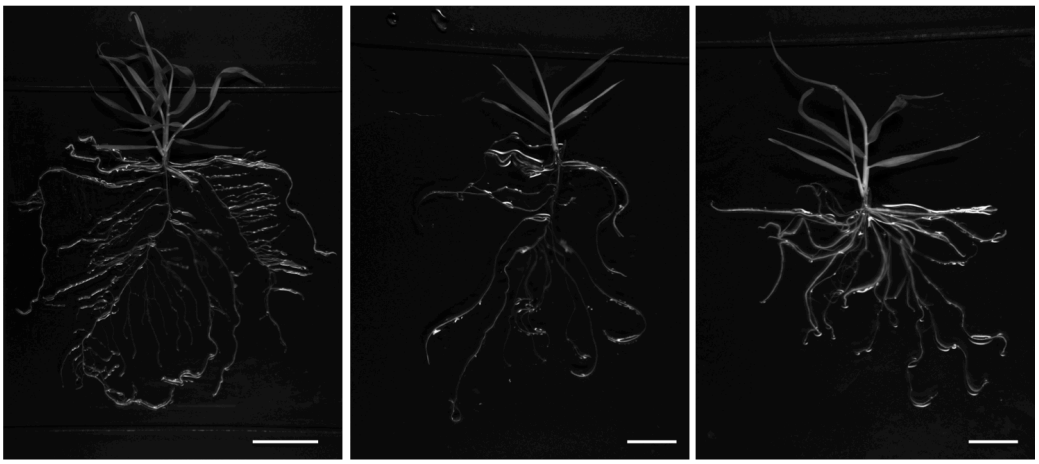

Figure 5: Brachy root morphologies on glass slide (MS+P, MS-P, soil extract). Scale: 3 cm. Pictures taken with gelimager.

3. Weight roots and shoots: after taking the picture of the root morphology, blot the roots dry on tissue paper (do not apply pressure, change tissue paper multiple times). Record weight of root and shoot, give mg amounts (e.g. 123 mg).

4. Freeze roots and shoots: freeze roots and shoots in tubes, store at -80˚C.

5. Phosphate quantification: grind roots and shoots in liquid nitrogen until they are a fine powder. Weight approximately 25 mg of tissue into a new tube for phosphate quantification (note exact weight). Keep remaining powder for metabolite analysis. For phosphate analysis, see Dangl lab protocol, next page.

6. Prepare material for Northen lab:

- weekly pictures of plants in Ecofab
- final picture of root morphology on glass slide
- root and shoot weight table
- phosphate content root and shoot

7. send to Northen lab on dry ice:

- frozen exudates
- aliquots of root and shoot tissue
- data logger (wrap in plastic so that it does not cool too much)

Northen lab downstream processing:

- root parameter quantification from pictures
- metabolomics on exudates, maybe on root extracts?
- if possible, redo colorimetric phosphate assay for the entire dataset

Extraction and Quantification of Cellular Inorganic orthoPhosphate (P_i)

1) Pre-weigh safe-lock tube where the tissue will be collected.
2) Harvest tissue and weigh the tube again to determine fresh weight.
   - Aim for 5-50 mg of tissue.
3) Add 800 µL of 1% glacial acetic acid (99% water) solution to the tube with sample
   - Ensure that the plant tissue is submerged.
   - Can store samples at –80°C before proceeding with quantification.
   - You can adjust the volume and modify the dilution factor in step (9) accordingly (e.g., adding 400 µL gives a dilution factor of 5).
4) Freeze-thaw the sample three times to break the cell wall. Ensure the tube is tightly capped to prevent bursting. Use liquid nitrogen and a water bath (we use 65°C) or dry oven.
5) Dispense 120 µL of a freshly prepared molybdate/ascorbic acid solution (see recipe below) into each well of a 96-well plate. (Optional: use a repeater pipet with a 1 mL tip.)
6) Add 80 µL of sample to a well and mix by pipetting up & down ~3 times.
   Include standards at known nmol amounts (see below) on the same 96-well plate.
7) Incubate 60 minutes at 37°C in a dry oven. The sample will acquire a blue color.
8) Measure the absorbance at 820 nm, using a plate reader.
9) Calculate the free P_i concentration in your sample based on a standard curve of OD_{820} vs. Pi amount (nmol), accounting for the dilution factor and tissue fresh weight.
   - (OD_{820} × 10) ÷ (slope × weight) = nmol per mg fresh weight

Molybdate/ascorbic acid solution (based on Ames, BN. *Methods Enzymol.* 1966)

Reagents:

(A) 10% (w/v) ascorbic acid. Store at +4°C for up to one month.
To prepare 10 mL of solution (final concentration is 0.568 M) using sodium salt:
1.13 g of (+)-sodium L-ascorbate
Add ultrapure water up to 10 mL
(Note: excess of sulfuric acid in solution B compensates for use of sodium salt.)

(B) Ammonium heptamolybdate – sulfuric acid solution. Store at room temperature.
To prepare 500 mL of solution (final conc. is 3.4 mM heptamolybdate, 1.1 N acid):
484 mL of ultrapure water
15.7 mL of concentrated sulfuric acid (~35 N)
2.1 g of ammonium molybdate tetrahydrate [$(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$]

Mix: 1 part of (A) to 6 parts of (B), and keep on ice for the experiment day.
For one 96-well plate, mix 2 mL of (A) with 12 mL of (B).

Orthophosphate standard curve solution

Prepare the following diluted K_2HPO_4 solutions, and follow steps (6) to (8) above.
Final concentrations (µM): 1000, 800, 600, 400, 200, 100, 80, 60, 40, 20, 10, 8, 6, 4, 2, 0.
80 µL gives this much (nmol): 80, 64, 48, 32, 16, 8, 6.4, 4.8, 3.2, 1.6, 0.8, …
The lower limit of quantification is approximately 2 µM, which is 0.16 nmol P_i in the well.