Module 1

M. truncatula as biological material

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The following protocols deal with diverse aspects of handling *M. truncatula* in the laboratory and greenhouse, for the propagation of genotype lines, as well as for various experimental purposes including studies on interactions with *Rhizobium*, *Glomus* or microbial pathogens. These protocols have been established for several Jemalong genotypes and the genotype R108-1. Some of these protocols have been tested on several other *M. truncatula* ecotypes and shown to work well with minor adjustments.

1. SEED GERMINATION

Legumes set seed within pods, the typical fruit structure in this wide plant taxon. In *M. truncatula*, ripe pods are compact spiky coils that need to be broken in order to release seed. There can be up to 12 seeds per pod (5-8 on average), depending on the growth conditions of the mother plant. *M. truncatula* seeds display embryonic dormancy during the 3-4 months following pod abscission from the mother plant. In addition, all seeds, whether dormant or not, are protected by a hard coat that needs to be at least partially ruptured to allow seed imbibition and germination. More specific treatments include seed vernalization (in order to reduce the time to flowering), and rescue of immature embryos.

2.1. Seed storage and extraction from pods

1. Store dry, intact pods at room temperature in strong paper envelopes or in screw-cap plastic vials with punched holes in the cap for air circulation. Seeds normally retain good viability for several years (3 at least) under such conditions.

2a. For small-scale extraction, gently crush individual pods using pliers and pull them lengthwise so that most of the seeds pop out.

2b. For large scale extractions, a commercial seed mill with rubber covered beaters works efficiently (for example Hege 16, Hege Maschinen, Germany ; ISV, Gif-sur-Yvette, France(Fig. 1). Alternatively, a corrugated rubber mat can be used. Crush the pods on the rubber mat using a plasterer's hawk with handle.

3a. To remove large pod fragments from the seeds use a combination of sieves. The remaining debris can be separated from the seeds by their differential sedimentation in a constant and adjustable air flow (Fig. 1).

3b. Alternatively, particularly in the case of small scale extraction, sort out seeds from pod debris by hand. Transfer the seeds to a large beaker and blow away the remaining particles with a hair-drier.

Specific material :

- steel pliers
- corrugated rubber mat (40 cm x 40 cm)
- plasterer's hawk with handle (any tool with a hard flat surface and a handle can be used), 18 cm x 27 cm
- set of sieves
- hair dryer

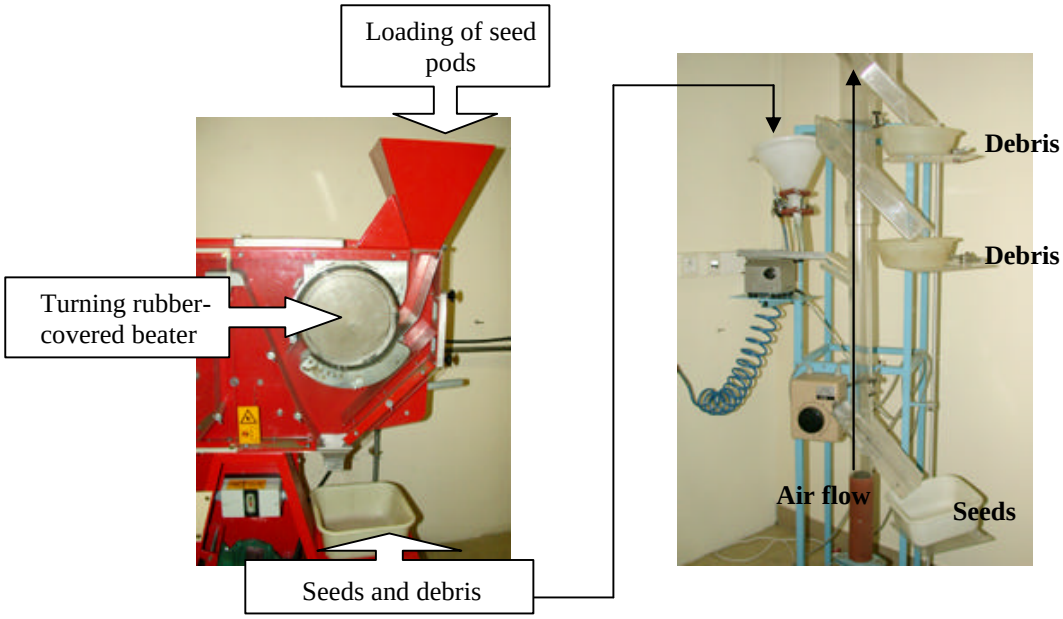

Figure 1. Commercial seed mill and separation of seeds from the debris.

1.2. Germination of non-dormant seeds

The seed coat, and especially its hydrophobic, waxy outer tegument, must be scarified in order to allow the penetration of water and O_2 that trigger germination. Scarification can be performed either mechanically by abrasion or chemically with sulphuric acid.

1a. *Mechanical scarification* : place seeds on a fine grade sand paper sheet and rub them gently with another piece of sand paper until there are visible signs of abrasion. Proceed to step 3.

1b. *Alternative chemical scarification* : soak seeds in a glass vial containing a few milliliters of concentrated, **anhydrous** sulfuric acid with intermittent agitation. Monitor the progress of the chemical scarification by observing the gradual appearance of small black spots on the tegument surface. When there are ~5 spots per seed on average (usually after 5-12 min), proceed to washing step 2.

2. Carefully remove all excess H_2SO_4 with a Pasteur pipette and quickly resuspend the seeds in a large amount of water (elimination of H_2SO_4 is **extremely important** in order to avoid local heating that may damage the seeds - the use of chilled water is beneficial for this step). Decant water. Rinse seeds a further 3 times with water.

3. *Seed sterilization* : it is recommended to use sterile glassware and tools under a laminar flow hood. Different sources of active chloride work efficiently :

sodium hypochlorite (38 g/l solution ; 12° CHL = 4 x dilution of standard commercial bleach) ;

calcium hypochlorite (8% w/v solution ; preferably from Aldrich) ;

commercial reagents : Pasti-chlor (Argos Ref. 613 ; dissolve 1 tablet in 400 ml water and add 1 drop of detergent, e.g. TIPOL). Bayrochlor (Bayrol, Ref. F 31822) can be used similarly as Pasti chlor.

For the Jemalong ecotype, add 5 ml of sodium hypochlorite 12° CHL to scarified seeds and leave them for 2 mins. Remove the bleach and extensively rinse the seed 5-6 times using sterile water.

For the R108 genotype, add any of these reagent to scarified seeds (except that sodium hypochlorite is used at 6° CHL) at least for 15 mins with gentle agitation.

4. *Seed germination* : Transfer the decanted seeds onto soft agar (see Appendix) in Petri plates. Spread uniformly up to 50 seeds per 10 cm diameter plate with the aid of sterile forceps and place them inverted in the dark at 14-20°C. Germination rates should be 80-90 % within 24-48 hours. Alternatively, when straight radicles are not required, place the seeds in water-containing Petri dishes at 20°C in the dark. In shallow water, the seeds germinate rapidly and the radicle growth is about 5-10 mm within 24 hours.

Remarks :

- The duration of the H_2SO_4 treatment is a critical parameter and has to be adjusted for each batch, depending on the seed origin and condition : usual time is 5-10 min, while some batches may need up to 20-30 min treatment. This is recommended for large seed samples.
- Sterilization time is critical (particularly for the Jemalong ecotype) since over-treatment with bleach results in root meristem damage and abnormal radicle growth. Germinating seeds are also very sensitive to traces of chlorine that may remain in the media or containers.
- In order to detect contamination (very rare !) sterilized seeds can be germinated on a rich nutrient substrate (e.g. C medium).
- For many routine applications, it is not mandatory to sterilize the seed. In this case, scarified seeds can be germinated on 0.8 % (w/v) water-agar or water–agarose, or even on non-sterile, water-saturated Whatman discs in Petri plates (in the latter system it is recommended to seal the plates with Parafilm to avoid radicle dessication) or in water.
- Seeds germinated in inverted Petri dishes have straight radicles.
- For optimal synchronization, seeds can be stored at 4°C for 1-2 days before transfer to a 20°C incubator. Storage of germinating seed at 4°C for up to 3 days has no noticeable effect on further growth (see below for vernalization effects).

***Specific material* :**

- sand paper
- concentrated, anhydrous H_2SO_4 (tightly close the stock bottle after each use)
- sterile water
- sterile C medium or water-agar 10 cm Petri plates (20 ml/plate)
- laminar flow hood
- 14-20°C incubator

1.3. Release of seed embryonic dormancy

Seed embryonic dormancy appears when the pod has just matured (~1 week after pod falling) and lasts for about 3-4 months. The germination potential of dormant batches is variable. They show delayed and asynchronous germination, sometimes as low as 10-20 % after 5 days.

We have identified two alternative treatments that **fully release embryonic dormancy** and result in **rapid and synchronized germination** :

Cold pre-treatment of imbibed seeds

Maintain inverted Petri plates with scarified and sterilized seed (see above) at 4-6°C in the dark for at least 7 days, before transferring the inverted plates to a 20°C incubator for germination. Most of the seeds should germinate in the first 24 h.

Remarks :

- Emerging radicles may already be visible after this 7-day cold treatment.
- Note that it is important to measure the precise temperature on refrigerator and cold-room shelves, since this can vary as a function of location.
- Although this one-week treatment at 4°C leads to a limited vernalization of seeds (see below), it does not significantly alter plantlet growth and symbiotic behavior, for example during nodulation.

Cytokinin treatment

1. Following scarification and sterilization, incubate seeds in 10 ml of 1 µM benzylaminopurine (BAP) solution for 3 h (time for full seed imbibition).
2. Rinse the seeds once for 10 min in deionized water. Transfer the seeds to Petri plates as above. Seedlings generated in this way display normal growth and nodulation.

Seedling vernalization

In *M. truncatula*, non-vernalised seedlings develop into vigorous plants that abundantly ramify and sprawl before the onset of flowering ~6 weeks after germination in optimal growth conditions (see below). However, for several specific applications (crossings, high throughput generation cycling...), it is possible to obtain plants that both show reduced vegetative growth and start flowering earlier in their development. This is achieved by so-called seed vernalization, i.e. leaving imbibed seeds on Petri plates at 4°C for a lengthy period of time (≥1 week).

Remarks :

- One-week and 2-week vernalization reduce the time to flowering down to 35 and 25 days, respectively (time for anthesis of the first flower).
- Vernalization in *M. truncatula* also results in dramatic changes in plant development which increase with the duration of the cold treatment : after 2-weeks vernalization, growth is much less vigorous with thinner stems and smaller leaf area. Shoot ramification is also strongly reduced, and the first flowers appear on the 5^{th}–6^{th} node (the 1^{st} node is defined as that bearing the first unifoliate leaf). Normally, the first flowers appear on the 10^{th}–12^{th} node in non-vernalized plants.
- For practical purposes, note that the overall time to flowering including the 4°C vernalization treatment remains around 6 weeks.
- With even longer vernalization periods (> 2 weeks), it is possible to further reduce the time to flowering down to ~20 days, but overall plant growth is poor.

1.5. Germination of immature embryos

Germinating immature embryos can help to save time in certain experiments, for example when the F1 and F2 progenies resulting from crosses are required as rapidly as possible. Alternatively, it may be essential to rescue maturing embryos if the continued survival of the mother plant becomes uncertain. It is possible to efficiently germinate immature embryos without nutrient supply 21-25 days after pollination onwards (pods fall 30-35 days after pollination under standard growth conditions). Rescue of embryos at earlier developmental stages requires specific culture media and conditions.

1. Remove a pod from the mother plant at the appropriate stage (=25 days after pollination) : the pod color should have already turned yellow-green or whitish, and the radicle of the embryo should have lost its chlorophyll and become white.

2. Open the pod carefully by hand, as the immature seeds are still soft and fragile.

3. Dissect the seeds with a scalpel and fine forceps under a stereomicroscope by removing as much as possible of the waxy outer layer of the coat and the underlying white membrane (endosperm) that surrounds the embryo.

4. Transfer the embryos into a shallow layer of sterile water (beaker) at 20°C. A high germination rate should be observed within 24-48 h (immature embryos are not dormant).

Specific material :
- scalpel (Feather blade #11) and fine forceps
- stereomicroscope
- 20°C incubator

2. GROWING *M. truncatula*

M. truncatula can be successfully grown using a number of substrates (moist paper, agar, nutrient mist, soil / inert substrates) and cultivation systems (pouches, tubes, plates, aeroponic chambers, growth chambers, greenhouse...). Certain systems are appropriate for short-term biological experiments and can be performed on seedlings or plantlets in small-size growing units (pouches, agar tubes and plates...). Other systems are devised for physiological experiments that require plants in optimal growth conditions (aeroponics, growth chambers) or for seed multiplication / manual crossings that require flowering plants that can complete their life cycle and set seed in abundance (solid substrates, growth chambers or greenhouse). The following protocols describe the most frequently used growth systems.

2.1. Settings of growth rooms/greenhouse

(*temperature, day - night/hygrometry/light intensity/light-dark photoperiod)*
- **in vitro growth room :** 25°C/-/80-100 µE.m^{-2}.s^{-1}/16 h–8 h.
- **growth chamber for aeroponics :** 22°C/75 %/200 µE.m^{-2}.s^{-1}/16 h–8 h.
- **growth chambers at ISV :** 22°C/70%/300 µE.m^{-2}.s^{-1}/16 h–8 h.
- **growth chamber for seed multiplication :** 25–21°C/-/120–250 µE.m^{-2}.s^{-1}/16 h–8h.
- **greenhouse :** 22°C/60%/115-150µE.m^{-2}.s^{-1}/16 h-8 h

2.2. Growth pouches

Growth pouches are designed for studies on seedling root biology, allowing both vigorous growth and easy observations on root systems. They comprise a flat paper wick enclosed in a thin transparent plastic envelope open at the top. The upper part of the wick is folded in such a way to hold the germinating seeds and allow the roots to grow down through holes in the fold and along the wick surface (pouch maintained in upright position). Root growth can be easily monitored through the transparent pouch. *M. truncatula* seedlings do particularly well in this system provided the following parameters are respected.

Preparation of the pouches

1. Slightly pull out the paper wick to 1-2 mm below the top edge of the pouch.
2. Add 7 ml of Fahräeus liquid medium with a pipette to the paper wick and allow the liquid to be absorbed.
3. Autoclave pouches in packs of ≤ 10 wrapped in aluminium foil and maintained in a vertical position under pressure between two flat surfaces to avoid deformation of the pouches. Autoclave separately aluminium covers (see specific materials) that are used to shield roots against light.

Culture of seedlings in pouches

1. Place pouches in a vertical stand under a sterile hood (avoid strong air flow in hood).
2. Open the wick with the help of sterile forceps. One by one transfer germinated seeds from a Petri dish to the pouch fold using a second pair of forceps, and gently insert the radicles into pre-made slits in the paper.
3. When all seedlings have been added, wrap the lower part of each pouch (or set of pouches) in an aluminium cover. Place groups of pouches vertically inside a transparent plastic box, push up against the back wall of the box, and apply gentle pressure to the pouches with a glass plate. Close the lid and position the box in a tilted position (~30° from vertical position) in an *in vitro* growth chamber (see below for details).
4. Young shoots should emerge from the pouch within 3 days. Cotyledons which are caught in the fold can be released with forceps at this stage.
5. Add sterile water to pouches as required (usually not before 5-7 days of culture).

Remarks :

- Pouches can take up to 12 germinated seeds. However, 4-6 plantlets/pouch is more reasonable for experiments lasting up to 2 weeks.
- The optimal stage for planting germinated seeds in pouches is when radicles are 5-10 mm long. Longer roots are more fragile and risk dessication during manipulation.
- It is also critical for success that radicles remain in close contact with the moist paper at the beginning of growth (first 24-48 h). Tilting of the box and pressure of the glass plate are crucial for this.
- The amount of liquid present in the pouch is critical (6-7 ml is optimal), as *M. truncatula* roots are very sensitive to excess water (plagiotropic and/or restricted growth). For this reason be

careful when replacing evaporated water or when adding liquid inoculum. Pouch dessication can be slowed down by the presence of imbibed paper towels inside the box.

- Young primary roots of *M. truncatula* grow rapidly in this system : 20-35 mm/day. Ramification initiates around day 4-5, usually when the primary apex has reached the bottom of the pouch. After 1 week root growth becomes spatially constrained; however, root systems keep growing and ramifying for a further 1-2 weeks without further nutrient supply. When necessary, up to 1 mM NH_4NO_3 can be added as "starter nitrogen", without noticeable effect on nodulation by *S. meliloti*.
- Sterilization of pouches can be omitted in many routine applications that require only short growth periods (3-10 days). A major advantage of avoiding autoclaving is that the plastic remains perfectly flat and clear.

Specific material

- "CYG seed growth pouches" (16.5 cm x 17.5 cm) with vertical stand can be purchased from Mega International (Minneapolis, Minnesota, USA, fax : 1 612 924 0701, email : tatox@aol.com)
- aluminium covers are prepared by folding lengthwise a piece of aluminium foil (30 cm x 45 cm), so that the mask protects most of the pouch from light. Masks can be recycled many times
- transparent plastic boxes (24 x 36 x 14 cm^3)
- Fahräeus medium (see Appendix)

2.3. Agar tubes/plates

M. truncatula seedlings can be grown on agar plates that are recommended for spot inoculation or local treatment of the roots with different reagents under sterile conditions. The growth is, however, strongly affected by the quality of agar. For example, never use Difco Bacto Agar for *M. truncatula* since the roots grow away from the agar and plant development is rapidly halted. Kalys agar (HP 696-7470, Kalys, 39 Avenue Jean Lebas, 59100 Roubaix, France) works well for *M. truncatula.* The Kalys agar is used at 0.7 % concentration for solid plates in various nutrient media or water, depending on the purpose of experiments. Usually 10-20 seedlings arranged in a row can be grown on square agar plates of 12 cm x 12 cm. Addition of the ethylene synthesis inhibitor AVG can be beneficial to plant growth (see Smith and Long, 1998).

Seedlings can also be grown on Fahräeus agar slants (20 ml) in glass tubes (60 ml, 2 cm diameter; 1-2 seedlings/tube) closed with air-permeable plugs.

2.4. Aeroponic caissons

Hydroponic culture appears not to be very successful for *M. truncatula*, since the roots of this species do not grow well in the presence of excessive moisture (probably due to oxygen shortage). In contrast, *M. truncatula* grows well in aeroponic conditions. The aeroponic caisson system is suitable for any growth stage, from seedlings to large plants, and yields vigorous plants in excellent physiological condition.

The aeroponic caisson consists in a large plastic chamber with a perforated lid on top and a defensor/humidifier which sits on the bottom and sprays the nutrient solution upwards as a fine mist. Plantlets are placed in holes through the top lid and roots grow in a permanent and homogeneous nutrient mist. Two variant systems of aeroponic caissons are used in our two laboratories. They can be easily made in any workshop. The model used in the LBMRPM INRA/CNRS in Toulouse has been engineered from large wheeled waste bins. The removable lid can be perforated in a variety of hole densities. A 40 x 40 cm^2 area with ~1 500 holes (4 mm diameter) works well for seedlings. Technical details can be obtained from the LBMRPM upon request. The model used at ISV is shown in Fig. 2. For long term experiments 40-80 plants can be grown on a surface area of about 1600 cm^2.

Setting up the aeroponic system

1. Although the aeroponic system is generally not axenic, it is essential to sterilize the inside of the growth container prior to each experiment.
   For the LBMRPM system, the inside of the growth container and the lid are sterilized with diluted bleach for several hours. Extensively rinse after bleach treatment and allow aeration for 24 h to eliminate traces of chlorine. The liquid is removed with a vacuum cleaner with a long hose attachment.

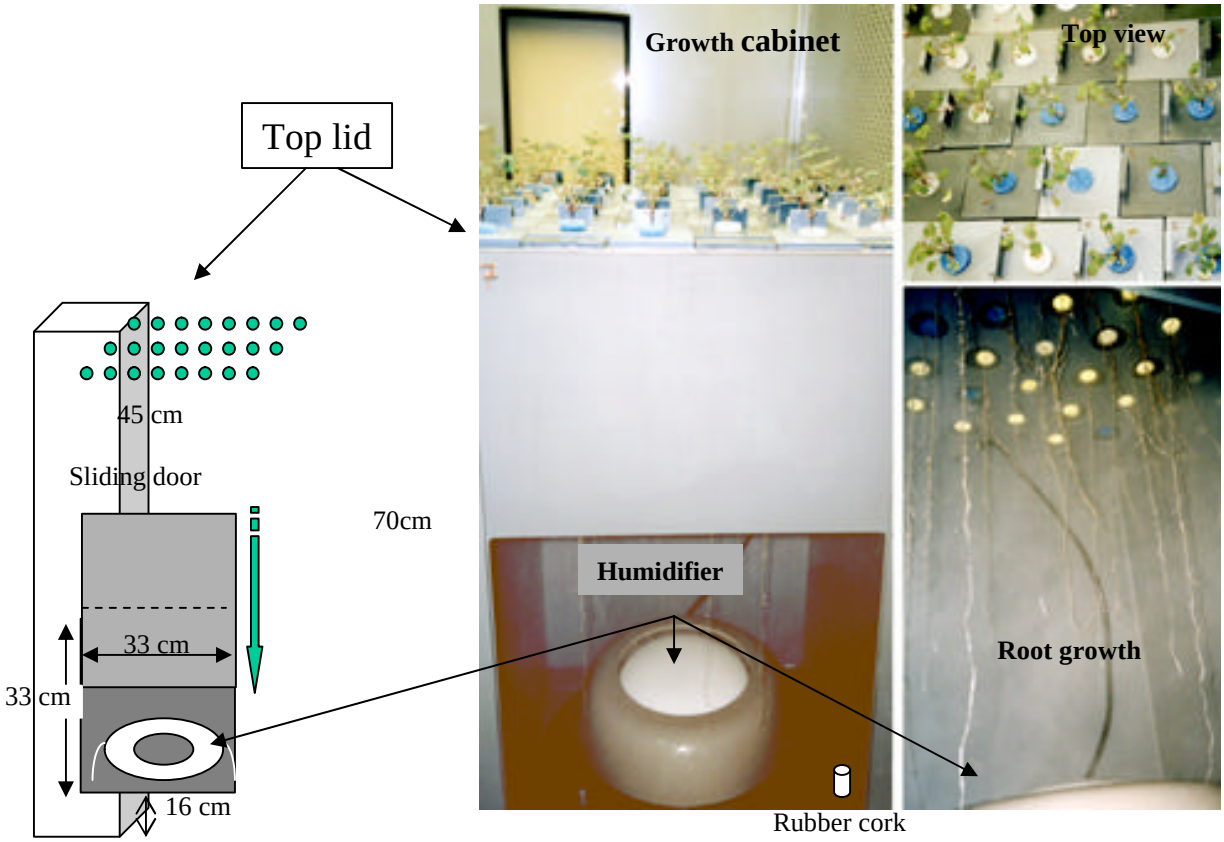

Figure 2. Aeroponic caisson used at ISV.

For the ISV system, plastic parts of the humidifier and the growth chamber are immersed in bleach for 1 day and rinsed thoroughly with de-ionised water. After drying they are stored in sealed large plastic bags. Aeration allows to eliminate traces of chlorine. The electronic part of the humidifier is placed in plastic bag and sterilised by spraying with surface bactericide (e.g. U2) and kept sealed until use. Several hours prior to an experiment, the aeroponic system is installed in a growth chamber and sterilised by evaporation of 70 % ethanol. That is followed by washes with sterile water and running the system for 1-2 hours with sterile water or nutrient medium. Evacuation and changes of liquids on the ISV model is facilitated by removal of the rubber cork plugging the bottom hole of the aeroponic growth factories.

2. Prepare the lid as follows :

2a. LBMRPM system : cover all the perforated area with strong black tape, overlapping the strips to obtain a waterproof seal. Puncture the tape above holes in the lid with a scalpel blade (2 perpendicular incisions).

2b. ISV system : cut discs from a 1cm thick synthetic sponge/foam that fit into the lid holes (2cm diameter is used most frequently) and then cut them along the radius.

3. Add 8-10 liters of nutrient medium and start the humidifier. Close the chamber.

4. In the case of 2a : the germinated seeds are planted with the help of forceps through the tape and lid holes so that the cotyledons rest gently on the tape, and the radicle below is exposed to the mist in the chamber.

In the case of 2b : seedlings at the hypocotyl-root junction are surrounded with the disc and placed into the holes.

Transfer the caisson to the growth room.

Remarks :

- If evaporation of nutrient medium causes the formation of white salt deposition on the hypocotyls in the LBMRPM system, wash each hypocotyl with sterile water. Otherwise plantlets will die. This can be done preventively every other day.
- The level of the nutrient solution is readjusted by adding water or nutrient solution. Ideally the nutrient medium should be renewed once a week to prevent problems due to contamination.

Specific materials

- Aeroponic caisson
- Humidifier (Defensor France)
- Nutritive solutions

2.5. Culture in pots in growth chambers

Substrates

M. truncatula grows best on well drained substrates such as an autoclaved mixture of sand and all-purpose compost (1:3, v/v). Satisfactory growth can also be obtained on 100 % sand, perlite, or vermiculite. Seedlings are planted in small pots (400 ml ; for example 8 x 8 x 7 cm^3). Non-vernalized plants need to be then transplanted into larger pots (~2 l).

Watering and fertilization

Take care not to overwater the pots, otherwise the root will rot (due to oxygen shortage). Let the soil partially dry out before watering again. Moreover *M. truncatula* is quite resistant to water shortage. Progressive, non-reversible leaf wilting is the most obvious over-watering symptom but by this stage it is usually too late !

De-ionized water is recommended since the accumulation of salts and chlorine from tap water can cause wilting symptoms and death of plants, especially on slow-growing plants.

Fertilizer can be added 1x/week to rapidly growing plants, such as NPK (6:3:6) supplemented with micronutrients (see Appendix). High-phosphate fertilizers appear to be somewhat detrimental in our hands (LBMRPM). At ISV , the plants are watered by automatic irrigation as it is described for the greenhouse (see below).

Specific applications

- Parent plants for crosses (see "Crossings")
- Seed multiplication (see below)

2.6. Culture in greenhouse

M. truncatula germinated seeds/plants can be cultivated on universal soil, vermiculite, perlite or sand depending on the experiments. Plants grow most rapidly on soil, and also very well on perlite. Perlite-grown plants are also ideal for nodulation assays. Plantlets obtained by *in vitro* culture are usually first planted in water-soaked sand or vermiculite in plastic boxes (45 cm x 30 cm x 7 cm) and later individually transferred to clay pots containing soil or vermiculite and watered with Nutrient solution (see Appendix). Irrigation can be performed automatically (3 days/week, 1 minute irrigation twice a day). For nodulation assays nitrogen-limited Solution I medium is used (see Appendix).

Materials

Vermiculite

This originates from laminar mineral. Treatment leading to expansion produces foliated inert, neutral (pH 7.0), hydrophil and isolating particles. (http://www.puteaux-sa.fr, Ref. 885191)

Perlite

This is made of volcanic rocks by expansion. The white granules with hard surface are inert, neutral (pH 6.8), aerated, hydrophilic and insulating. (http://www.puteaux-sa.fr, Ref. 885161).

Soil

Universal soil (peaty-moss) P13, pH 5.7 is used for *M. truncatula* (e.g. product of STERCKX, Belgium).

2.7. Diseases/Pests

Root rot will occur if there is overwatering (see above). Molds appear on leaves when the hygrometry is too high. Growth rooms with high light intensities and efficient air cooling have dry atmospheres during the day part of the cycle (~30 % relative humidity) that normally prevent the common fungal diseases (oidium, etc...). In the growth rooms and greenhouses, thrips and red

spider mites are the most common pests feeding on *M. truncatula* and can both cause severe symptoms. When required, treat plants once a week with insecticides and/or fungicides

2.8. Seed multiplication

In growth chambers (see above), *M. truncatula* Jemalong has a generation time of approximately 3 months. This is somewhat shorter for R108.

- Non-vernalized plants appreciate sufficient space to spread (up to 1 m^2/plant), and under optimal conditions can yield several thousands seeds/plant within 4 months. Pots are placed in large white trays to recover the ripe pods and prevent mixing of genotypes.
- When only moderate amounts of seed/plant are sufficient, it is more convenient to vernalize the mother plants (see above) so as to reduce plant size at the flowering stage. Plants vernalized for 2 weeks can yield ≥ 500 seeds. It is possible to grow them in high densities (50-100 plants/m2) owing to limited ramification by using a customized system similar to the Aracon devised for *Arabidopsis* multiplication : transparent plastic mineral water bottles (one on top of the other with the narrow neck removed) are progressively placed over the elongating primary stem. A plastic cup with a central hole is placed over the pot (8 x 8 x 7 cm^3) at the seedling stage and will collect the detached, mature pods before final harvest.

In the greenhouse, plants grown in soil/vermiculite in larger pots display vigorous growth and abundant seed production. The branches of each plant can be fixed to a rod in vertical position in order to save greenhouse space and facilitate pod harvest. When many plants are grown in close vicinity, the aerial parts should be wrapped with a loose net when the green pods start to turn yellow to prevent mixing of detached pods from different plants. For a limited number of plants, plants can be grown without the net and pods collected daily as they mature on plants : the yield will be higher and there is less risk of fungal infection.

3. VEGETATIVE MANIPULATIONS

3.1. Propagation by cuttings

M. truncatula genotypes can be easily propagated by making cuttings from a single plant. The limiting step is the generation of adventitious roots at the base of the cuttings. Three variants of the method can be used depending on the substrate for rooting.

Rooting in water-soaked perlite

1. Autoclave small glass pots (e.g. yoghurt jars) filled with tap water-soaked perlite and covered with aluminium foil. Just before use, perforate the cover foil with a sterile forceps or needle (5 holes/jar).
2. Choose branches from the donor plant in order to generate branch tips with the apical bud and at least 4 nodes bearing opened leaves. Section the stem in the middle of the internode.

3. Immediately transfer the cut branch tips to a beaker of water in order to avoid dehydration and cavitation of the xylem.
4. Trim the stem at right angles with a razor blade just below (1-3 mm) the oldest node, which appears to be the prefered site for adventitious root emergence, and remove the corresponding leaf.
5. Insert cuttings (up to 5) through the holes in the water-perlite jar, and place the flask back in the growth chamber/greenhouse. Adjust the level of water as required.
6. Adventitious roots will emerge from the base of the cutting within 8 days and long branched root system develops within 2 weeks.
7. The cuttings with ≥ 5 mm roots can be transfered to soil or to an aeroponic chamber. Shoot growth will resume within a few days.

Remark :
- Vermiculite can be used instead of perlite. Root growth is as efficient as in perlite, however it is more difficult to separate the roots from the vermiculite particles since they grow into them.
- Jemalong cuttings just incubated in water (no solid substrate) will form adventitious roots within 8-15 days.

Rooting in soil (Jiffy bags)

1.-2.-3.- as 2.–3.–4. above ; leave the stalk of the lower leaf to anchor the cutting in the Jiffy bag (Jiffy-7 peat pellets, AS Jiffy products, Norway). Remove any flower or axillary branch.
4. Wet the base of the cutting and dip it in rooting hormone powder (Rootone F, Umupro). Eliminate excess powder by tapping.
5. Plant the cutting 2-3 cm deep into a sterile Jiffy saturated with water.
6. Lay the Jiffies on their sides (with the cuttings in a horizontal position) in a transparent plastic box (35 cm x 23 cm x 14 cm high ; can take up to 15 cuttings) and place the closed box in a growth chamber (~100 $\mu E.m^{-2}.s^{-1}$; 16 h/8 h). Avoid overheating inside the box.
7. Keep the box closed for 1 week to maintain 100 % hygrometry, unless molds develop on the leaves. After one week, slightly open the lid and add water as required. Some leaves will turn yellow and fall off.
8. Roots begin to grow out of the Jiffies from the 10^{th} day after planting. Around 15-18 days after planting, most cuttings should bear roots growing out of the soil.
9. Jiffies with rooted cuttings can be planted vertically at this stage into larger pots, and no longer need air hygrometry control.

Remarks about the first two rooting methods
- It is recommended to start from healthy and actively growing plants in order to increase the success rate (normally ≥ 80 %).
- Cuttings can also be generated from 4 node shoot fragments without the apical bud.
- Propagation of flowering branches yields plants that maintain flowering and resume flower bud formation as soon as the cutting has recovered normal growth rate.

Rooting in agar (axenic conditions)

This protocol is suitable for maintenance and propagation of clonal material grown on agar media in axenic conditions (see above). Plantlets grown in vitro on SHb10 agar medium (Chabaud et al., 1996) turn yellow and die after 3-4 months. For optimal maintenance, cuttings should be renewed every two months.

1. Under a sterile hood, section the shoot at an internode to generate a cutting with an apical bud and one fully developed leaf.
2. Lay the cutting **horizontally** in a Magenta box containing fresh agar medium, so that the stem section makes direct contact with the medium. Do not plunge the base of the stem into the agar.
3. Adventitious roots appear after 10-15 days on ≥ 90 % of cuttings. If necessary, reposition the cutting to optimise contact of the young roots with the medium.

3.2. Grafts between shoot and root system

Grafting is a powerful experimental method to study the effects of organ genotype on other distant parts of a plant (e.g. shoot/root). The following protocol is adapted for reciprocal grafting of young plantlets in axenic conditions.

1. Grow plantlets for ~3-4 days in sterile growth pouches until roots are ~8 cm long.
2. All the following steps must be carried out in sterile conditions. Remove the front plastic sheet and the paper fold from the pouch. Add sterile water to the roots throughout the grafting procedure to prevent dessication.
3. Select pairs of plantlets with similar hypocotyl diameter. Carefully separate the roots from their paper support with the aid of a scalpel blade.
4. Transversely section the hypocotyls at the lower end of the green chlorophyll zone using a fresh and clean razor blade (this operation must not crush the sectionned tissues). Transfer the resulting scions (aerial parts) and root stocks to sterile water in Petri dishes until needed.
5. With care successively insert a root and a scion into both ends of a polyethylene capillary tube (1 cm long, 0.8-0.9 mm internal diameter) and bring both explants in **close contact**. Two forceps are necessary for this delicate operation that is better performed on a hard surface.
6. Transfer the grafted plantlet to Fahräeus medium agar in square plates (24 x 24 cm^2). Check that contact between the two explants is maintained. Cover the graft tubing and its ends with low-melting agarose (1.5 %, 30°C) in order to immobilize the graft on the agar plate. This also helps to prevent explant dehydration.
7. Partially seal the plate with Parafilm. Wrap the lower part of the plate into a black plastic sheet to protect roots from light. Place the plate in a slanted position in an *in vitro* growth chamber (24°C ; 100 $\mu E.m^{-2}.s^{-1}$; 16 h photoperiod).
8. Growth of the rootstock resumes after an average lag period of 1 week (5-14 days). During this recovery phase, the scion often generates adventitious roots at the graft junction which are easily recognized and should be regularly cut off in order to promote rootstock growth.

<u>*Remarks*</u> :

- It is **critical** that the **graft junction be kept tight until the graft has sealed**. This helps the rapid development of the tissue connecting both vascular systems. Both hypocotyl portions

should be aligned on the same axis and the two section planes should be perfectly parallel. With some experience, most of the grafts will grow after a short lag phase.
- For nodulation studies, inoculation with *Sinorhizobium meliloti* can be performed a few days after root growth has resumed.

Specific material (sterilized)
- transparent, stiff polyethylene tubing, 0.8 mm internal diameter.
- square plates (24 x 24 cm^2) containing 200 ml Fahräeus medium with 15 g/l agar.
- 15 g/l sterile low-melting agarose in water

4. CROSSINGS

M. truncatula is a self-fertile, autogamous species where self-pollination occurs before flower opening (cleistogamy). After floral induction, all branches form 1-3 flowers at each node, thus following an indeterminate pattern. Under normal growth conditions most flowers set seed and generate pods until the quantity of developing pods on the plant leads to abortion of newly formed flowers.

Culture of parent plants

1. Vernalize seeds for 15 days at 4°C.

2. Plant seedlings into small pots (e.g. square pots 8 x 8 x 7 cm^3) containing a mixture of sand and compost (1:3, v/v) and transfer them to a growth chamber (25°C, 100 $\mu E.m^{-2}.sec^{-1}$, 16 h photoperiod). Water plants regularly with **deionised** water and include complete fertilizer (NPK 6:3:6, see Appendix) when needed (once per 1-2 weeks).

3. Such vernalized plants start to flower after ~ 25 days and are easy to handle due to their small size. Systematically remove (at least once a week) developing pods resulting from natural selfing in order to maintain full pod-forming potential. Trim old branches as needed. Parent plants maintained in these conditions can be used for repeated crosses over several months.

Manual crossings

This manual crossing procedure for *M. truncatula* is similar to method 3 described in Pathipanawat et al. (1994). It has been established using the cultivar Jemalong, and the same basic technique can be used for other genotypes / ecotypes for which the procedure should be easily adapted with minor modifications.

Pollen sampling

1. Select and remove flowers at the optimal male stage (just past anthesis) from the male parent according to external morphological criteria : at this stage, petals are 1-2 mm longer than the calyx teeth, and a slight central depression is visible on the shaft of the standard petal.

2. Dissect flowers under a stereomicroscope with a cold white light source, using curved extra fine forceps and avoiding pollen dispersion. In appropriate flowers, where anthesis has just occured, the released pollen appears turgescent, moist and sticky and is packed around the stigma.

3. Extract whole sexual columns and store them up to 1 h inside a Petri plate on the edge of a wet filter paper, avoiding contact between pollen and paper.

Choice of female flowers and crossings

1. Choose flowers on the female parent : the optimal female stage is just before anther bursting and pollen release, slightly earlier than that for male flowers.
2. Handle female flowers with care under a stereomicroscope. Sever sepals and incise the standard petal longitudinally (~ 0.5 mm below its central line) using a scalpel.
3. Carefully remove one by one the 10 non-opened anther bags with an extra fine forceps, and visually check for the absence of released pollen on the stigma (if pollen is present discard the flower). Clean the forceps with paper after removing each anther bag.
4. Dab the stigma with the tip of a freshly harvested sexual column in order to saturate the sticky stigma surface with exogenous pollen.
5. Gently replace the pollinated pistil under the standard petal.
6. Label the branch tip bearing the cross-pollinated flower, and insert into a ~ 25-ml clear plastic vial containing 1 ml water (to maintain high hygrometry). Hold the stem in place with a cotton wool plug.
7. Keep the female plant under indirect light until the protecting vial is removed 24-48 h later. Successful crossing leads to the development of a small coiling pod that becomes visible 2-4 days after artificial pollination.
8. Ripe grey-brown pods containing hybrid seeds are obtained within 4-5 weeks after crossing. They should be harvested before natural abscission or alternatively tied to the stem with surgical gauze.

<u>*Remarks*</u> :

- With some training, it takes ~5 min for a single cross, and 1 h for 6-8 crosses including pollen sampling and handling female plants.
- The efficiency of this method is good, with an average of 80 % cross-pollinated flowers giving pods with ≥ 5 viable seeds/pod. Three conditions appear critical for optimizing success rates : (i) Healthy parent plants (e.g. thrips feed upon pollen !) ; (ii) Choosing the optimal developmental stage of both the pollen and the receptor pistil ; (iii) Saturation of the stigma surface with exogenous pollen. When these conditions are fulfilled, close to 100 % crosses are successful and result in pods containing 10-12 seeds, as is the case for normal self-pollinating flowers.
- We have made use of a GUS reporter gene fusion as a genetic marker of exogenous pollen, in order to distinguish GUS-positive true F1 hybrids from GUS-negative self-progeny. We have thus verified that, in our experiments, the frequency of residual self-pollination resulting from incomplete pollen removal is very low (less than 1 % F1 seeds).
- This crossing protocol was initially established using several lines of the ecotype Jemalong. Significant variations in the optimal stage for crossing and in the rate of success were observed between various *M. truncatula* ecotypes and appear to mainly depend on the characteristics of the maternal ecotype and the genetic distance between parental lines.

Specific material

- low magnification stereomicroscope and cold white light source installed on a bench with sufficient working space for handling trays of plants ;
- extra fine forceps for microscopy (Dumoxel n° 7, Dumont, Switzerland) ;
- fine scalpel with a new blade (Feather #11) for each series of crosses ;
- small Petri dish (5 cm diameter) with a piece of folded, wet Whatman paper to store pollen.

Other material

fine scissors, sterile water, transparent polystyrene vials (3 cm diameter, 7 cm height), cotton wool, self-adhesive labels

5. *M. truncatula* CELL SUSPENSION CULTURES

Cell cultures are widely used in cell biology as well as in biochemical and molecular biological and cell cycle studies. The advantage of these cultures is that they provide practically non-limited materials from relatively homogeneous cell types/lines. Moreover, they can be a source of protoplasts used for transient expression of transgenes. In the following a protocol is given for the preparation of cell cultures from *M. truncatula* ecotype Ghor (Hanh Trinh and Patricia Durand, unpublished data).

1. Scarify seeds with sand paper and sterilise them with Bayrochlor for 20 min.
2. Wash seeds 2-3 times with sterile water, transfer them in sterile water in a Petri dish covered with aluminium foil and gently agitate overnight at room temperature.
3. Place the sterilised seeds under a laminar flow hood onto sucrose-free SHM2/2 solid medium in Petri dishes and incubate at 4°C for 48 hours.
4. Transfer the seeds on saccharose-containing (20 g/L) medium for germination and incubate them in constant light at 24°C for 4 weeks to obtain sterile seedlings.
5. For callus formation, culture small pieces of root or cotyledon tissue on solid MSO2 (SM) medium in the dark for 4 weeks.
6. Cut the calli in about 5 mm pieces and transfer them in liquid MSO2 (LM) medium and cultivate them with agitation (120 rpm) at 24°C, in constant light .
7. Filter the callus cultures through a 200 µm sterile mesh. Collect the flow-through fraction containing smaller cell aggregates and cultivate them in fresh LM medium.
8. Repeat the filtration and transfer to fresh medium each week until fine microcallus suspensioncultures are obtained.
9. Subculture the microcallus suspension cultures weekly in LM.

6. INOCULATION OF *M. truncatula* WITH *Sinorhizobium meliloti* AND NODULATION ASSAYS

Inoculation of *M. truncatula* roots with *S. meliloti* bacteria results in the formation of nitrogen-fixing root nodules. Nodulation is negatively regulated by the presence of combined

nitrogen in the growth media/substrate. Therefore, nodulation assays have to be performed in nitrogen-free or nitrogen-limiting conditions. Although nodulation is very effective and can be obtained from a wide range of initial bacterial density, the standard or most frequently used conditions are given for the different inoculation procedures.

6.1. Inoculation of growth pouches

1. Grow a culture of *S. meliloti* in 5 ml TY medium supplemented with appropriate antibiotics for 16-24 h at 28°C. Bacteria should be in late log phase/early stationary phase (OD_{600} = 0.4-1.0). Alternatively, fresh bacteria grown on agar TY plates can be used.
2. Dilute the bacterial culture in sterile water at room temperature down to 0.001-0.005 OD_{600} (~ $1\text{-}5.10^6$ bacteria/ml). If the culture medium contains antibiotics, it should be diluted at least 100 fold, otherwise the bacteria must be washed by centrifugation and resuspended in sterile water just before inoculation.
3. Insert a sterile 2-ml pipette between plastic cover-sheet and paper to inoculate each root system in the pouch with 0.1-0.2 ml diluted bacterial suspension. This is better done when lateral roots have not emerged yet, i.e. before 4-5 days of seedling growth in the pouch. The pouch can be tilted so that the inoculum will flow along the root down to the tip and inoculate the region of the root that is susceptible to rhizobial infection.
4. In the pouch system, infection threads are formed in root hairs from around 36-48 h post-inoculation. Developing nodule primordia and young nodules on wild-type genotypes become visible through the transparent pouch from 4-5 days post-inoculation onwards.

Remarks :
- Rhizobial cultures should be fresh. Storage of bacteria at 0-4°C before inoculation delays the kinetics of nodulation by at least 24 h.
- A high initial density of rhizobial cells is not required since these bacteria proliferate on contact with the receptive parts of the roots. Higher densities of cell suspensions (≥ 0.1 OD_{600}) may trigger defense reactions resulting in poor nodulation.
- It is important not to add more than a total of 1 ml inoculum to each pouch, as *M. truncatula* roots are very sensitive to an excess of liquid in this system (see above).
- The spatio-temporal pattern of nodulation can be precisely followed by marking (best to use permanent ink marker) on the transparent cover-sheet the position of growing root tips at regular intervals (24 h for example). Be careful not to press too close to the root tip.
- In general young emerging laterals are the most efficiently nodulated.

6.2. Inoculation of agar plates/tubes

The operating principle is identical to that described above for pouches. Smaller inoculum volumes (≤ 0.1 ml/root) are usually sufficient for plate assays.

Bear in mind that growth of *M. truncatula* plantlets on agar plates is highly dependent on the agar type. This may slow down the growth and evoke stress effects. This probably explains why various stages of the rhizobial interaction (infection, nodulation...) are delayed and often partially inhibited. Addition of the ethylene synthesis inhibitor AVG can be beneficial (see Smith and Long, 1998).

The agar plate assay is a convenient system to study local root responses by using spot inoculation techniques. One possibility is to mix equal volumes of bacteria/other reagents and 0.7% agarose and to lay a small droplet on the nodulation-sensitive root zone or other root regions using a micropipet.

6.3. Inoculation of aeroponic tanks/factories

Nodulation is very rapid in this system, with nodules first visible as tiny bumps as early as 3 days after inoculation.

6.3.1. Inoculation of seedlings

1. Sterilised and germinated seeds are grown in square Petri dishes on 0.7 % Kalys agar-water/Solution I plates.
2. When the root growth is about 5 cm, insert 1 or 2 seedlings/hole of the top lid of a running factory filled with 8 liters of Solution I. If the humidity in the growth-chamber is below 70 %, cover the aerial part of the seedlings with small glass jars to avoid desiccation. The cover can be removed after 1-2 days.
3. For inoculation, add 8 ml of OD_{600}=1.0 *S. meliloti* grown in TA complete or GTS minimal medium until early stationary-phase and washed with Solution I.

6.3.2. Inoculation of plants

1. Grow plants to the desired size in an aeroponic tank containing nitrogen-rich medium (see above). Typical culture times vary between 1 and 3 weeks. For fast inoculations, it is possible to grow the seedlings directly on nitrogen-free medium.
2. Replace the complete medium by nitrogen-free medium and grow the plants for an additional period to induce N-starvation (≥ 2 days).
3. Inoculate the tank by adding to the growth medium 1/1 000 v/v of an early stationary-phase culture of the desired rhizobial strain. This corresponds to a final concentration of ~10^6 bacteria/ml. Agitate the tank several times to disperse the inoculum in the growth medium.
4. Nodulation is very rapid in this system, with nodules first visible as small bumps 3 days post-inoculation.

Remark :
Remember that aeroponic systems are not axenic. Furthermore, if other aeroponic tanks in the same growth room are already inoculated, beware of possible airborne rhizobial contamination ; it is therefore recommended to avoid opening tanks inside the growth room. Nitrogen-rich growth medium will generally inhibit symbiotic interactions with such contaminants - however they will be initiated as soon as nitrogen is removed. Thus, premature nodulation often indicates rhizobial contamination.

6.4. Inoculation of pots/trays

Plants can be grown in pots containing soil or other solid substrates (sand, perlite, vermiculite ; see above). For reliable nodulation assays, these substrates need to be previously sterilised. Inoculation is carried out by pouring a relatively large volume (~ 100 ml/liter of substrate) of diluted bacterial suspension on the soil surface of the pot/tray.

7. INOCULATION OF *MEDICAGO TRUNCATULA* PLANTS WITH ARBUSCULAR MYCORRHIZAL FUNGI

In this procedure, *M. truncatula* seedlings are grown until they have one trifoliate leaf. They are then transplanted into turface or sand and inoculated with arbuscular mycorrhizal fungal spores. The symbiosis will develop over the following 4 weeks.
We have used this protocol to colonize *M. truncatula* with *Glomus intraradices, G. versiforme, G. caledonium* and *Gigaspora rosea*.
To ensure that the symbiosis develops under as clean conditions as possible, we routinely sterilize or surface sterilize all components.

7.1. Preparation of growth materials and pots

Items required :
Chlorox (bleach)
Fine sand (autoclaved)
Coarse sand or Turface (autoclaved). Turface is a trade name for calcined clay.
Plant pots (11cm diameter plastic pots)

Procedure

a. The cones are surfaced sterilized before use. Soak cones in 10 % chlorox for about 15 min. Rinse well by submerging in deionised water. Repeat the rinse at least 3 times. Leave to dry. They can be stored in a clean plastic bag until needed.
b. The sand and turface are rinsed at least 3 times with double distilled water and autoclaved before use.

7.2. Preparation of *M. truncatula* seedlings

Items required *:*
M. truncatula A17 seeds
Concentrated Sulphuric acid
Double distilled water
Chlorox and Tween 20
Sterile double distilled water
Sterile filter paper circles that will fit in petri plates
Parafilm
Aluminium foil
Beakers and pipettes
4°C room

Procedure

a. a. Immerse 20 *M. truncatula* seeds in conc. H_2SO_4 for 10 minutes. This should be carried out in a fume hood.
b. Remove the acid and rinse the seeds 3 times with double distilled water. It is important to ensure that the seeds do not overheat in this step.
c. Immerse seeds in 30 % Clorox + 0.1% Tween 20 for 5 minutes.
d. Remove the chlorox and rinse the seeds 3 times with sterile double distilled water.
e. Spread seeds on damp sterile filter paper in a petri-plates. Seal the edges of the plates with parafilm and then wrap the plates in aluminum foil and keep at 4°C for 4 days.
f. Remove the seedlings from the cold and leave at room temperature for 2 days. Then remove the foil and leave at room temperature for an additional day. The seedlings will have germinated and will turn green after exposure to light.
e. Plant the germinated seedlings into sand or turface and allow them to grow until they have the first trifoliate leaf. During this time, they should be watered with a low phosphate fertilizer such as ½ strength Hoaglands solution with 20 µM phosphate.

7.3. Surface sterilisation of fungal spores

The fungal spores should be sterilised just before use. There are many protocols for surface sterilisation. We use the following method.

Items required:
AM fungal spores (e.g. *G. intraradices*)
Chloramine T/Tween 20 solution :
(Dissolve 0.4 g Chloramine T in 20ml of water. Add 50 µl Tween 20)
Streptomycin sulphate (200 µg/ml – Filter sterilized)
Sterile distilled water

Procedure

a. Place 200 spores in 20 mL of fresh Chloramine T/Tween 20 solution. Stir gently for 15 minutes. Allow spores to settle then remove the solution. Repeat. Allow spores to settle and remove the solution.
b. Add 20 mL of streptomycin sulphate. Incubate at 4°C for at least one hour but the spores may be left in streptomycin overnight. Do not leave the spores in streptomycin for longer than one day as it may reduce germination.
c. Remove streptomycin and rinse five times with sterile distilled water. This is easily performed by filling the tube with water, allowing the spores to settle to the bottom, then removing water with a pipette.
d. Save the water from the last wash. It can be used for the mock-inoculation of the control plants.

7.4. Inoculating *M. truncatula* plants with AM fungal spores

Items required :
M. truncatula plants (stage – first trifoliate leaf)
Sterilised sand, turface and pots
Sterilised spores

Procedure

a. Fill 2 plant pots approximately 2/3 full with sterile turface or coarse sand. Then add a 1cm layer of fine sand. Place 10 seedlings into each pot, spreading the roots out onto the fine sand. One pot will be inoculated with spores. The other pot will be a control and will receive the last spore wash.
b. Pipette 200 spores onto the roots of the plants in one of the pots. Cover the roots with turface or coarse sand. Pipette an equivalent volume of spore washing water onto the control plants in the second pot. Place both pots in a growth chamber. (The conditions in our chambers are as follows : daylength-16 hour days, Temperature-25°C light intensity-200-230 μE). It is useful to cover the plants with a dome or plastic bag until they recover from transplanting.
c. Fertilize as needed (usually 1-2 times per week) with a low phosphate fertilizer. The symbiosis should be well developed within 4 weeks.
d. To harvest the roots, invert the pots and shake gently. The contents will fall out. Place the turface and roots in a large volume of water and shake gently. The turface will fall off the roots. The root can then be stained to enable observation of the mycorrrhizal fungus.

7.5. Staining roots to observe the mycorrhizal fungus

In order to observe the mycorrhizal fungus within the roots, it is usually necessary to stain the roots. Trypan blue is a suitable stain for a wide range of AM fungi.

Stock solutions

- 10% (w/v) potassium hydroxide
- 2% (w/v) Trypan Blue
- Lactoglycerol solution (combine 300 ml Lactic acid, 300 ml Glycerol, 400 ml double distilled water)

Procedure

a. Submerge the roots in potassium hydroxide (10 %) and heat at 90°C for 20 min.
b. Decant the potassium hydroxide and rinse the roots twice with deionized water.
c. Prepare Trypan blue staining solution by mixing 25ml of Trypan blue stock and 1000 ml Lactoglycerol
d. Cover roots in Trypan blue staining solution and place at 90°C for 3-5 min (DO NOT LEAVE THEM LONGER –they will turn completely blue)

e. Decant the stain into a waste bottle and place the stained roots in glycerol.
f. Mount roots in glycerol on microscope slides for microscopy (NOTE – Do not mount in lactoglycerol, it destroys the microscope). The fungus will be stained blue and should be clearly visible within the roots. If the fungus has not stained enough, repeat the staining step. If the roots have stained too much, place them in lactoglycerol and they will destain.
g. The roots from the mock-inoculated controls should be stained and examined. These serve as a control and will indicate the cleanliness of the growth conditions. Obviously they should not contain any mycorrhizal fungi.

8. INFILTRATION OF *M. truncatula* LEAVES WITH PATHOGENIC BACTERIA LEAVES

This technique provides an easy and convenient way to study compatible and incompatible plant defense reactions induced by pathogenic bacteria. Compatible reactions are characterized by water-soaked lesions which later on result in yellowing and wilting of the whole leaf. The incompatible reactions, in contrast, lead to localized necrosis in the infiltrated area. About 30 µl of bacterial suspension, or 10 mM Mg_2Cl for the control, are injected in the leaves of 6-weeks-old plants with a hypodermic syringe. Compatible (*Xanthomonas campestris* pv. *alfalfae*) and incompatible (*Pseudomonas syringae* pv. *pisi*) bacteria are grown in YDA medium overnight at 30°C, washed and resuspended in 10 mM Mg_2Cl at a concentration of 10^9 bacteria/ml. Use gloves during infiltration of bacteria. Place the leaf on the forefinger with the lower face upward and introduce the needle below the surface. Do not perforate the leaf.

9. REFERENCES

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APPENDIX

COMPOSITION OF PLANT CULTURE MEDIA

Soft C (Campbell) medium

For 1 liter of C medium, add :

- 0.5 g K_2HPO_4
- 0.2 g $MgSO_4$
- 0.1 g NaCl
- 10 g mannitol
- 2.5 g yeast extract
- 0.5 g casamino acids
- 15 g agar

Autoclave the medium. For soft C medium, add 100 ml sterile water to 250 ml C medium.

Fahräeus medium (modified from Vincent, 1970)

The following formula corresponds to liquid, nitrogen-free Fahräeus medium used in growth pouches. Combined nitrogen (NH_4NO_3 or KNO_3) can be added to the desired concentration. For Fahräeus agar, add 15 g/l agar before medium sterilization.

Stock solutions	**Stock concentration**	**Volume (ml)** (for **1 liter** of 1x medium)	**Final Concentration**
Macronutrients[1]			
$MgSO_4$, 7 H_2O	0.5 M	1.0	0.5 mM
KH_2PO_4	0 .7 M	1.0	0.7 mM
Na_2HPO_4, $2H_2O$	0.4 M	2.0	0.8 mM
Fe-EDTA[2]	20 mM	2.5	50 µM
Micronutrients[3]			
$MnSO_4$, $CuSO_4$, $ZnSO_4$ H_3BO_3, Na_2MoO_4	1mg / ml each	0.1 each	0.1 µg / l each

[1] : sterile macronutrient stock solutions are stable at room temperature. Store at 4 °C once bottles have been opened.

[2] : prepare separate solutions of 5.6 g/l $FeSO_4$ and 7.4 g/l Na_2EDTA by heating at 50 °C and mix.

[3] : store micronutrient solutions at –20 °C.

- Adjust pH to 6.5.
- Distribute into 250 or 500 ml flasks and sterilize in autoclave (120 °C, 20 min).
- $CaCl_2$ (1 M stock solution) must be added to the medium (1 mM final concentration) after autoclaving and just before use, since it co-precipitates with phosphate ions. Alternatively, add 70 µl of 100 mM $CaCl_2$ directly in the middle of each pouch.

Aeroponic nutrient medium (LBMRPM INRA/CNRS)

Stock solutions[1]	**Stock concentration**		**Volume (ml)** (for **10 liters** of 1x medium)	**Final Concentration**
$CaCl_2$	222 g/l	2 M	5 ml	1 mM
$MgSO_4$, 7 H_2O	123 g/l	0.5 M	5 ml	0.25 mM
K_2SO_4	60.9 g/l	0.35 M	15 ml	0.5 mM
Fe-EDTA[2]		20 mM	25 ml	50 µM
Micronutrient mix			10 ml	
H_3BO_3	2 g/l			30 µM
$MnSO_4$, H_2O	1.8 g/l			10.6 µM
$ZnSO_4$, 7 H_2O	0.2 g/l			0.7 µM
$CuSO_4$, 5 H_2O	0.08 g/l			3.2 µM
Na_2MoO_4, 2 H_20	0.25 g/l			1 µM
$CoCL_2$, $6H_2O$	0.02 g/l			84 nM
Phosphate mix[3]		1 M	50 ml	5 mM
KH_2PO_4	46.7 g/l			
K_2HPO_4	131.9 g/l			
NH_4NO_3 [4]	80 g/l	1 M	50 ml	5 mM

[1] : sterile stock solutions are stable at room temperature. Store at 4 °C once bottles have been opened.
[2] : prepare separate solutions of 5.6 g/l $FeSO_4$ and 7.4 g/l Na_2EDTA by heating at 50 °C and mix.
[3] : buffers the 1x medium at pH 7.0.
[4] : to be added for "High Nitrogen" aeroponics medium.

- Add the stock solutions to 10 liters of sterile, de-ionized water in the order of the list. Mix well between each addition, and especially after the addition of phosphate.
- This medium should be used fresh (on the same day). Do not store it at cold temperature, since salts would precipitate.

Nutritive solution for greenhouse culture (ISV)

Stock solution:

N.P.K. 18.6.26 fertiliser	4 kg
nitric acid	1 liter
deionised water upto	80 liters

N.P.K. 18.6.26 (purchaised from Duclos) is composed of 18 % N (12% nitric and 6% ammonium), 6% P (phophoranhydride), 26% K (Potassiumoxide) and contains 2% MgO as well as oligo elements (0.025%B, 0.005% Cu, 0.06% Fe, 0.025% Mn, 0.0025% Mo, 0.005% Zn).

The nutritive solution is obtained by dilution of stock solution with the help of a pump (Dosatron) which injects 16 ml of stock solution in liter of water. Irrigation is done automatically during 3 days/week, with 1 minute irrigation twice daily.

Nitrogen-limited Solution I (ISV)

This nitrogen-limited medium is used in nodulation assays both in aeroponic tanks and in combination with perlite, vermiculite or sand with manual irrigation.

Preparation of the components of the stock solution

A

A1.	KNO_3	20.2 g/L
A2.	KH_2PO_4	27.2 g/L
A3.	$CaCl_2 \times 2H_2O$	73.0 g/L
A4.	$MgSO_4 \times 7H_2O$	24.6 g/L
A5.	K_2SO_4	43.5 g/L

Dissolve each component (A1-A5) separately in 1 liter of deionised water.

B

$EDTA_2Na_2Fe$ (Sigma E6760) 8.2 g/1 liter of deionised water

C

H_3BO_3	11 g
$MnSO_4 \times H_2O$	6.2 g
KCl	10 g
$ZnSO_4x7H_2O$	1 g
$(NH_4)_6Mo_7O_{24} \times 4H_2O$	1 g
$CuSO_4 \times 5H_2O$	0.5 g
H_2SO_4	0.5 ml

Dissolve the components in the given order in deionised water in 1 liter of final volume. Heating facilitates the solubility of components.

Stock solution
Mix in a 10 liter container :
250 ml of solution A1
250 ml of solution A2
250 ml of solution A3
250 ml of solution A4
250 ml of solution A5
250 ml of solution B
13.5 ml of solution C
with deionised water upto 10 liter of final volume.

Dilution of the Stock solution :
Add 200 ml of stock to 7.8 liters of deionised water.

Media for *in vitro* and cell cultures

MSO2 medium
MO404 Murashagi and Skoog basal medium (Sigma) supplemented with Gamborg's vitamines (Sigma), 30g/L saccharose, 250 mg/L casein hydrolysate pH 5.8.
After autoclaving add the phytohormones : 2,4-D (2mg/L) and kinetin 250 mg/L. For solid medium, add 7g/L Kalys agar.

SHM2/2
SHM2/2 : half strength of (N6 major salt, SH minor salt and vitamins, 20g/L sucrose), and 7g/L Kalys agar (HP 696-7470 Kalys, France) (Kamaté et al. 2000).

SH minor (100 ml)

$MnSO_4$.H2O	1 g
$H3BO_3$	500 mg
$ZnSO_4$ x 7H2O	100 mg
KI	100 mg
$NaMoO_4$ x $2H_2O$	10 mg
$CuSO_4$.x $5H_2O$	20 mg ($CuSO_4$=12.8 mg)
$CoCl_2$ x $6H_2O$	10 mg

SH vitamins (100 ml)

Nicotinic acid	500 mg
Thiamine.HCl (vitamine B1)	500 mg
Pyridoxine.HCl (vitamine B6)	500 mg

N6 major 1L

$MgSO_4$ x 7H2O)	1.85 g
KNO3	28.3 g
$(NH_4)_2SO_4$	4.63 g
$CaCl_2$ x 2H2O	1.66 g
KH_2PO_4	4.00 g

Complete fertilizer for soil-grown plants (LBMRPM INRA/CNRS)

We use a NPK (6:3:6) formulation supplemented with micronutrients ("Engrais plantes d'intérieur", Substral ®, France) :

- 6 % total N (2.7 % ammoniacal N, 3.3 % nitric N), 3 % P_2O_5, 6 % K_2O ;
- -micronutrients : 0.01 % B, 0.001 % Mo, 0.005 % Cu, 0.01 % Mn, 0.002 % Zn, 0.03 % Fe-EDTA.

TIME SCHEDULE

Module 1.

9h30-9h45 Introduction

9h45-11h45 Visiting the greenhouse/growth rooms/growth chambers
showing *M. truncatula* at different growth conditions
preparation of perlite, vermiculite, sand, soil
pests/diseases/treatments
collection of seed pods
sorting the seeds from the seed pods and selection of seeds from debris

11h45–12h30 Student lab
Seed sterilisation (mechanical & sulphuric acid)
Germination

12h30–13h30 Lunch

13h30-15h30 Student lab
Growth of seedlings on agar plates
Growth of seedlings : in pouches
Growth of seedlings : in aeroponic tanks
Inoculation of *M. truncatula* with *Sinorhizobium meliloti*

15h30-15h50 Break

15h50-16h50 Conference room
Infection of *M. truncatula* with *Glomus*
Crossing (Video presentation)

16h50-19h30 Student lab
Crossing-practical demonstration
Vegetative multiplication
Grafting
Infiltration of *M. truncatula* leaves with pathogenic bacteria
Cell cultures