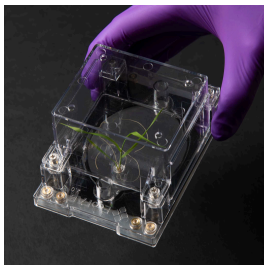
Sep 30, 2024

Use of EcoFAB 2.0 for reproducible plant-microbe interaction experiments



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We use this protocol and it's working

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Abstract

We conducted a multi-laboratory experiment across five labs to demonstrate reproducible microbiome formation, metabolite production, and plant growth using the EcoFAB 2.0 system. This protocol provided standardized instructions for the participating labs to implement the EcoFAB 2.0 system effectively, ensuring consistency in plant-microbiome experimentation. It includes annotated videos detailing each experimental step, utilizing a model bacterial community and the model grass *Brachypodium distachyon* in a hydroponic EcoFAB 2.0 setup.

Image Attribution

A Fabricated ecosystem (EcoFAB 2.0) is a standardized device for reproducible aseptic plant growth, sampling of the growth medium, and determination of plant-microbe interactions. It can accommodate small model plants such as Brachypodium distachyon and is compatible with automated systems found in most laboratories. Photo credit: Thor Swift/Berkeley Lab (CC BY-NC-ND 4.0).

Troubleshooting

Materials and equipment

1 **Materials sent by the organizing lab (LBNL)**

- Material needed for 40 EcoFAB (See EcoFAB 2.0 assembly protocol)
- Sterilization pouches
- Murashige & Skoog (MS) Basal Salts (Caisson Labs MSP01-1LT).
- Micropore tape (UV-treated)
- 15 mL centrifuge tubes, sterile
- 2 mL microcentrifuge tubes containing 2 stainless steel beads, sterile
- ~80 *Brachypodium distachyon* Bd21-3 seeds
- 17-member SynCom inoculation solution in 20% glycerol, stored at -80˚C
- 16-member SynCom inoculation solution in 20% glycerol, stored at -80˚C
- Mock solution (½ MS + 20% Glycerol)
- Sterile plates, Petri Dishes, 100×15 mm, BD Falcon (sleeve of 25)
- 3g Phytoagar (Bioworld**,** Plant Media, #40100072)
- 2x Box of 50 Acrodisc Syringe Filters with Supor Membrane, Sterile - 0.2 µm, 32 mm (Pall corporation, #4652)
- 25 kraft paper envelopes 2¼ x 3½ inch
- HOBO Pendant Temperature/Light 64K data logger (#UA-002-64)
- HOBO Pendant MX Temperature/Light Bluetooth Data Logger (#MX2202)
- 2 x Vacuum Filter System 1000 mL, 0.22 µm PES Membrane, Sterile (Corning, #431098) – note a vacuum line will be needed for the filtration

> Note
>
> SynCom is shipped separately on dry ice using the fastest available service (e.g., DHL Medical Express, Aeronet Worldwide). Upon receiving the shipped material, check it for shipping damage. MS medium pouches should be stored at 4°C, and SynCom and Mock solutions at -80°C until use.

Additional materials needed

- Bleach solution diluted to 6% w/v NaOCl (preferably, to avoid additives in bleach, we use sodium hypochlorite solution VWR, #BDH7038-4L)
- 70% Ethanol
- Sterile Milli-Q water
- Sterile 10 mL serological pipettes and matching pipettor
- Sterile 1 mL filter tips and matching 1 mL pipette
- Sterile 50 mL tubes
- Sterile 15 mL tubes
- Sterile 1.5 mL tubes
- Sterile needles gauge ≥18, length ≥25mm (e.g., DB PrecisionGlide needles #305175)
- Sterile 10 mL syringes
- Kimwipes

- 48x LB agar plates with no antibiotics
- Medium and fine-tip forceps
- Scissors
- Timer
- Ruler
- Glassware (e.g., 1L beaker and bottle) for making plant growth media and storing milli-Q water – make sure all glassware is well washed and baked at 400˚C for 60 min to remove any carbon contamination. Remember to remove tape/marker/pour rings before baking. Alternatively, rinse glassware 3x with Methanol (LC-MS grade, ≥99.9%) 10-20 % of container volume and let it fully dry.

Equipment to be used

- Laminar flow hood
- Biosafety cabinet (BSC)
- Flatbed scanner and Camera
- Freezer (-80˚C) and Lyophilizer
- Plant growth chamber that is set to 14h light at 26°C / 10h dark at 20°C and Photosynthetic photon flux density (PPFD) to 110-140 µmoles/s/m2 at the plant level. If you can control relative humidity, please set it to 70%. Confirm your growth chamber setting with the PPFD meter and light/temperature sensor. and insert the values following a link

Note

To ensure accurate growth conditions, we sent you two HOBO data loggers. Before the experiment, place the Bluetooth logger #MX2202 into the chamber for 7 days and send us the readout following the **guidelines**. Once we verify your chamber settings, place the other logger, #UA-002-64, into your chamber at the start of the experiment. The red light should be blinking at an interval of 4 sec. This device will monitor temperature and light and is pre-programmed to take a measurement every 10 minutes for a maximum of ~200 days. Please keep a log of the starting date of the experiment in your lab.

Experimental procedure

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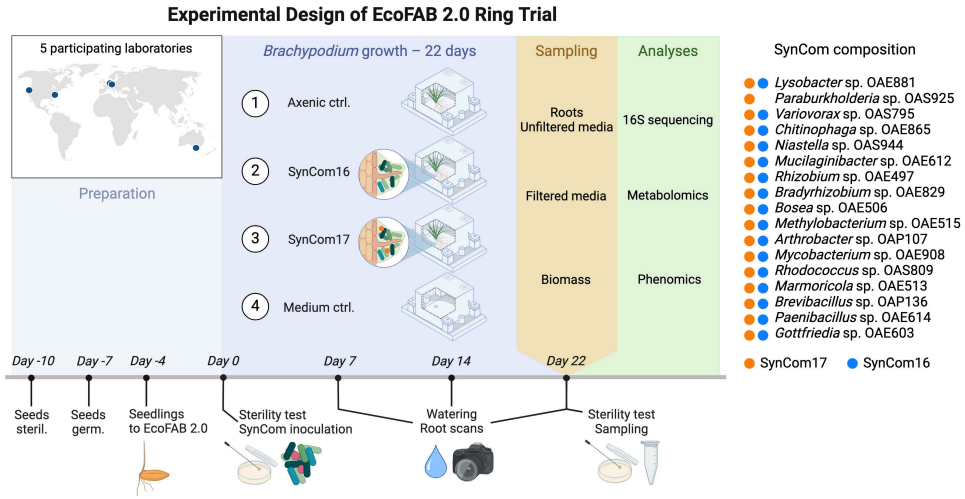


Figure 1: Timeline and experimental layout for the EcoFAB 2.0 ring trial. The experiment utilized a 17- or 16-member synthetic bacterial community (SynCom) (+/- *Burkholderia* sp. OAS925). Five laboratories on three continents conducted identical experiments to demonstrate reproducible root exudation and microbiome formation in EcoFAB 2.0.

Timeline (Figure 1)

- In advance: Prepare media and supplies, confirm growth chamber parameters
- Day -10: Sterilize *Brachypodium* seeds, and stratify at 4˚C in a fridge
- Day -7: Germination of *Brachypodium* seeds in the growth chamber
- Day -4: Setup EcoFABs, image seedlings, and transfer them to EcoFAB
- Day 0: Test EcoFAB sterility and inoculate SynCom into EcoFAB.
- Day 7: Add sterile Milli-Q water, scan roots
- Day 14: Add sterile Milli-Q water, scan roots
- Day 22: At the end of the experiment, test EcoFAB sterility, add sterile Milli-Q water, scan roots, sample all growth media, sample and weigh roots, sample and weigh shoots, and store materials.

Layout (Figure 1)

Brachypodium will be grown in EcoFABs (4 treatment groups x 7 replicates = 28):

1. Axenic plants mock-inoculated
2. Plants Inoculated with 16-member SynCom
3. Plants Inoculated with 17-member SynCom
4. Technical control: ½ MS medium in EcoFAB with no plants, mock-inoculated

Measurements:

- Images of roots and supply of evaporated water are done on days 7, 14, and 22 post-inoculation.
- Sterility tests of uninoculated EcoFABs are done on days 0 and 22.
- After 22 days, we will measure root and shoot biomass, collect unfiltered growth media and root

tissues for 16S sequencing, and sample unfiltered media for metabolomics.

Note

The bacterial isolates are part of the model synthetic community and are available via the DSMZ strain collection listed in the publication below.

Citation

Coker J, Zhalnina K, Marotz C, Thiruppathy D, Tjuanta M, D'Elia G, Hailu R, Mahosky T, Rowan M, Northen TR, Zengler K
(2022)
. A Reproducible and Tunable Synthetic Soil Microbial Community Provides New Insights into Microbial Ecology..

https://doi.org/10.1128/msystems.00951-22 LINK

3 **Prepare in Advance**

- **Assemble and sterilize the EcoFAB 2.0:** Follow the EcoFAB 2.0 assembly protocol guidelines and sterilize the devices in individual sterilization pouches (Figure 2, Video 1).

Citation

Peter Andeer, Vlastimil Novak
. Assembly and sterilization of EcoFAB 2.0 for plant growth experiments. protocols.io.

https://protocols.io/view/assembly-and-sterilization-of-ecofab-2-0-for-plant-c5gty3wn
LINK

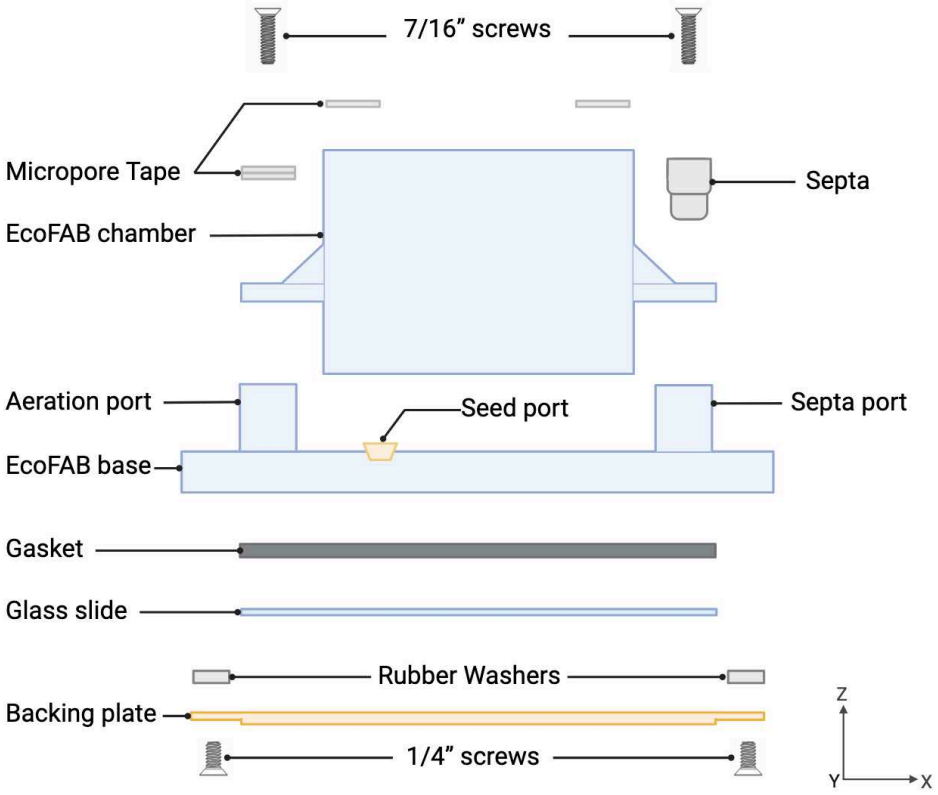


Figure 2: A side view of EcoFAB 2.0 typical assembly with indicated parts.

Video 1: EcoFAB 2.0 assembly

--Work in sterile conditions for the steps below--

- **Prepare ½ MS media:** Dissolve 2.165 g of MS powder (MSP01-1LT) in 1 L Mili-Q water and adjust pH to 5.7 with KOH. Sterile filter the media using the 0.2 µm PES membrane

vacuum filtration units. For easy handling, aliquot the media in sterile 50 mL tubes and store it at 4°C.

- **Prepare 4 plates for seed germination:** 1.5% w/v Phytoagar in ½ MS (1.5g/100mL) autoclaved and poured on plates in a sterile hood. Wrap and store plates at 4C°.
- **Make 48 LB agar plates for sterility tests:** Use 31 plates on Day 0 (28 for all EcoFABs + 3 for neg. control) and 17 plates on Day 22 (14 for control EcoFABs + 3 for neg. control).
- **Label your EcoFABs and tubes** according to the standardized codes X-EF-S, where "X" is the letter assigned to the specific laboratory (lab A-E), "EF" is the EcoFAB number (1-7 for Axenic treatments, 8-14 for SynCom16, 15-21 for SynCom17, and 22-28 for Medium Ctrl.), S is sample type ("Root" for root samples in 2mL tubes, "Med" for filtered medium samples in 15mL tubes, and "16S" for unfiltered medium samples in 1.5mL tubes).

4 **Day -10: *Brachypodium* seeds surface sterilization**

- **Dehusk dry seeds:** Select seeds that are uniform in size, shape, and color. Remove the lemma with either fingernails or tweezers (Figure 3). The embryo sits at the base of the seed (lemma side), taking care not to damage it. Prepare 40 seeds (germination rate is usually between 80-100% depending on the age of seeds) for the 21 plant-containing EcoFABs

--Work in sterile conditions for the steps below--

- **Sterilize seeds:** Put no more than 25 dehusked seeds into a 2 ml Eppendorf tube. Add 70% ethanol, shake for 30 seconds, and remove the supernatant. Add 1 ml of 6% w/v sodium hypochlorite, shake for 5 minutes, and remove the supernatant. Wash 5 times with 1 mL sterile milli-Q water.
- **Plate seeds:** Using sterilized forceps, place sterile seeds on 1.5% Phytoagar plates (10 seeds per plate) with the embryo (lemma side) facing toward the lid. Wrap the plate with micropore tape and place it in the dark at 4˚C

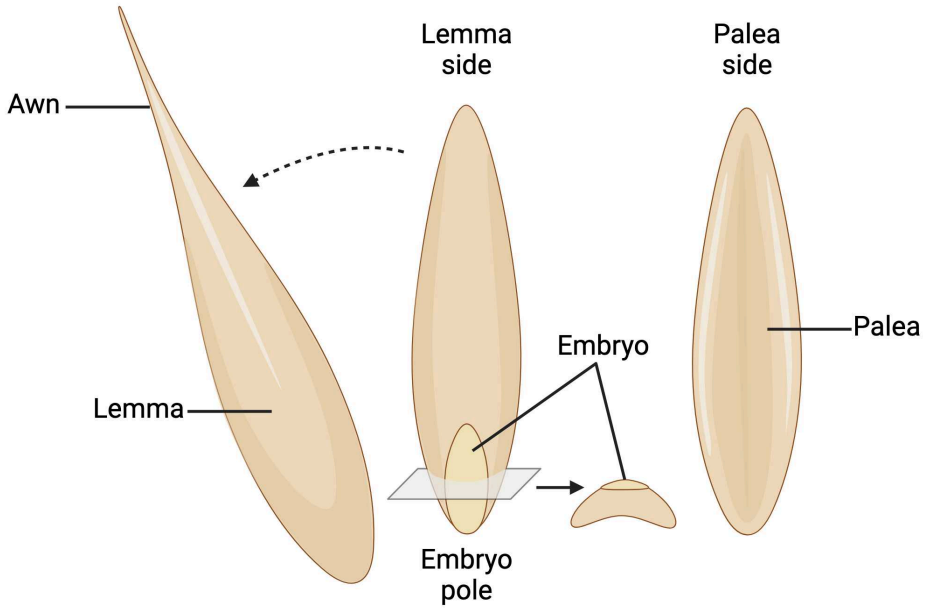


Figure 3: *Brachypodium distachyon* seed anatomy and de-husking. The lemma covers the convex side of the seed, while the palea covers the concave side. Before surface sterilization, the lemma is removed, revealing the lighter-colored embryo at the embryo pole.

Video 2: *Brachypodium distachyon* seed dehusking, surface sterilization, and plating.

Note

The seed sterilization procedure was adjusted from Haas and Raissig (2020).

Citation

Haas, A. S. and Raissig, M. T. (Invalid date)
. Seed Sterilization and Seedling Growth on Plates in the Model Grass Brachypodium distachyon.
Bio-protocol.

https://doi.org/10.21769/BioProtoc.3700 LINK

5 **Day -7: *Brachypodium* seeds germination**

- **Transfer plates** from the fridge, place them vertically under an angle into a growth chamber (Figure 4), and ensure the embryo poles point down and face the lid.

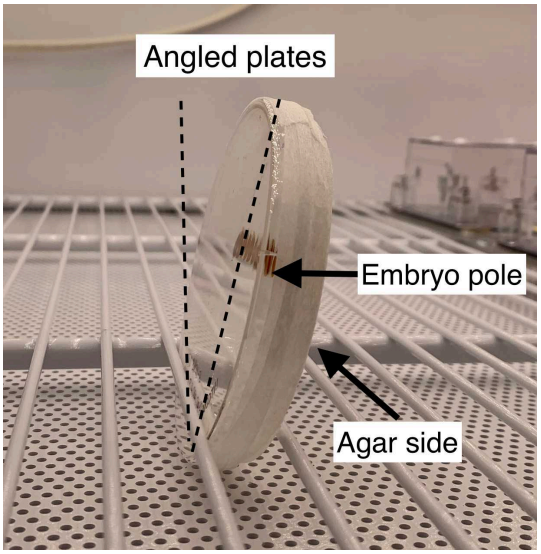


Figure 4: Position of plates with seeds during germination.

6 **Day -4: Transfer *Brachypodium* seedlings to EcoFAB 2.0**

- **Retrieve the germination plates** from the growth chamber
- **Image the plates with seedlings**: Take a picture of the plates with a camera (Figure 5)

--Work in sterile conditions for the steps below--

- **Prepare EcoFABs:** Tighten the screws at the back of the EcoFAB and tape the port 1 and air vents according to the assembly protocol. Slowly add 9 mL of ½ MS via port 2

into EcoFAB using a serological pipette. Be careful for the medium not to overflow from the seed port. Seal port 2 with septa.

- **Transfer the seedlings:** Select seedlings that are all uniform in size. Using sterilized forceps, gently grab the seedlings by the seed. Gently insert the root into the plant reservoir port so that most of the root is submerged in the growth medium and the root points towards the opposing sampling port (Figure 5). Take care not to damage the root. If the seedling is damaged, replace it with another. Save the seedling plates to check for contamination at the end of the experiment.
- **Close the EcoFAB 2.0** and place it with the used seedling plates in a growth chamber.

Figure 4*:* An example of *Brachypodium* seedlings vertically germinated for 3 days, ready for transfer to EcoFAB 2.0. Note that shoots and roots grow from embryos, which must face away from the agar to prevent the growth of shoots into the media.

Video 3: EcoFAB 2.0 setup and seedling transfer.

7 **Day 0: Add microbes to EcoFABs 2.0**

--Work in biosafety cabinet for the steps below--

- **Check sterility**: Temporarily remove the septa. Plate 50 µl of growth medium from each EcoFAB on an LB plate, wrap with a parafilm, and incubate at room temperature. After 7 and 22 days, check for colonies. Be sure to include control plates.
- **Add Microbes and new growth medium:** Thaw SynCom solutions on ice and dilute 100 µL in 9.9 mL sterile ½ MS (in 15 mL tubes). Mix well. Pipet 1 mL of diluted SynCom solution into EcoFAB (Figure 6). If necessary, add sterile Milli-Q water to the refill line (Figure 7). Re-seal the port slowly with the septa.
- **Add mock solution to plant and medium controls:** repeat the process above with the mock solutions.
- **Tilt to move bubbles:** Tilt the EcoFAB slightly to move air bubbles away from the seed port to the septa before returning to the growth chamber.

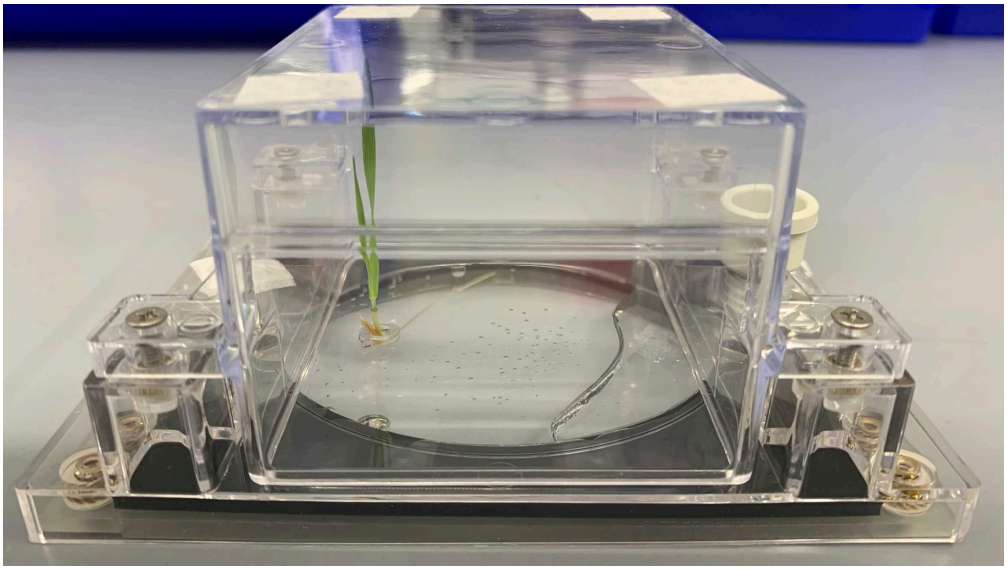


Figure 5: *Brachypodium* seedling before SynCom inoculation (Day 0). Notice that the medium covers the plant roots while the bubble sits opposite the seedling (septa side).

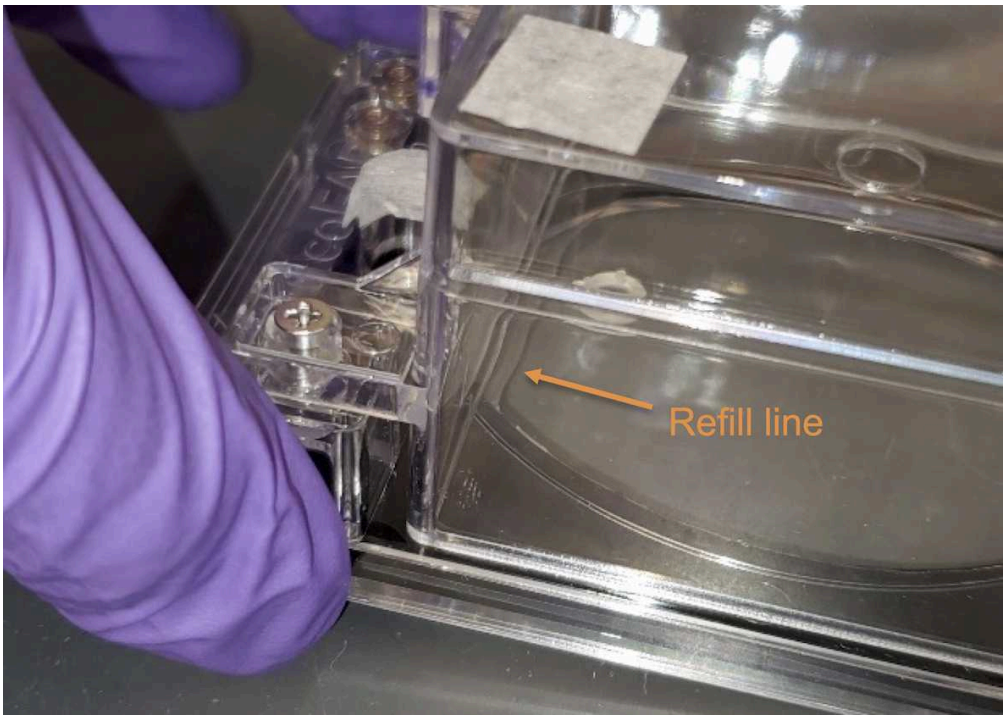
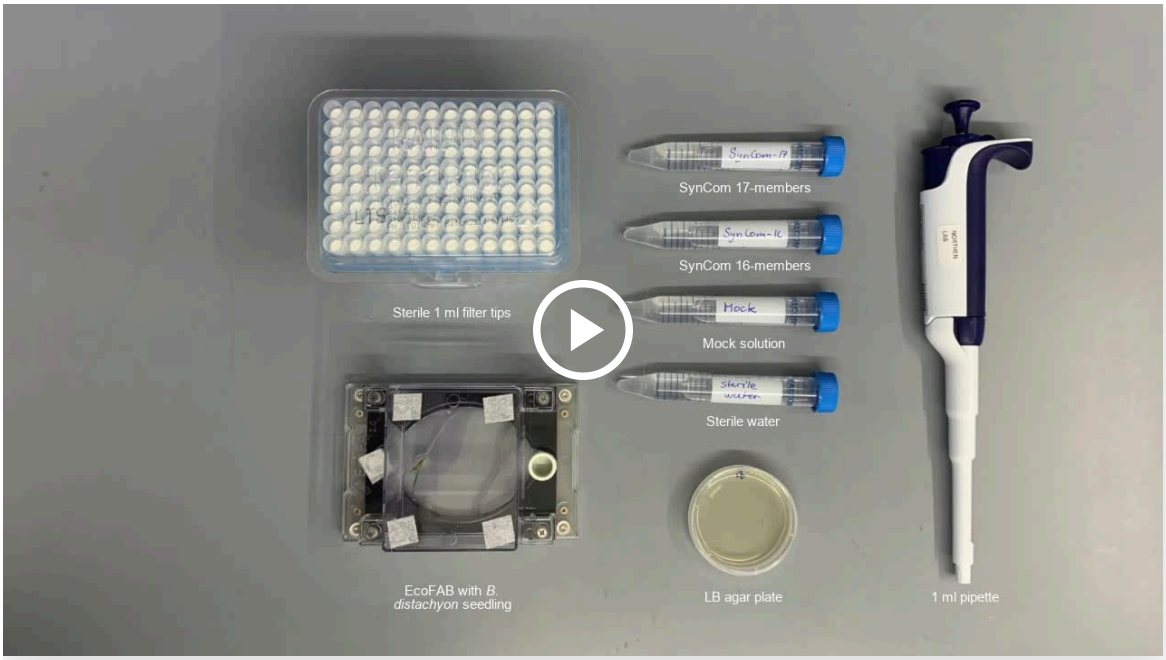


Figure 7: EcoFAB is refilled weekly with sterile Milli-Q water to the refill line so that the seed port is completely covered.

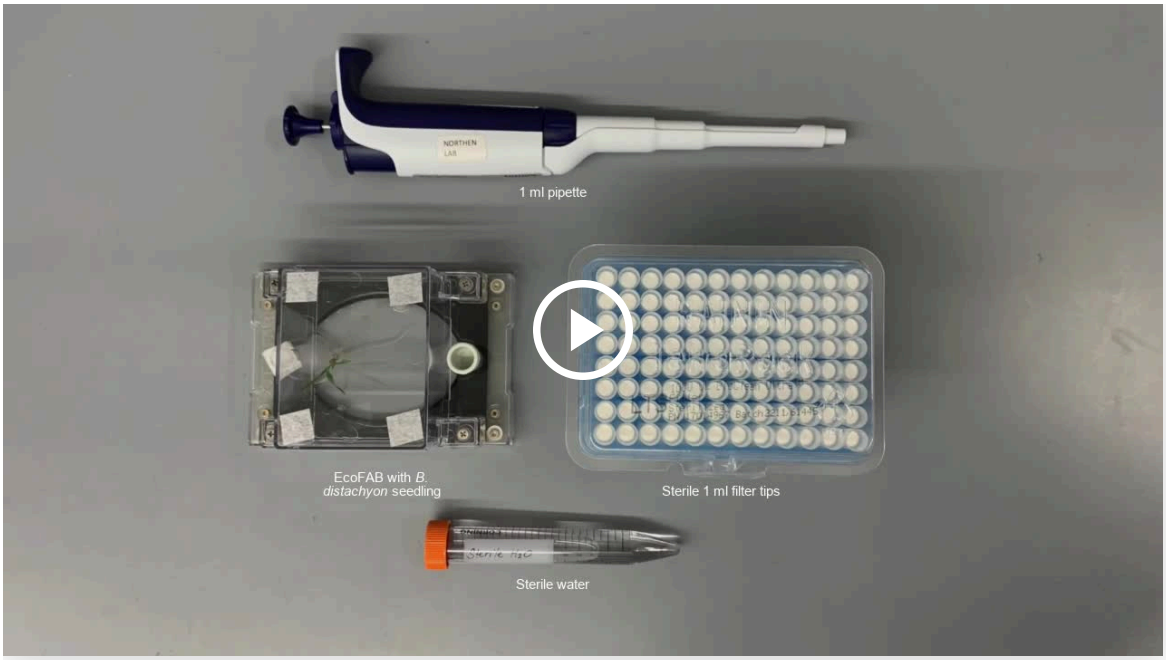


Video 4: Sterility test and SynCom inoculation into EcoFAB 2.0.

8 **Day 7: Image EcoFABs and refill growth media**

- **Fill with Milli-Q water:** In a laminar flow hood, remove the septa and fill EcoFAB with sterile Milli-Q water to the refill line (Figure 7). Reseal port 2 with the septa.
- **Image the EcoFABs**: Scan each EcoFAB to make the root morphology visible. Return the EcoFABs to the growth chamber.

- **Tilt to move bubbles:** Tilt the EcoFAB slightly to move air bubbles away from the seed port to the septa before returning to the growth chamber.
- **Rotate the EcoFAB position within the growth chamber** to minimize the effects of light intensity differences



Video 5: Water refill of EcoFAB 2.0.

9 **Day 14: Image EcoFABs and refill growth media**

- **Repeat steps of day 7:** Fill with Milli-Q water to the refill line (Figure 7), image the EcoFABs, tilt to move bubbles, rotate the EcoFAB position within the growth chamber

10 **Day 22: Sample EcoFABs — end of the experiment**

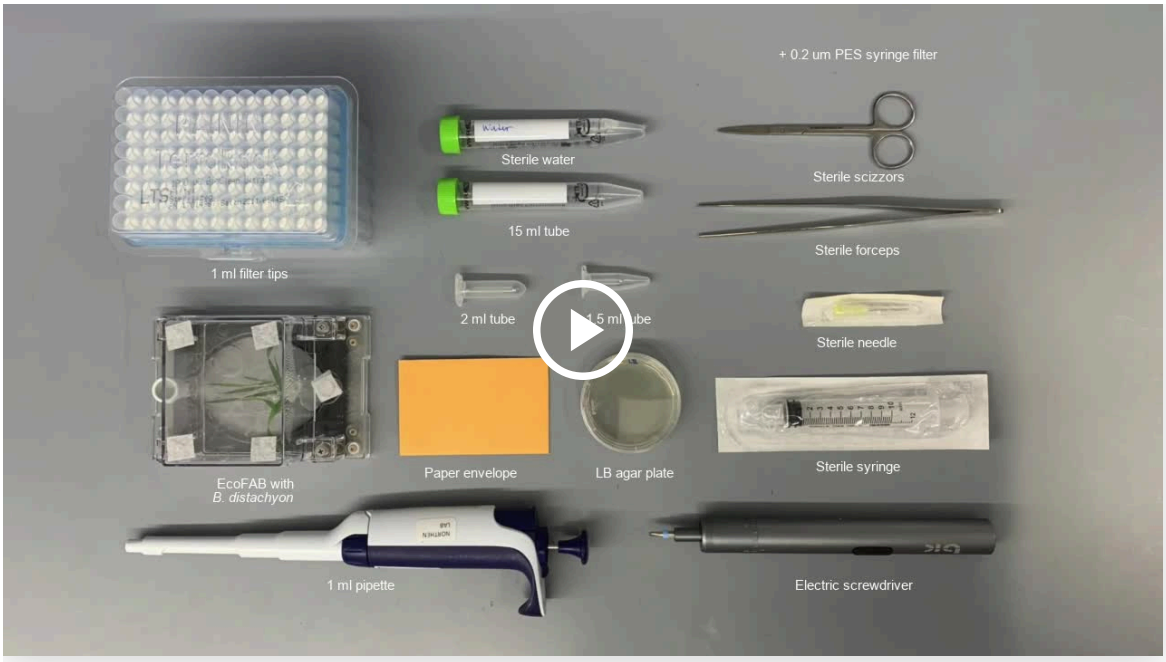
--Work in biosafety cabinet for the steps below--

- **Check sterility:** Temporarily remove the septa from port 2. Plate 50 µl of growth medium from axenic and technical control EcoFABs on an LB plate, wrap with a parafilm, and incubate at RT. After 7 and 22 days, check for colonies. Be sure to include control plates.
- **Sample unfiltered medium for 16S:** Pipette 200 µl of growth medium from each EcoFAB into a 1.5 ml tube and store at -80˚C.
- **Fill with Milli-Q water:** Fill EcoFAB with sterile Milli-Q water to the refill line (Fig. 5). Reseal port 2 with the septa.
- **Image the EcoFABs:** Scan each EcoFAB so that the root morphology is visible (scanning can be done outside of the BSC).
- **Sample growth medium for metabolomics:** Uncap the septa. Draw all EcoFAB growth medium with a sterile syringe attached to a needle. Remove the needle, attach a 0.2 µm filter to the syringe, and filter the spent medium into a 15 mL tube (if clotting occurs, replace the filter). Store the filtrate at -80°C.

- **Harvest shoots and roots** from the EcoFAB. Remove the top chamber and discard the seed residue. Gently pull plants slowly up through the seed port, taking care not to damage the plant. Cut the root from the shoot.
- **Sample roots for 16S.** Using sterile tweezers, place roots in pre-weighted 2 mL safe-lock tubes with steel beads, weigh the tube, and subsequently snap-freeze in liquid nitrogen. Sterilize your tolls between each sample to avoid cross-contamination.

---From here on, there is no need to work sterile anymore. ---

- **Collect shoots FW phenotypes.** Weigh shoots immediately before plants begin to dry. Take pictures of the shoot next to a ruler using a scanner (or a digital camera). Place shoots in individually labeled paper envelopes and freeze them before lyophilizing.
- **Weight shoot DW** out of the envelopes after lyophilization for 3 days.



Video 6: EcoFAB 2.0 sampling and harvest

Deliverables

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Note

Designating one institution (in this trial, Lawrence Berkeley National Lab, LBNL) as a central hub for processing samples and analyzing data will help minimize potential variability.

Generated data for the LBNL

- Weekly root scans in EcoFAB 2.0
- Plant biomass table
- Pictures of LB agar plates

- Pictures of plant shoot phenotypes

Data and pictures should be submitted to the cloud (in this trial, Google Drive) utilizing a common template spreadsheet and file directories to allow for fast sharing of large amounts of data, minimize mistakes, and facilitate downstream analysis.

Samples shipped to LBNL

- Frozen filtered exudates (15 ml tubes on dry ice)
- Frozen root tissue (2 ml tubes on dry ice)
- Frozen unfiltered media samples (1.5 ml tubes on dry ice)
- HOBO data loggers

Downstream processing by LBNL

- Root parameter quantification from pictures
- Metabolomics on exudates
- 16S sequencing on roots and medium

Citations

Step 2

Coker J, Zhalnina K, Marotz C, Thiruppathy D, Tjuanta M, D'Elia G, Hailu R, Mahosky T, Rowan M, Northen TR, Zengler K. A Reproducible and Tunable Synthetic Soil Microbial Community Provides New Insights into Microbial Ecology.
https://doi.org/10.1128/msystems.00951-22

Step 3

Peter Andeer, Vlastimil Novak. Assembly and sterilization of EcoFAB 2.0 for plant growth experiments
https://protocols.io/view/assembly-and-sterilization-of-ecofab-2-0-for-plant-c5gty3wn

Step 4

Haas, A. S. and Raissig, M. T.. Seed Sterilization and Seedling Growth on Plates in the Model Grass Brachypodium distachyon
https://doi.org/10.21769/BioProtoc.3700