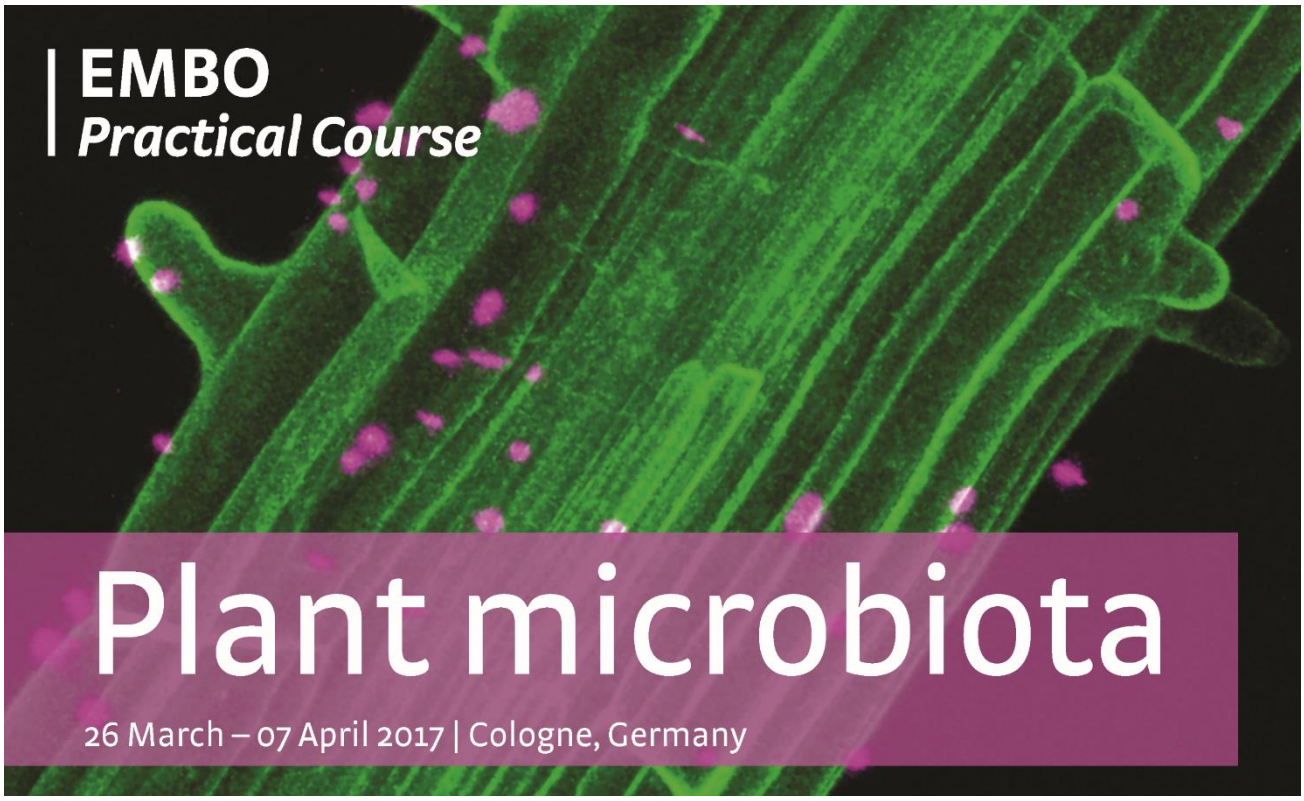

PROTOCOLS

(Week 2)

Fungal isolation from roots (normal plating method)

1. Clean roots (remove soil as much as possible)
2. Wash roots 3 times in sterile water or 10 mM $MgCl_2$ (5 min each). Rotate the 12 mL falcon tubes.
3. Wash roots in 80% EtOH (1 min). Rotate the 12 mL falcon tubes
4. Wash roots in 3% NaClO (Sodium hypochlorite) in water (1min). Rotate the 12 mL falcon tubes
5. Wash roots 3X in water or 10 mM $MgCl_2$ (5min). Rotate the 12 mL falcon tubes
6. Transfer roots in petri dishes and cut the roots in 0.5 cm pieces
7. Put pieces on different agar media without antibiotics (Mm-,PGA/PDA-,MEA- and CDA-)
8. After 2-3 days start transferring fungi to PDA plates supplemented with antibiotics

Preparations of bacterial culture collections (traditional and limiting dilution methods)

Materials (**limiting dilution method)**

- 10 mM MgSO4 (45 ml per sample)
- 10% TSB (Tryptic Soy Broth, 250ml per sample)
- Scalpel and forceps
- Metal beads ϕ = 1-3 mm (3 per sample)
- 50 ml tubes (11 per sample)
- Screw-cap 2ml tubes
- Tissuelyser/Precellys device
- 96-well plates
- Multichannel pipet (100-200 µl)
- Multichannel pipet reservoir
- **TSA plate (50%) (only for traditional plating method)**
- **Sterile glass beads 3 mm (only for traditional plating method)**

Protocol

1. Extract plants from the pot, remove excessive amounts of soil attached to the root
2. For following steps, use 4 cm-long root fragments collected 1 cm down from the rosette
3. Transfer root fragments to 50 ml tubes containing 15 ml 10 mM $MgSO_4$, wash on rotary shaker at 80 rpm for 10 to 20 min
4. Repeat washing 3 times in total, each time in new 50 ml tube containing 15 ml 10 mM $MgSO_4$
5. Transfer root fragments to 2 ml tubes containing 500 µl 10% TSB medium and 3 metal beads, homogenize on Precellys for 30 sec at 5000 rpm
6. Combine root slurry with 30 ml 10% TSB medium in 50 ml falcon tube, mix and wait 15-30 min for plant material to sediment
7. In 50 ml falcon tubes, using 10% TSB, prepare the intermediate dilutions of root homogenate: 1:10 to 1:10k, as well as plating dilutions: 1:20k, 1:40k, 1:60k. Avoid uptake of the plant material.

(traditional plating method)

8. Plate 50 µl of the diluted slurry on 500 petri dishes (TSA 20%) using sterile beads (6-10/plate).
9. Incubate at 18-20°C for 3-10 days
10. Transfer all colonies in 96-well plates supplemented with 150 µl TSB 20% to obtain > 3,500 colonies

11. Incubate for 10-14 days. Pipet 6 µl in a PCR plate (taxonomy) and add 100 µl glycerol 60% to the wells (stock -80°C)

(limiting dilution method)

8. Inoculate 3 96-well plates with each of the dilutions of root homogenate: 1:20k, 1:40k, 1:60k. Use 160 µl per well (15,4 ml per plate)

9. Seal the plates and incubate for 15-20 days at RT without shaking

Preparation calcined clay systems

Collect the required number of Magenta boxes (including lids) and autoclave (wet run). Dry in an oven after autoclaving.

Fill a large plastic beaker with calcined clay till 600 ml (corresponding to approximately 380 g clay) and fill up to 1200 ml with MilliQ water. Mix thoroughly with a large spatula.

Let it stand to settle the clay and discard supernatant.

Repeat this steps 4 more times (in total 5 times). The supernatant needs to be clear after the last wash.

Transfer the washed clay in a flat plastic tray (do not overfill to keep the thickness of the clay layer under 5 cm) and cover with aluminum foil.

Autoclave twice (wet cycle – intermittent time between runs 12 hours).

Dry the autoclaved clay in an oven (65-80°C) until the clay is fully dry (can take several days).

Fill the sterile Magenta boxes under the laminar flow with 100 g calcined clay and autoclave the filled boxes (dry run).

The whole preparation process takes 1-2 weeks (drying step is bottleneck).

Preparation FlowPots systems

Workflow FlowPots

MONDAY	TUESDAY Inoculate Fungi on PGA / PDA	WEDNESDAY	THURSDAY	FRIDAY
MONDAY	TUESDAY Inoculate bacteria Prepare soil and autoclave for the first time; oven for 24 hours	WEDNESDAY Rinse soil and autoclave for the second time Oven for 24 hours	THURSDAY Soil 24 hours on the bench	FRIDAY Sterilize seeds and store at 4°C Take soil sample Build flow pots Autoclaving 45min Let it stay on the bench over weekend
MONDAY Flowpots flush with H_2O Harvest fungi Homogenize Store in fridge	TUESDAY Harvest Fungi Take second soil sample **Inoculation**	WEDNESDAY	THURSDAY	FRIDAY

Material

- 50ml syringes cut in half – dispose in a plastic box covered with an aluminum foil
- Glass beads in a bottle
- Mesh cut in ca 8x8cm
- Cable binder (20cm) – close it and put it in a pipet tip box
- Tygon tubing – before: incubate tubes in 70% EtOH in a beaker overnight under the fume hood (let the EtOH evaporate) and then autoclave.
- Microbox (Combiness) with a tip rack inside and cover the box with aluminum foil
- Microbox lids (Combiness) wrapped in aluminum foil
- 600ml beaker (waste) covered with alu
- 1/2MS + MES (2,2gMS/l, 0,5gMES/l pH 5,7 with KOH) – Use MS without MES and with vitamins
- $MgCl_2$ (10 mM)

- MilliQ water
- 4 days before inoculation: sterilize seeds and stratify for 4 days

Autoclave all the material (dry cycle)

Autoclave $MgCl_2$ and MS solution (wet cycle)

Soil preparation

- Mix peat with vermiculite at the ratio of 2:1. For about 12 FlowPots you need 600 ml peat and 300 ml vermiculite.

- Transfer the soil mixture in a plastic tray, moisten with MilliQ water (but don't overflow) and cover with aluminum foil.

- Autoclave 25 min (wet cycle)

- Keep at room temperature for 24 hours.

- Moisten the soil again and autoclave a second time (25 min – wet run).

- Keep at room temperature for 24 hours.

Optional: Take a soil sample to test contamination by plating a soil wash on TSA plates.

Building FlowPots

Add a layer of glass beads (3 mm) into a cut syringe.

Fill the syringe with soil mixture using a sterile spoon till the border. Add sufficient soil by slightly pressing by do not compact.

Close the top with a mesh and cable binder.

Place the FlowPots inside a Combiness box (without lid) and place the box inside a sun bag. Close the bag with autoclave tape, but allow air exchange.

Autoclave for 45 min (wet run).

Keep the FlowPots in the bag for a few days after autoclaving (room temperature) (e.g. Friday till Monday/Tuesday).

Wrap the lids of the Combiness boxes in aluminum foil and autoclave dry.

Flushing of FlowPots with water

One day before inoculation flush the pots with 50 ml of autoclaved MilliQ water to remove the toxic components.

Adapt the luer lock of the syringe to the tygon tubing/connector, that is connected to the FlowPot. Fill the open syringe with the water and press it through the FlowPot (keep upside down and collect the drain in a beaker).

Depending on your time schedule, you inoculate immediately the FlowPots or close the system and keep the box overnight till inoculation (not longer).

Optional: Use 1 FlowPot and take a soil sample for plating on TSA plates (check for contamination).

Inoculation of gnotobiotic systems

Preparation of bacterial suspension

Optional: Use a 96 pin replicator to transfer a small aliquot of culture on a large square agar plate to assess the viability, purity and colony morphology of the individual SynCom members.

Optional: Spin microtiter plate with culture collection to pellet cells (4000 xg – 15 min). Remove supernatant and resuspend the pellet in 200 µl 10 mM $MgCl_2$.

Pipet all individual wells together (200 µl each) in a 50 ml falcon (or a selection of you want to test a specific SynCom). Spin falcon to pellet cells (4000 xg – 15 min). Remove the supernatant and resuspend in the same volume using 10 mM $MgCl_2$.

Take 2 cuvettes. Add to cuvette1 1 ml $MgCl_2$ and to cuvette 2 750 µl $MgCl_2$ and 250 µl suspension (pipet well to homogenous well). Measure OD600 of the suspension. OD600 is a measure of the numver of cells per ml: OD 1 corresponds to 2*10^8 cells/ml (this correlation is dependent on many factors!).

In a fresh 50 ml falcon, make 10 ml of a bacterial suspension at OD600 0,5 using the above measured OD600 by combining the right amounts of bacterial suspension and 10 mM $MgCl_2$.

From the initial bacterial suspension, aliquot 300 µl suspension in a lysis tube to analyse the input community structure.

Optional: Make a ten-fold dilution series till -7 of the final 50 ml falcon and plate 50 µl of the dilutions on round agar plates to estimate the bacterial concentration and diversity in the input.

Preparation of fungal isolates

Use two-week old cultures grown on PGA without antibiotics.

Harvest 50 mg of fungal mycelium into a pre-weighted 2 ml screw-capped tube with one big metal bead. Carefully remove the agar medium from the mycelium (using scalpel or pipet tip).

Add 1 ml $MgCl_2$ and grind the mycelium in the tissue disruptor (program 4 – 10 min).

Optional: the mycelium can be stored overnight at 4°C but this is not advised.

Optional: Transfer 10 µl of each grinded mycelium on a PGA plate to check the viability.

Assemble 900 µl of each fungal grinded mycelium together ending up with a 50 mg / ml mycelium suspension. Aliquot 300 µl in a lysis tube to analyse the input community structure.

Inoculation of Calcined clay system

We will inoculate 1 box with a bacterial SynCom, 1 box with a fungal SynCom and 1 box with a combined SynCom.

Take 2 fresh 50 ml falcons and add 35 ml ½ MS medium to each falcon. Add to each falcon the inoculum:

Box 1: 500 µl bacterial suspension + 100 µl $MgCl_2$
Box2: 100 µl grinded mycelium suspension – with a P1000 pipet + 500 µl $MgCl_2$
Box 3: 500 µl bacterial suspension + 100 µl grinded mycelium suspension – with a P1000 pipet

Mix by inverting and pour both falcons onto the clay.

Close the Magenta box and mix everything together by vigorous shaking. Get the calcined clay down by slamming the box on the desk.

The final concentration of bacteria and fungi in the clay is 2*10^6 CFU and 0,05 mg mycelium per gram clay respectively.

Optional: Also inoculate control boxes by mixing 600 µl 10 mM $MgCl_2$ to 35 ml ½ MS medium.

Inoculation of FlowPot system

We will inoculate 2 FlowPots with a combined SynCom.

Take 1 fresh 50 ml falcon and add 50 ml ½ MS medium. Add the inoculum to the falcon by pipetting 500 µl bacterial suspension and 100 µl grinded mycelium suspension (with a P1000 pipet) into the falcon. Mix by inverting.

Flush the FlowPots with the inoculum solution using a 50 ml syringe as described above for the water flusing.

Optional: Also inoculate control FlowPots by mixing 600 µl 10 mM $MgCl_2$ to 50 ml ½ MS medium.

Sowing of the gnotobiotic systems

Sterilize seeds using the method of your choice (depends on the plant species and seed-size/type) and stratify if necessary.

Transfer the seeds to the systems using a sterile tooth pick or by adding sterile water to the seeds and pipetting. In the Magenta boxes we sow 3 seeds per corner and in the FlowPots 10 seeds spread over the surface. After germination, we thin the seedlings to 4 and 3 per system respectively.

Close the systems. Seal the Magenta boxes with Micropore tape and incubate in a light cabinet for 6-8 weeks and 5-6 weeks respectively.