Growing *M. truncatula*: choice of substrates and growth conditions

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1 Introduction

Medicago truncatula can be successfully grown using a number of supports and substrates (moist paper, agar, nutrient mist, liquid medium, soil / inert substrates) and corresponding cultivation systems (pouches, tubes, plates, aeroponic and hydroponic tanks, pots, trays, in growth chambers or in the greenhouse) (Table 1). Some systems are appropriate for short-term biological experiments that can be performed on seedlings or plantlets in small-size growing units (pouches, agar tubes and plates). Other systems are adapted for physiological experiments that require plants in optimal growth conditions (e.g. 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 greenhouses). This chapter presents the most frequently used growth systems for *M. truncatula*.

Table 1 - Various appropriate "substrate x culture room" combinations for growing *M. truncatula*.

Substrate **Culture room**	agar	paper (pouch)	sand / perlite / vermiculite	soil	aeroponics	hydroponics
In vitro growth chamber	**+**	**+**	**(+)**			
classical growth chamber			**+**	**+**	**+**	**+**
Greenhouse			**+**	**+**	**(+)**	**(+)**

+ : appropriate combination; **(+)** : combination working under certain conditions

A Temperature, light intensity and photoperiod settings for growth chambers and greenhouse

The commonly applied environmental conditions for the culture of *M. truncatula* in "controlled" environments such as the growth chamber and greenhouse have been surveyed (Moreau, Salon and Munier-Jolain, unpublished data), and the ranges of the different environmental conditions are listed below:

Day temperature: …… 20 to 25 °C

Night temperature: …….. 15 to 21°C

Photosynthetically Active Radiations (PAR): … 200 to 600 µmol.m^{-2}.s^{-1} (growth chamber)

Photoperiod (light/dark): …… 14/10 or 16/8 hours

As examples, the settings applied in routine at LIPM, Toulouse are as follows

(temperature, day - night / hygrometry(where appropriate) / light intensity (PAR)/ light-dark photoperiod):

- growth chamber for aeroponics**:** 22 - 22°C / 75 % / 200-350 µmol.m^{-2}.s^{-1} / 16 h – 8 h (HQL 400 De Luxe mercury vapor bulbs, Osram);
- growth chamber for seed multiplication**:** 25 – 21°C / - / 200-300 µmol.m^{-2}.s^{-1}/ 16 h – 8h (mixed lighting: alternating HQL 400 De Luxe mercury vapor and NAV-T 400 High Pressure sodium bulbs, Osram);
- glasshouse: 22 °C constant/ - / 120 - 300 µmol.m^{-2}.s^{-1} / 16 h - 8 h;

- for *in vitro* growth rooms, the settings are a fixed 24h temperature and a lower PAR: 25°C constant/ - / 80-100 µmol.m^{-2}.s^{-1} / 16 h – 8 h (mixed lighting: alternating Osram L30W/77 and General Electric F30W/54 tubes).

Further comments relevant to the impact of environmental factors on M. truncatula growth and development can be found in ***Appendix 1*** *at the end of this chapter.*

B General recommendations for seedling manipulation and plant transfer

Seeds become quite sensitive to desiccation in air as soon as they germinate, the most exposed part being the radicle. Moreover, the longer the root, the more there is a risk of rapid water stress. WARNING: seedling root meristems can suffer irreversible damage by exposure to only 1 min of air flow in a sterile hood. Therefore, a general recommendation is to submerge germinated seeds in sterile water in Petri plates (see below Fig. 3c, and Chapter "Seed storage and germination") while awaiting transfer to any growth system. Obviously precautions in relation to strong/warm air flows should also be taken for seedlings and during transplantation of older plantlets (e.g. from pouches or aeroponics to soil). Finally, seedlings/plants transferred from the cold room (4°C) to strong light conditions in the growth room should be kept for at least several hours in a moist environment.

With respect to minimizing contamination during culture, it is highly recommended to remove the teguments of germinated seeds before planting when using seed batches which are contaminated by fungal spores (more details in Chapter "Seed storage and germination").

C Growth pouches

Growth pouches are designed for studies on seedling root biology, allowing both vigorous growth and easy observations of the 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 support 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 (Fig. 1a). *M. truncatula* seedlings grow particularly well in this system provided the following guidelines are respected. However, it should be borne in mind that a greater level of variability in root development and architecture (lateral branching) can be observed between individual plants and between pouches in this system as compared with plants grown in aeroponic tanks (see below).

Preparation of the pouches

1. Slightly pull out the paper wick to 1-2 mm below the top edge of the pouch.

2. Using a pipette add 6 - 7 ml of Fahräeus liquid medium (see Appendix 2) to the paper wick and allow the liquid to be absorbed. Flatten the paper wrinkles by hand pressure.

3. (*Optional step, see remark below*) Autoclave pouches in packs of ≤ 10 wrapped in aluminium foil and maintained in a vertical position under pressure between two flat surfaces to avoid heat deformation of the pouches. Autoclave separately aluminium covers (see specific materials) that are used to shield roots from light.

Culture of seedlings in pouches

1. Place pouches in a vertical stand (supplied by the manufacturer) under a sterile hood. Submerge germinated seeds in sterile water for the time of transfer as indicated above (§B, General recommendations for seedling manipulation and plant transfer).

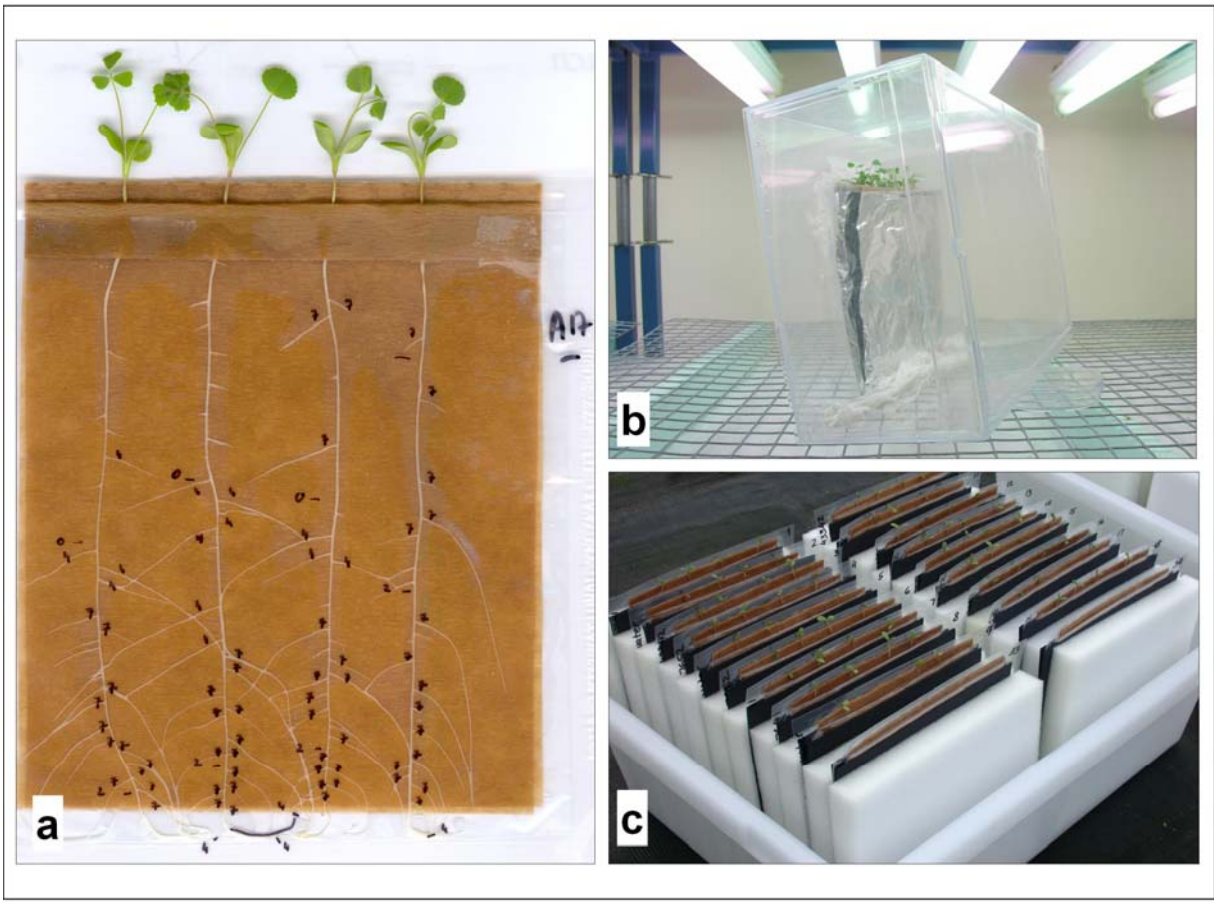

Figure 1 - Growth pouches
a: Growth pouch holding 4 plantlets, photographed 12 days after seedling transfer from germination plates. Root systems are shielded against light during growth. Thanks to the transparent plastic pouch, root development can be followed daily. Here, the position of primary root apices was marked at 2, 4 and 6 days (marks 0, 2 and 4, respectively) and the presence of lateral roots was marked at 2, 6 and 9 days (marks 0, 4 and 7, respectively) after seedling transfer **b**: Stack of pouches wrapped in an aluminium cover and placed vertically inside a transparent plastic box. Note the tilted position of the box on the *in vitro* growth chamber shelf, and the wet white paper wick to maintain air humidity inside. **c**: Alternative device used at the ACNFP to maintain pouch sets in a proper position during culture (see text).

2. Open the wick fold with the help of sterile forceps. One by one transfer germinated seeds from the Petri dish to the pouch trough using a second pair of forceps, and gently insert the radicles into pre-made slits in the paper.

3a. When all seedlings have been added, wrap the lower part of each pouch (or set of pouches) in an aluminium cover. Add an additional empty water-saturated pouch both to the front and the back of each stack of pouches to minimize drying of the outer pouches. 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 (to be kept for at least 2 d). Close the lid and position the box in a tilted position (~30 ° from vertical position) in an *in vitro* growth chamber (Fig. 1b).

3b. *Alternatively*, use a folded piece of black cardboard (bottom folded, unsealed sides) to cover each pouch, this will facilitate the observation of root development. Square foam pieces, 2x15x16.5 cm, can be placed between the pouches to apply homogenous pressure to the pouch surface (Fig. 1c; communicated by S. Whitehand and J. Lichtenzveig).

4. Young shoots should emerge from the pouch within 3 days. Cotyledons which are caught in the fold should be released with forceps at this stage, but take care not to damage the fragile seedling.

5. Compensate for water evaporation by adding sterile water to pouches as required (usually not before 5-7 days of culture).

Remarks

- Pouches can take up to 12-15 germinated seeds. However, 4-6 plantlets/pouch is more appropriate for experiments lasting up to 2 weeks in order to avoid excessive lateral root overlap.

- The optimal stage for introducing germinated seeds into pouches is when radicles are 5-10 mm long. Longer roots are more fragile and more sensitive to desiccation during manipulation.

- It is also very important that radicles remain in close contact with the moist paper at the beginning of growth (first 24-48 h). This is why tilting of the box and pressure of the glass plate are recommended (see §3a).

- The amount of liquid present in the pouch is critical (6-7 ml is optimal) since *M. truncatula* roots are very sensitive to excessive water and respond by plagiotropic and/or restricted growth. For this reason be careful not to over water when replacing evaporated water or when adding liquid inoculum (see Chapter “Rhizobial inoculation and nodulation of *Medicago truncatula*”). Pouch desiccation can be slowed down by the presence of imbibed paper towels inside the transparent box.

- Young primary roots of *M. truncatula* grow rapidly (20-35 mm/day) in this system. Lateral roots appear around day 4-5, usually corresponding to the time when the primary apex reaches the bottom of the pouch. Although root growth becomes spatially constrained after 1 week root systems continue growing and ramifying for a further 1-2 weeks without further nutrient supply. When necessary, up to 0.5 mM NH_4NO_3 can be added as "starter nitrogen" (no noticeable inhibitory 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 materials

- "CYG seed growth pouches" (16.5x17.5 cm) and associated aluminium vertical stands can be purchased from Mega International (Minneapolis, Minnesota, USA; technical information available at www.mega-international.com).

- aluminium covers are prepared by folding lengthwise a piece of aluminium foil (30x45 cm) and then transversely in 3 parts, so that the mask protects most of the pouch from light. Masks can be recycled many times.

- transparent plastic boxes (24x36x14 cm).

- Fahräeus medium (see Appendix 2).

D Agar plates / tubes

Agar plates

M. truncatula seedlings can be grown on sterilized agar plates propped vertically (at an angle of ~ 70°) that are appropriate for localized treatments such as spot inoculation or treatment of the roots with different reagents under sterile conditions. However, seedling growth is significantly modified under these conditions, due most likely to the combination of growth on the top of a solid surface and of various (inhibitory) chemical compounds present in agar. Root growth rate, as well as nodulation, is slower than in the pouch system (see above), and primary roots also tend to grow obliquely. *M. truncatula* roots on agar are thicker and form longer root hairs, displaying a "Thick Short Root" stress phenotype (Fig. 2a, 2b). For this reason, addition of the ethylene synthesis inhibitor AVG can be beneficial for root growth (see Smith and Long, 1998), despite the fact that AVG inhibits, in addition to ACC synthase, other pyridoxal-5'-phosphate-dependent enzymes.

The source of the agar is of crucial importance. 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, Roubaix, France; www.kalys.com/bioGB.htm) and Becton-Dickinson Bacto-Agar (ref. 214010) work well for *M. truncatula.* Agar is used at 0.7-1.5 % concentration for solid plates in various nutrient media or water, depending on the objectives of the experiment. Agar can be replaced by the gellan gum Phytagel as a solid support (Sigma; 0.4-0.5 % (w/v) for culture in a vertical position; e.g. Kosuta *et al.*, 2003; Olah *et al.*, 2005), since this can reduce stress effects on growing seedlings - however care should be taken to make sure that the growth medium is compatible with the Phytagel (see Doner and Bécard, 1991).

Up to 10-15 seedlings arranged in a row can be grown on square agar plates of 12x12 cm (Fig. 2a). Various nutrient media can be used (see Annexe 2 for Fahräeus medium). Agar should be poured as slants so that it only partially covers the dish surface when contact of aerial parts with growth medium is to be avoided (e.g. when leaf toxic chemicals are present in the medium). This has also proved an efficient remedy to reduce the spread of residual fungal contamination of seedlings (see Chapter “Seed storage and germination”). Finally, drying out of the agar can be limited by using a perforated Parafilm seal - the perforations are important to allow plant-released ethylene to diffuse away. Alternatively, a surgical tape can be used (e.g. 3M Micropore 1530-0).

Pouch paper overlays on agar plates

Growing seedlings/young plants on pouch paper laid on top of agar growth medium combines the ease of access to root systems of agar plate cultures with a significant reduction of the stress responses associated with direct agar contact. This method has proved to be an excellent system for studying nodulation, Nod factor responses, mycorrhization and nematode infection on composite plants with transformed roots (Boisson-Dernier, Andriankaja *et al*., 2005; see also Chapter “Rhizobial inoculation and nodulation of *Medicago truncatula*”).

Water or medium-saturated pre-cut pieces of pouch paper (see below) are laid onto solid agar plates. The paper can usually be limited to the zone supporting the root system, thus allowing better illumination of the leaves within a stack of plates.

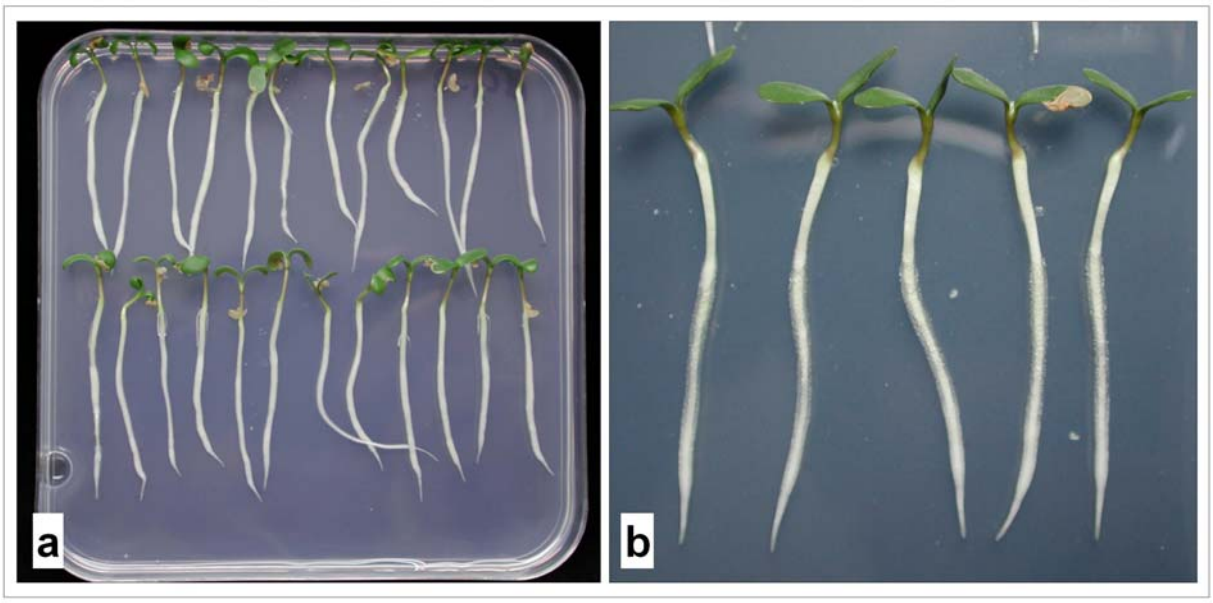

Figure 2 - Culture of seedlings on agar plates
a: Depending on the goal and duration of the experiment, either 1 or 2 rows of 10-15 seedlings / row can be grown on square agar plates (12x12 cm). Here, 2 rows of seedlings had been cultured for 4 days in an *in vitro* growth room. Note that root growth rate is slower than in the pouch system, and that primary roots also tend to grow obliquely. **b**: Close-up showing the "Thick Short Root" phenotype of these seedlings, generally attributed to stress effects; the TSR roots are relatively thick and form long root hairs.

Agar slants in tubes

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. This system can for example be very useful in nodulation assays (Chapter “Rhizobial inoculation and nodulation of *Medicago truncatula*”, and relevant figure therein).

Culture conditions

Both agar plates and tubes should be placed vertically in an *in vitro* growth chamber with moderate light intensity to avoid above-optimal temperatures inside the closed plates / tubes. Shielding roots from direct light (for example, by using dark plastic liners wrapped in aluminium foil) may also be a useful precaution against physiological stress.

Specific material

- Square Petri dishes 12x12 cm with 50 ml of agar growth medium or 24x24cm dishes with 200 ml agar are generally used.
- For paper / agar plate overlays, plain "38# seed germination paper" (the same as that used in Mega International pouches) can be directly purchased from Anchor Paper Company (www.anchorpaper.com) and cut to custom sizes. We recommend cutting dimensions of 4.6x18 inches (with the paper crepe running lengthwise, i.e. vertically when in the plates). This is convenient for covering 4-5 square 12x12 cm Petri plates after saturation with growth medium.

E Aeroponic caissons

Aeroponic growth of *M. truncatula* is both rapid and vigorous and has long been established and used in a number of studies on *Mt* root biology. The aeroponic caisson system is suitable for any growth stage, from seedlings to large plants, and yields vigorous plants in excellent physiological condition (Fig. 3a, and 3h-j). Roots can be readily sampled from the caisson, and the composition of the nutrient liquid medium can be easily controlled and modified. Moreover this system limits variability between individual plants grown in the same caisson, provided that care is taken to ensure uniform lighting and uniform access of roots to the nutrient mist (see § A and Appendix 1).

The aeroponic caisson comprises a large plastic chamber with a perforated lid on top and a mist generator at the bottom which circulates the nutrient solution as a fine mist throughout the container. Plantlet roots are inserted through holes in the upper lid and the roots subsequently grow in a permanent and homogeneous nutrient mist. The model used in the LIPM, Toulouse, (originally designed by René Odorico, LIPM) has been engineered from large wheeled waste bins (Fig. 3a, 3b). The removable lid can be perforated with a variety of hole densities. A 40x40 cm^2 area with up to 1,500 holes (4 mm diameter) works well for seedlings (Fig. 3d). For long term experiments 40-80 plants can be successfully grown with this size of caisson. Aeroponic caissons can be constructed in any workshop, and technical details can be obtained from the LIPM upon request. Variants of such a system have been developed in other laboratories, including the ISV (CNRS, Gif/Yvette, France; http://www.isv.cnrs-gif.fr/embo01/manuels/index.html) and laboratories in the U.S. (e. g. protocol contributed by Doug Cook's lab at http://medicago.org/documents/Protocols/AeroponicChamber081001.html).

Setting up the aeroponic system

1. Although the aeroponic system is not rigorously axenic, it is essential to sterilize, prior to each experiment, both the inside and the lid of the caisson with diluted bleach (250 ml commercial sodium hypochlorite at 9.6 % active chlorine in 10 liters) for several hours. Extensively rinse with sterile de-ionised water after bleach treatment. The liquid is removed with a vacuum cleaner with a long hose attachment and disinfected hose tip. Allow aeration in a clean environment for 24 h to eliminate traces of chlorine. Prior to experiments sensitive to microbial contaminations, it is recommended to sterilize the lid, rubber seals and the upper edges of the caisson once again using 70 % ethanol. Then, perform the following steps using as clean materials and conditions as possible (tools, hands, germination plates and the outer surface of the adhesive tape …). For risks and detection of rhizobial contamination, see also Chapter "Rhizobial inoculation and nodulation of *Medicago truncatula*".

2. Prepare the lid as follows : cover the entire perforated area with strong adhesive tape to obtain a waterproof seal. Puncture the tape above the holes in the lid with a scalpel blade (2 crossed incisions works best to avoid damaging the delicate hypocotyl).

3. Add 8-10 liters of nutrient medium (see Appendix 2) and start the humidifier. Close the tank.

4. Since seedlings are very sensitive to air desiccation (see §B), use germinated seeds with sufficient radicle length (i.e. ≥ 1 cm) so that they will be immediately exposed to the mist in the chamber once inserted into the caisson lid. It is advisable to submerge germinated seeds in sterile water in Petri plates (Fig. 3c) while awaiting transfer to the caisson. Plant the seedlings with the help of forceps through the tape and lid holes so that the cotyledons

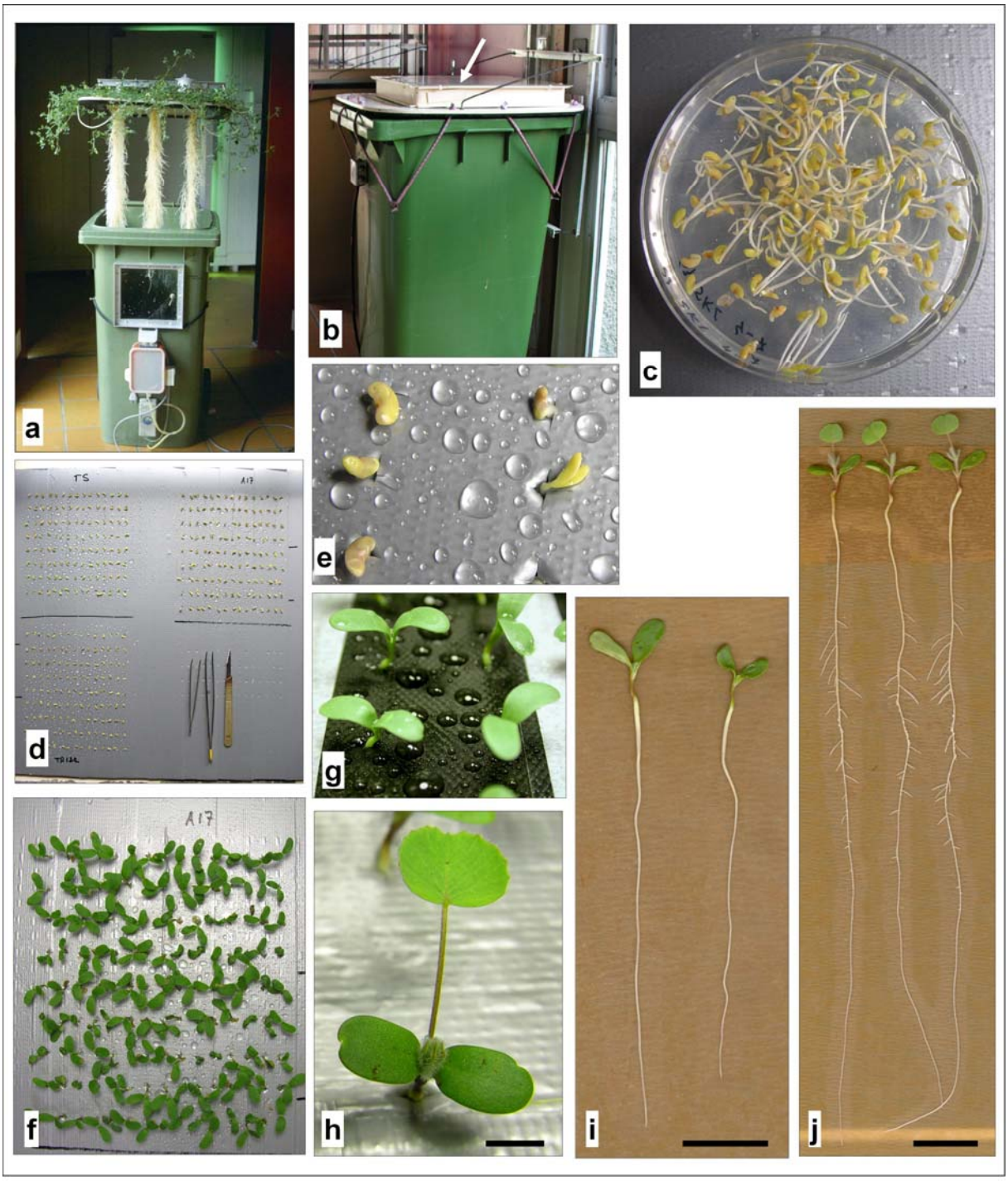

Figure 3 - Aeroponic culture
a: Front-view of an aeroponic "caisson" containing 4 week-old *M. truncatula* plants (3 rows of 5); the lid can be lifted and suspended above to allow access to the large and fast-growing root systems. This aeroponic system was originally designed by René Odorico at LIPM-Toulouse. **b**: Side-view of an aeroponic caisson with lid closed and seedlings covered with a tight fitting cover which includes an upper transparent plexiglass window (white arrow). **c**: Petri plate containing a batch of germinated seeds submerged in sterile water, just before transfer. **d**: Top-view of lid covered with punctured adhesive tape into which 380 germinated seedlings have just been transplanted. **e**: Detail of the aerial part of the seedling; seed

(Figure 3, continued)
teguments have been removed and sterile water regularly sprayed during planting. **f**: Top-view of seedlings after 3 days of aeroponic culture. **g**: Seedlings after 4 days of aeroponic culture, showing fully expanded cotyledons. **h**: 6 day-old seedling shoot bearing its first, monolobed leaf; bar = 5 mm. **i**: 3 day-old seedlings after removal from aeroponic caisson; bar = 2 cm. **j**: 7 day-old seedlings after removal from aeroponic caisson; the microsymbiont *Sinorhizobium meliloti* was inoculated at the time of planting and young nodules are visible between the lateral roots; bar = 2 cm.

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are 1-2 mm above the tape (Fig. 3d, 3e). Removing the loose seed teguments at this stage will allow more homogeneous cotyledon expansion. Regularly spray a mist of sterile water onto the seedlings during this operation to maintain high humidity.

5. Protect the seedlings with a tight fitting, disinfected cover with a transparent plexiglass window (Fig. 3b). Transfer the caisson to the growth room. The cover, which maintains a moist atmosphere is necessary for at least the first 48 h of culture until the primary roots are sufficiently long. If the lid surface dries out despite the cover, spray additional sterile water under the cover as needed during this initial period. The appearance of seedlings at various times of aeroponic culture is illustrated in Fig. 3f-3j.

Remarks

- Check that the glue of the adhesive tape is water resistant, otherwise leaks will quickly appear and unwanted chemicals be dissolved in the medium. Light-grey or clear-colored tape will absorb less heat from the incident light.
- If evaporation of nutrient medium causes the formation of white salt deposits on the hypocotyls, wash each hypocotyl by spraying sterile water from a water bottle. Otherwise plantlets will die. This can be done as a precaution every day.
- The level of the nutrient solution is readjusted by adding sterile deionised water (preferable to nutrient solution). Ideally the nutrient medium should be renewed once a week to prevent problems due to differential nutrient consumption, release of root exudates and potential microbe proliferation.

Specific materials / chemicals

- Aeroponic caisson (technical specifications available from LIPM upon request)
- Transparent plastic cover
- Humidifier (Defensor, France)
- Nutritive solutions (see Appendix)
- Bleach : dilute commercial sodium hypochlorite solution (9.6 % active chlorine) 40 times on the day of use. Commercial bleach in original sealed flasks is chemically unstable and should not be used beyond 3 month-storage. Alternatively, make up a fresh, filtered calcium hypochlorite solution of equivalent active chlorine content.

F Hydroponic cultures and assays in liquid medium (S. Ruffel, M. Lepetit)

M. truncatula can also be grown and nodulated with success in hydroponic systems. Although growth of *M. truncatula* roots is generally hampered in water-saturated environments (e.g. see §C & G), this negative effect (probably due to oxygen limitation) can be satisfactorily overcome by vigorous aeration of the liquid in hydroponic cultures. One advantage of hydroponics is to allow a better control of ion concentration around the root than aeroponics. The system described below is used routinely in the B&PMP lab (Montpellier, France; S. Ruffel, P. Tillard, and M. Lepetit, unpublished).

After chemical scarification and cold treatment (Chapter "Seed storage and germination"), *M. truncatula* seeds are germinated in the dark at 21°C between two sheets of filter paper on a PVC holder, itself on the top of a tank filled with tap water (Fig. 4a). In order to maintain humidity, the edges of the filter papers are immersed in water, and a plastic plate is placed on the top of the tank. Seeds germinate between the two paper sheets and young seedlings appear after 3 to 6 days.

When seeds have a root of about 4 cm in length, individual plantlets are transferred onto a polystyrene raft (2 cm thick) floating on HY nutrient solution (see Appendix 2; generally 36 plantlets / 10 liter solution). Each plant is maintained on the raft using a punched cylindrical piece of polystyrene (Fig. 4b). Vigorous aeration of the nutrient solution is achieved using an aquarium pump and a pumice stone diffuser (Rena; www.rena.net). The following environmental growth conditions are routinely used: light/dark cycle 8h/16h, 22°/20°C, 85% hygrometry, 250 $\mu mol.m^{-2}.s^{-1}$ PAR. Nutrient solutions are renewed once a week.

For inoculation with *Rhizobium*, plants are grown for 1 week on HY nutrient solution and subsequently on HY nutrient solution devoid of nitrogen, adjusted to pH 7.0 and containing a rhizobial suspension (OD_{600} = 0.015). Nodules appear during the first week after inoculation and N_2 fixation initiates during the second week. Nodulated plants are maintained on aerated N-free HY medium adjusted to pH 6.0, renewed once a week, until the end of the culture (Fig. 4c).

Note: Short-term experiments (less than 24 h) can be performed on *M. truncatula* seedlings with roots growing in vials containing Fahräeus medium without aeration. For example Charron *et al.* (2004) developed such a system to follow root hair development under conditions of minimal stress.

G Culture on solid substrates in the growth chamber or the greenhouse

M. truncatula can be grown on solid particulate substrates for various types of experiments, as well as for seed multiplication (see below) and crossings (see Chapter "Vernalization, crossings and testing for pollen viability"). Various protocols for *M. truncatula* culture on solid substrates are available in the literature and also at www.medicago.org/documents/Protocols/cultivate.html. Since it is not possible to directly compare their respective efficiencies, the authors' aim in this section is to indicate basic culture principles and standard methods used in their laboratories.

Growth containers can be tubes, pots or trays. The most usual culture conditions are either those of the growth chamber with high light intensities or the greenhouse. Growing plants in the greenhouse differs slightly from that in the growth room, mostly due to seasonal variations in environmental growth conditions. In temperate/continental areas, the best growing season in the greenhouse is from the end of winter to late spring (February - end of May in Northern

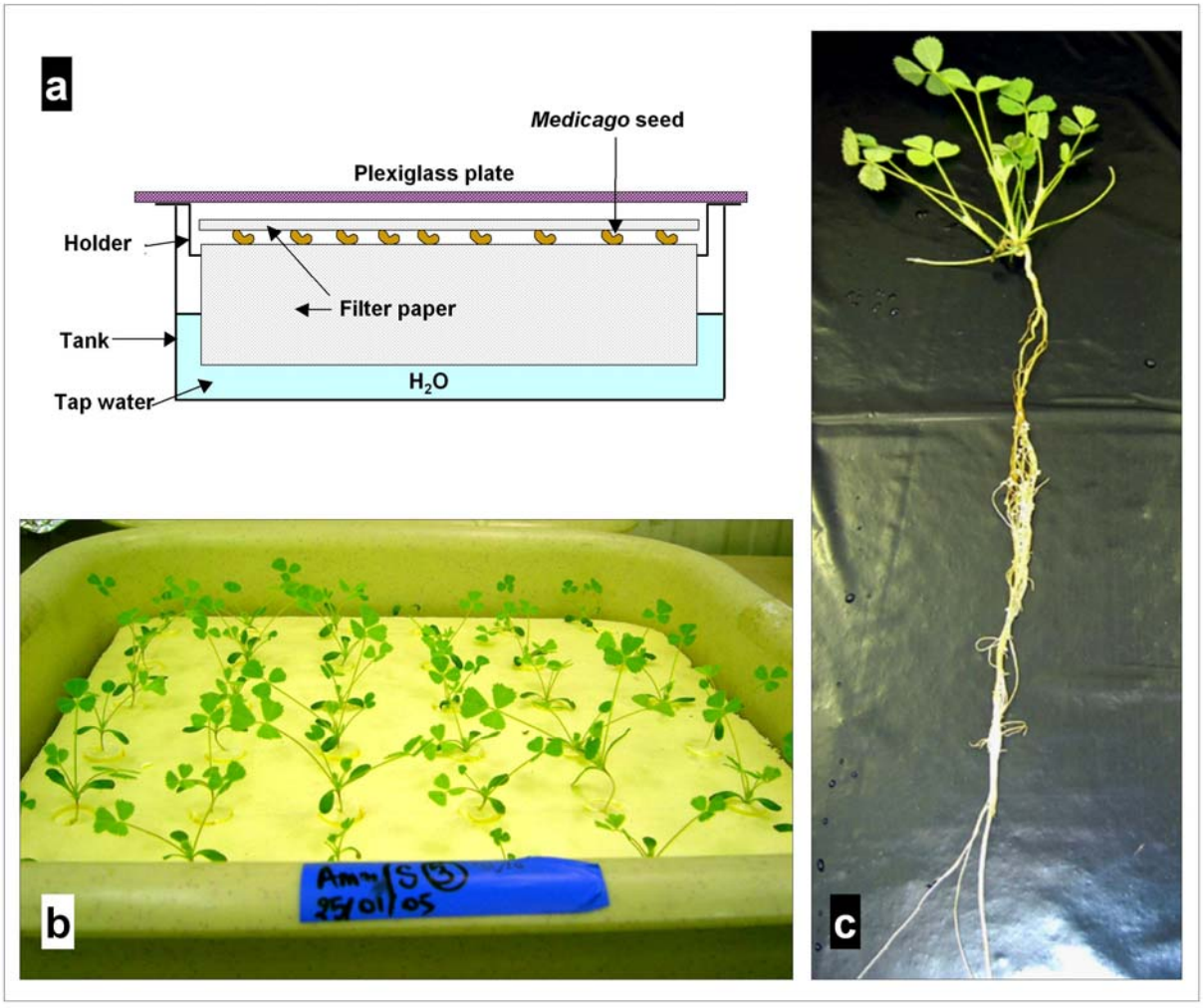

Figure 4 - Hydroponic culture

a: Germinating seeds for hydroponic culture. **b**: Polystyrene raft floating on HY nutrient solution and accommodating 36 plantlets / 10 liter solution. Each plant is maintained on a raft by a punched cylindrical piece of polystyrene. **c**: Appearance of a 4 week-old plant 2 weeks after rhizobial inoculation.

hemisphere). Mid-summer is the worst, mostly due to elevated temperatures leading to etiolated growth. In winter, slow growth can be partially overcome by supplementing light and precautions must be taken against the effects of high humidity (fungal attacks, risk of root rot).

Substrates

M. truncatula grows best on well drained, fairly dense substrates such as mixtures of sand and soil (Fig. 5a, 5b). Ratios of 1:2-1:3 (sand:soil, v/v) offer a good compromise between drainage and water/nutrient retention characteristics. A mixture of perlite and soil (1:3, v/v) also works well, especially when substrate humidity risks are high (e.g. saturating automatic irrigation or glasshouse culture during winter). Satisfactory growth can also be obtained on 100 % perlite, sand, or vermiculite. Perlite-grown plants are also ideal for nodulation assays. Soil-based

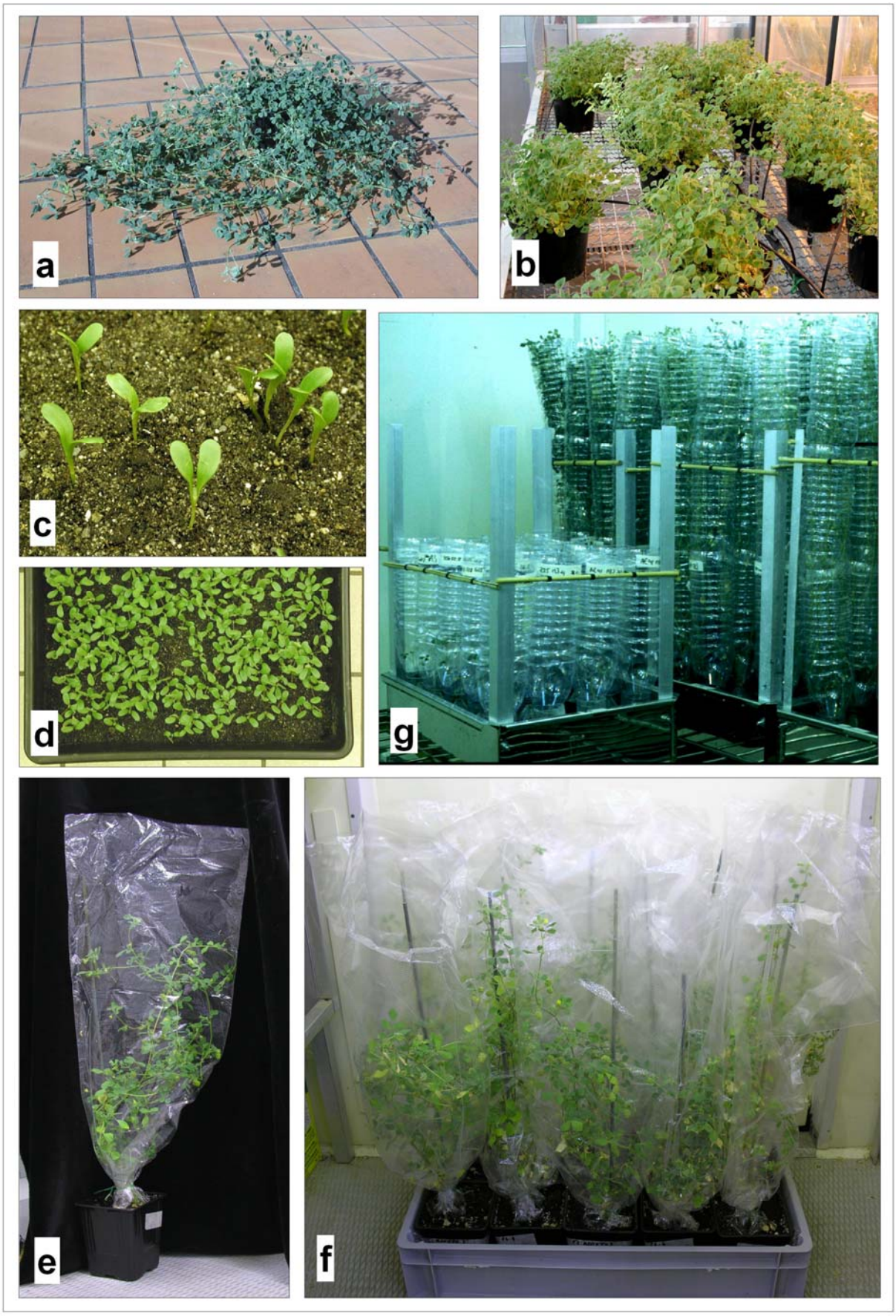

Figure 5 – Culture of *M. truncatula* on solid substrates and seed collection

(**Figure 5, continued)**

a: Non-vernalized, 6 week-old plant grown on a pot of soil/sand mixture. **b**: Glasshouse bench with pots containing 30 plants each; plants were vernalized for 7 days and grown for 3 weeks with automatic watering. **c**: High-density culture of *M. truncatula* in trays: first seedlings emerge 2 days after planting. **d**: 6 day-old high-density culture in trays. **e**: Simple and efficient system to safely collect mature pods for seed amplification; plants wrapped in an air-permeable sleeve are maintained in upright position by a vertical rod placed inside. **f**: Assembly of wrapped plants (here, 15 in a 40x60 cm tray) allowing recovery of progenies from many plants in parallel. **g**: Higher-density system for seed amplification: on the left, tray with young plants; on the right, three trays with 6 week-old plants growing inside transparent mineral water bottles.substrates should be avoided in *in vitro* growth chambers with reduced light intensities because of risks of root rot and fungal development. In general, solid substrate components should be autoclaved before use, except for some commercial soil mixtures.

- Soil : commercial, peat moss-based synthetic soils with a fine structure and slight acidity (pH 5.5 to 6) are particularly good. Usually, such commercial soil is already disinfected and does not need to be autoclaved before use. All-purpose compost batches should be tested before use, as *M. truncatula* may not grow well on excessively rich substrates.
- Sand : river sand with a range of granulometric sizes is convenient.
- Perlite is made by expansion of volcanic rocks. The white granules with a hard surface are inert, aerated, hydrophilic and insulating. Some raw batches of perlite may have a high pH (>8.0) that strongly inhibits *M. truncatula* growth. Therefore, it is critical to check perlite pH and, if necessary, to wash/neutralize it before use.
- Vermiculite originates from laminar mineral. Treatment leading to expansion produces foliated inert, neutral (pH 7.0), hydrophilic and insulating particles. As a matter of precaution, vermiculite should also be washed before use.

- other particulate supports (non comprehensive list):
  - expanded clay;
  - Turface (A.H. Hummert Seed, St.Louis, MO);
  - synthetic soil (Metro-Mix 360; Scotts-Sierra Horticultural Products Co., Marysville, OH, USA / or Sungro Horticulture www.sungro.com) (Wang and Grusak, 2005).
  - modified University of California mix (Matkin & Chandler, 1957) used to grow *M. truncatula* in the greenhouse at SARDI Waite campus, Adelaide (contributed by R. M. Nair). Procedure: 1,200 litres (2 bins) of Waikerie sand is sterilized at 100°C for at least 45 minutes. 750 litres of peatmoss is added and mixed for 5 min. Extra water can be added at this stage. After 20 min the following ingredients: 1,520 g calcium hydroxide (hydrated lime) and 2,000 g calcium carbonate (Ag lime) are mixed in for 5 min. This should produce 3 bins of UC base. This is left for at least 24 h to cool. Each bin of UC base is put into a mixer and 1.5 kg of mini osmocote is added and mixed for 5 min. The pH of the mix is maintained between 6 and 6.5.
  - modified *Arabidopsis* mix (a lighter soil-like substrate, light enough to use in growth cabinets used at CSIRO Plant Industry Floreat). Consists of 8 litres shamrock peat, 7 litres coco coir, 8 litres medium perlite, 20 litres fine vermiculite and 20 ml dolomite

mixed together; this mixture is relatively high in organic nitrogen and is not suitable for nodulation experiments.

Planting seedlings / plantlets

One to several seedlings can be planted and grown for 3-4 weeks in a small pot (for example 8x8x7 cm; 400 ml vol). Young plants (especially non-vernalized ones) then need to be transplanted into larger pots (up to 2 l). Seedlings are planted one by one by hand. Radicles should be quite long (2-4 cm) to ensure fast and deep rooting. First, saturate and pack down the substrate with water. Make a vertical hole in the substrate with a spatula and gently insert the whole seedling radicle and hypocotyl, leaving just the cotyledons above the surface. Close the hole by side pressure and with generous sprinkling of water, so that the seedling is firmly anchored in the substrate; otherwise it may be pushed upwards due to fast primary root growth. It is worthwhile to cover the pots with Saran wrap / a glass plate to maintain high humidity and avoid intense direct lighting for at least 2 days. After this, plantlets can easily withstand dry and windy air conditions that may be present in the growth chamber.

Some experiments require that many seedlings be planted in large pots or trays at high densities, e.g. to generate batch progenies from mutagenized populations, to screen plantlet phenotypes, etc… (the maximum seedling density leaving enough space for cotyledon expansion is around 4,000/m²). For rapidity, it is more convenient to sow seeds or seedlings by batches, and two practical points need special consideration: the homogeneity of plant distribution, and proper rooting into the substrate.

If the substrate is a sand/soil mixture, it should only be slightly packed down by water soaking to enable quick root penetration (see below). Dry seeds - after mechanical scarification - can be easily sown in homogeneous densities. In contrast, either wet seeds (after chemical scarification) or seedlings tend to stick to each other in clumps that will not grow well. To circumvent this, suspend the seeds/seedlings in liquid agar gel (0.10-0.15 %, w/v, adjust to the right concentration that prevents seed sedimentation; 100 ml for 50-100 seeds/seedlings; see also Penmetsa et *al.*, 2000 and http://medicago.ucdavis.edu/Protocols/EMS.html) and evenly pour the suspension onto the soil surface. In order to ensure efficient rooting, seeds/seedlings are then overlaid with a fine dry sand/soil mixture (1:1, sieved to eliminate larger particles; 10-15 mm thick). Evenly spread and gently pack down this top layer using a flat tool (e.g. a Petri dish) and humidify it with a fine water spray. In this way, growing hypocotyls cannot lift the cotyledons upwards through the top layer until the root is well anchored and resting on the substrate. Cover the container with Saran wrap / a glass plate to maintain high humidity - at this stage, it is possible to transfer the trays to the cold room for synchronisation of germination or seed vernalization. Once all viable seedlings have sprouted from the substrate (after 2-3 days, Fig. 5c), gradually remove (or puncture) the cover and maintain watering with a fine spray for the first week. This procedure yields a high proportion of well-germinated seed (Fig. 5d). Note that whenever more than 5 % of the emerged seedlings have their roots sticking out in the air it means that the top layer is too thin.

Watering and fertilization

M. truncatula prefers substrates that are not saturated with water and is quite resistant to transient water shortage. WARNING: it is **critical** not to over-water the plants, i.e. to avoid permanent water-saturation of the substrate, otherwise the root will rot, probably due to oxygen shortage. Progressive, non-reversible leaf wilting is the most obvious over-watering symptom but by this stage it is usually too late!

To avoid this problem, allow the soil to partially dry out between waterings. When pots are placed on water trays, excess water should not stagnate in trays more than 12 h consecutively, especially for slow-growing plants (vernalized or older plants) or under low light intensities. Plants in the same growth room but with varied developmental/physiological status probably need very different amounts of water - a situation that requires individual care for growing success. On the other hand, automatic irrigation is well suited for growing sets of plants at the same developmental stage. In this case it is critical that excess water is rapidly drained off (i.e. no water trays under the pots).

When water trays are used, the wash-through (if any) is not eliminated from the trays and therefore it is subsequently reabsorbed. The situation is similar when an automatic irrigation system does not deliver saturating amounts of water. In both cases, the substrate is actually not washed through. Thus, salts and chlorinated compounds from tap water (and possibly also excessive fertilizers and root exudates) accumulate over weeks in the substrate, and may lead to a decrease in growth rate, followed by wilting symptoms and death of plants (especially slow-growing ones). Therefore, use of ***de-ionized water*** is highly recommended for culture in pots with water trays. Alternatively, perform an extensive rinsing of the soil every 2-3 weeks by adding at least 1 pot-equivalent volume of water, followed by some fertilizing solution to restore nutrient availability. Whenever wilting symptoms caused by salt accumulation appear, they should disappear within 2 days after such a washing.

In conclusion, wilting symptoms can have very different causes (lack of water, salt stress, over-watering), and it is important to correctly interpret the situation in order to take appropriate steps.

Fertilizer (complete nutrient solution, unless specific mineral supply is required such as N- or P-limited formulas for nodulation or mycorrhization, respectively) should be added in reasonable amounts to the plants, thus preventing premature yellowing of older leaves which is a symptom of nutrient shortage. Depending on the growth set-up, watering system and user's preference, fertilization can be performed at regular intervals (e.g. once a week to fast-growing plants) or permanently. A number of commercial liquid concentrated formulas give satisfactory results, although some of them may cause progressive chlorosis symptoms after several weeks of use. At LIPM-Toulouse, the best, long lasting fertilization effects have been obtained by adding weekly an NPK (6:3:6) mixture supplemented with micronutrients ("Engrais plantes d'intérieur", Substral, KB; www.kb-jardin.com/page.php?rub=3&idproduit=46) with the following composition: 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, 0.002 % Cu-EDTA, 0.01 % Mn-EDTA, 0.002 % Zn-EDTA, 0.03 % Fe-DTPA). We have found that fertilizers enriched in phosphate (supposed to promote flowering) are somewhat detrimental in the long-term.

H Seed multiplication and collection

In growth chambers with high light intensity and in the greenhouse, plants grown on mixed substrates (soil and sand/perlite/vermiculite) in larger pots display vigorous growth (Fig 5a, 5b) and abundant seed production. In growth chambers, *M. truncatula* c.v. Jemalong has a generation time of approximately 10 weeks from seed to seed. More information about practical aspects of *M. truncatula* reproductive biology is available in Chapter “Vernalization, crossings and testing for pollen viability”.

Non-vernalized plants appreciate sufficient space to spread (up to 1 m^2/plant). Under optimal conditions, they can yield several thousands of seeds/plant within 4 months. When only moderate amounts of seed per plant are sufficient, it is more convenient to vernalize the

mother plants (see Chapter "Vernalization, crossings and testing for pollen viability") so as to reduce plant size at the flowering stage. Plants vernalized for 1-2 weeks yield at least 150 seeds and up to 500 can be easily obtained.

Pods readily fall from the plant once they are ripe. ***Therefore, we emphasize the need to be extremely cautious when growing different genotypes side-by-side***. Various systems can be used to collect fallen pods during culture and prevent mixing of progenies. When space is not limiting, aerial parts / whole plants can be placed in large trays (5-15 cm deep) and mature / detached pods harvested manually at regular intervals. For a limited number of plants, this is the simplest system giving the best yield/plant and the lowest risk of fungal/pest infection.

Vernalized plants can be grown in higher densities owing to their reduced shoot ramification (see Chapter "Vernalization, crossings and testing for pollen viability"). A system with a good compromise between population density (40x60 cm trays, 15 plants/tray in 11x11x12 cm pots, i.e. up to 60 plants/m^2), sufficient air circulation and error-free pod collection is shown in Fig. 5e-f. Before the first maturing pods turn yellow-grey, a rod is planted vertically in each pot and the whole plant is wrapped in an air-permeable, transparent plastic sleeve (e.g. "bakery perforated film", 300x700 mm; Sealed Air / Cryovac Corp.). The sleeve is tied around the shoot base in order to collect fallen pods at a distance from the humid substrate, and the upper part of the sleeve is cut wide open to ease shoot ventilation and elongation. The rod / sleeve assembly maintains all branches in an upright position.

Alternatively, a system with even higher plant densities (50-100/m^2) can be used, similar to the "Aracon" devised for *Arabidopsis* multiplication. It is well adapted to the production of numerous distinct progenies in moderate seed quantities, e.g. as needed following genetic screens (contribution of Fabienne Maillet and Jean Dénarié, LIPM-Toulouse). Transparent plastic mineral water bottles (one on top of the other with the narrow neck removed; ventilation holes punched on the sides) are progressively placed over the elongating primary stem. A plastic cup with a central hole is placed over the pot (8x8x7 cm) at the seedling stage and will collect the detached, mature pods before final harvest (Fig. 5g). This system, less ventilated and less accessible for phytosanitary treatments, should be used in growth rooms with a low hygrometry in order to prevent the spread of fungal diseases.

I Treatments against pests and diseases (Theo Pfaff & Emma Groves)

All plants can suffer from diseases or pests, and those growing in a greenhouse or growth room are of course not an exception. For the success of experiments, it is important that pests or fungi do not cause excessive damage or interfere with plant growth. For all treatments, the correct diagnosis of the pest problem is essential and it is not sufficient merely to know the broad category of the pest.

Below are listed the most common challengers along with the measures and treatments adopted by the ACNFP Murdoch (followed by those used at the LIPM-Toulouse, contributed by Jean-Luc Pariente), that keep such outbreaks under control.

The treatments should be applied in rotation to avoid the appearance of tolerance / resistance in pests. When required, treat plants 2-3 times with 1 week intervals to ensure killing the insects or fungi which were at the egg / spore stage during the first application. The procedure may differ depending on the type of facility (growth room or greenhouse). If biological control solutions are preferred, this of course precludes the use of synthetic pesticides during the same period. The advantage of biological control (very effective in most cases) is to avoid the health hazards associated with pesticide treatments. This approach is of course not aimed to totally eliminate the pests, but to keep them below nuisance levels.

Common pests and diseases

Aphids (*Aphis craccivora* (cowpea aphid), *Acyrthosiphon kondoi* (bluegreen aphid), *Therioaphis trifolii fm. maculata* (spotted alfalfa aphid), *Myzus persicae*, *Macrosiphum euphorbia*, *Aulacorthum solani*, among others)

Small sap-sucking insects that attack new growth and flower buds. They are found in clusters of green or brown insects on flower buds and young shoots. Best treatment is with Malathion Rogor or Mavrik. Small infestations can be effectively controlled with Confidor (200 g/l imidacloprid), a systemic pesticide applied either as a spray or preventive soil drench.

LIPM, Toulouse: Poliaxe (Imidaclopride), Pirimor (Pyrimicarbe), or biological control using *Aphidius colemani* (parasitoid insect)

Thrips (*Frankliniella occidentalis*)

Thrips are small sap sucking insects, just visible to the naked eye. They attack young plants and flower buds and can cause severe damage to plants if not controlled. Small brown marks on the petals, silver tracks on leaf surfaces, tiny black spots and distorted young foliage are indications of their presence. Thrips prefer sheltered positions and are often found feeding on pollen in flowers, on the underside of leaves or on the pot soil surface. Such living habitats combined with the bushy nature of large *Medicago* plants makes it difficult (if not impossible) to totally eliminate thrips with insecticides. Moreover, thrips multiply at a very fast rate so it is important to act quickly when the first signs of thrips infestation become visible. We apply insecticides such as Confidor or Malathion on a routine basis. Alternatively, Rogor or Mavrik can be used. Finally, as thrips can "overwinter" in dead or dying plant material and soil, ***it is good practice to remove plants as soon as possible as well as all decaying parts (fallen leaves, branches) from growing spaces.***

LIPM, Toulouse: Vertimec (Abamectin), Dicarzol (Formetanate), or biological control using *Amblyseius cucumeris* (predatory mite)

Fungus gnats (*Bradysia* spp.)

Most species of dark-winged fungus gnats feed on fungi and decaying organic matter and are not considered economic problems. However, we found that they attack healthy tissue of young *Medicago* plants causing injury to the roots. There are two stages when an infestation becomes visible. Normally you will be aware of an increasing population when you find them in the air-filter or flying around your head while you are potting plants. Otherwise you may observe the larvae (3 mm long, white with a black head), which are capable of ring-barking young seedlings and causing great damage, just bellow the soil surface and at the bottom of pots of 8 to 10 day-old plants. To control the population we recommend a combination of treatments. Pestigas (Pyrethrum aerosol), which is effective, fast working and non-persistent, controls the adult phase of the fly on contact. Do not apply Pyrethrum in high concentrations, because it is phytotoxic to leaves of *Medicago*. Confidor (imidacloprid) also works on fungus gnats on contact with adults; and it can also protect seedlings systemically against the larvae when added to the soil as a drench. Together with Pestigas two other methods of control can be employed. Firstly, a layer of white sand can be added on top of the pots containing soil to deter adult gnats from laying eggs. Secondly, we routinely drench all pots or soil mix with the Bt larvicide 'VectoBac12AS' (Synonym: "Gnatrol", Valent BioSciences Corp.), which attacks the larval stage of development. We apply 4.5 ml/l to the top layer of the pots. Over the long term you can control the fungal gnat's population by using 1.5ml/l, applied every 2-3 weeks.

LIPM, Toulouse: the most efficient is to target larvae with a vermicide containing Diazinon. Adults can be trapped on chromo-attractive plaques covered with glue.

Red Spider Mites (*Tetranychidae*)

These are tiny round arachnids with red/ black bodies visible to the naked eye. In the warmer months they are present as two-spotted mites with a pale colored body and two dark spots on their backs. They can become a problem when greenhouse conditions are warm and dry and plants are prone to drought stress. They cause chlorotic pinpricks on leaves and when infestation is severe leaves appear scorched and dry. The mites also spin a fine web-like structure over leaves of the *Medicago* plant and they are found mostly on the underside of leaves. We have found that application of a suitable specific targeting miticide such as Red Spider Miticide is sufficient to control outbreaks of Red Spider mites in our glasshouses. Lime Sulpher insecticide/fungicide (200 g/l sulphur) can be very effective in controlling spider mites (it is also a good powdery mildew control) which is best sprayed in the cooler part of the day to avoid phytotoxicity.

LIPM, Toulouse: Abyss (Bromopropylate).

Powdery Mildew (*Erysiphe trifolii* among others)

The name of this fungal disease is from the white, powdery appearance on the upper sides of leaves and stems. Powdery mildew is caused by a number of different fungi. There are all obligate parasites, surviving only on living tissue. The fungi produce masses of spores, which become airborne and spread to other plants and it becomes a serious problem for *M. truncatula* under humid and warm conditions. This fungal infection can be controlled with fungicides such as Mancozeb Plus, Benlate or Bravo. Lime sulfur insecticide/fungicide (200 g/l sulfur), Bayleton garden fungicide (50 g/l Triadimefon) or Tilt are very effective in controlling heavy outbreaks of powdery mildew. Other applications such as Copper oxychloride and Mancozeb (sulphur and manganese) are useful if applied at the first signs of infection. 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.

LIPM, Toulouse: treatment with a sulfur-based product (e.g. "Sovi Soufre", containing triturated / ventilated sulphur) is very efficient.

Whiteflies (*Trialeurodes vaporarium, Bemisia tabaci*)

Whiteflies have a wide host range and thrive on hundreds of cultured plant species including *M. truncatula*. Whiteflies develop on the undersides of leaves and feed on plants using piercing-sucking mouthparts, causing stunted growth, leaf yellowing, and reduced yields. Whiteflies also excrete honeydew, which promotes sooty mold that interferes with photosynthesis. They are able to reproduce quickly and spread rapidly and high densities of whiteflies can even lead to host death. Biological treatments with predatory (*Macrolophus*) or parasitoid (*Encarsia*) insects work very efficiently in greenhouses at the LIPM.

Additional cultural control:

Greenhouse soil is always steamed in order to prevent a possible outbreak of soil-borne diseases (e.g. *Pythium*, *Phytophthora* and *Fusarium*).

Table 2 - Pesticides and Fungicides used in the Greenhouse

Pesticides		
Trade name	**Active Ingredient**	**Comments**
Malathion	Diethyl-dimethoxyphosphinothioyl-thio-butanedioate	Organophosphorus insecticide
Rogor	Dimethoate	Systemic Insecticide
Mavrik	S-Kinoprene	Amino-acid derived insecticide
Confidor	Imidacloprid	Systemic Insecticide
Red Spider Miticide	Dicofol	Selective miticide
VectoBac12AS	Protein from *Bacillus thuringiensis* subsp. *israelensis*	Biological larvicide
Pestigas	Pyrethrum aerosol	Biological
Fungicides		
Mancozeb Plus	Mixture of Mancozeb & Sulphur	Systemic fungicides
Benlate	Benomyl	Systemic benzimidazole fungicide
Bravo	Chlorothalonil	Broad spectrum fungicide
Tilt	Propiconazole	Strong fungicide

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Appendix 1

Temperature, light intensity and photoperiod settings for the growth chamber and greenhouse (Delphine Moreau)

Impact of environmental factors on plant development and growth

A phenotype can be characterised using development variables (such as the number of initiated leaves, the number of "appeared" leaves (fully unfolded) or the date of the beginning of flowering; see Moreau *et al.*, 2006) or growth variables (such as plant biomass, leaf area or the quantity of nitrogen in the plant). Plant development and growth are strongly dependent upon environmental conditions:

- the main environmental factor governing plant development is temperature. Photoperiod can also have an impact, especially influencing the date of flower initiation (for review, see Kiniry *et al.* 1991);
- light is the main environmental factor governing plant growth: growth is affected by variations of Photosynthetic Active Radiation (PAR) intensity, PAR quality and photoperiod (for review, see Sinclair & Muchow 1999). Mineral (especially nitrogen) and water nutrition are also known to influence plant growth.

Thus, phenotypes are strongly dependent upon the environmental conditions for growth, making the choice of the temperature, light intensity and photoperiod settings of crucial importance.

Choosing temperature settings using an ecophysiological model

Plant developmental stages are best characterised by the number of appeared leaves on the first primary branch (B0). For stages prior to B0 emergence, plant development can be characterised by the number of leaves on the main axis (MA) (see Chapters "Morphology, development and plant architecture of *M. truncatula* " and "Phenotyping"). Since temperature is the main environmental factor affecting plant vegetative development, each developmental stage occurs at a given thermal time (Moreau *et al.* 2006). The model of vegetative development presented in Chapter "Mutant screening / phenology key" allows the prediction of the time necessary for the different developmental stages from temperature inputs. This model can be used along with measurements of actual temperatures to choose appropriate temperature settings.

Choosing light settings in order to avoid carbon limitation

For many experiments conducted in controlled environments, photosynthetically active radiations (PAR) are a limiting factor for plant growth. Indeed even the highest light fluxes in controlled environments are lower than those commonly observed in the natural habitats of *M. truncatula* (up to 1,500 µmol.m^{-2}.s^{-1}). Limiting light intensities can cause variable levels of carbon limitation and are likely to affect gene expression or plant phenotypes. If this is a concern, PAR values lower than 300 µmol m^{-2} s^{-1} should be avoided. Moreover, when choosing settings, it is recommended that high temperatures are associated with high PAR in order to avoid plant etiolation.

Importance of characterising the environmental conditions during plant culture

Gene expression is likely to vary according to the environmental conditions in which the plants are grown (Ndong *et al.*, 2001). It is therefore necessary first to ensure reproducible conditions between experiments and thus to precisely characterise the environmental conditions for each experiment in order to obtain reproducible data and to allow comparison

of data from different experiments. It is recommended that, as a minimum, temperature conditions are dynamically characterised throughout plant culture. For a precise characterisation, temperature can be regularly measured using several PT100 sensors (Pyro-Contrôle, Vaulx-en-Velin, France) distributed in the culture room, and the data can be stored in a data logger (DL2e; Delta-T Devices, Cambridge, England). To control temperature settings, simpler and cheaper measurements can be performed using Testo sensors (www.testo.com).

Appendix 2
Composition of Plant Culture Media

1. 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]	g/l	M		
$MgSO_4$, 7 H_2O	123.2	0.5	1.0	0.5 mM
KH_2PO_4	95.3	0.7	1.0	0.7 mM
Na_2HPO_4, $2H_2O$	71.2	0.4	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.
- Aliquot into 250 or 500 ml flasks and sterilize (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, in the case of pouches it is possible to add 1/100 V of 100 mM $CaCl_2$ directly in the middle of each pouch.

2. Aeroponic nutrient medium (LIPM formula; Lullien et *al.*, 1987)

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			10ml	
H_3BO_3	2 g/l	30 mM		30 µM
$MnSO_4$, H_2O	1.8 g/l	10.6 mM		10.6 µM
$ZnSO_4$, 7 H_2O	0.2 g/l	700 µM		0.7 µM
$CuSO_4$, 5 H_2O	0.08 g/l	3.2 mM		3.2 µM
Na_2MoO_4, 2 H_2O	0.25 g/l	1 mM		1 µM
$CoCl_2$, $6H_2O$	0.02 g/l	84 µM		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 (respecting the list order) to 10 liters of sterile, de-ionized water . Mix well between each addition, and especially after the addition of the phosphate.
- This medium should be used fresh (on the same day). Do not store it at cold temperatures, since the salts will precipitate.

3. Hydroponic nutrient solution HY

Stock solutions	**Stock concentration**		**Volume (ml)** (for **10 liters** of 1x medium)	**Final Concentration**
KH_2PO_4	13.6 g/l	0.1 M	100 ml	1 mM
$MgSO_4$, 7 H_2O	24.6 g/l	0.1 M	100 ml	1 mM
K_2SO_4	17.4 g/l	0.1 M	25 ml	0.25 mM
$CaCl_2$	14.8 g/l	0.1 M	25 ml	0.25 mM
Fe-Na-EDTA		100 mM	10 ml	100 µM
Micronutrient mix			10ml	
H_3BO_3	1.85 g/l	30 mM		30 µM
$MnSO_4$, H_2O	0.85 g/l	5 mM		5 µM
$ZnSO_4$, 7 H_2O	0.29 g/l	1 mM		1 µM
$CuSO_4$, 5 H_2O	0.25 g/l	1 mM		1 µM
Na_2MoO_4, 2 H_20	0.17 g/l	0.7 mM		0.7 µM
KCl	3.72 g/l	50 mM		50 µM
NH_4NO_3 [1]	80 g/l	1 M	0.3-100 ml	0.3-10 mM
KNO_3[1]	101 g/l	1 M	0.3-100 ml	0.3-10 mM

[1] : the concentration of the nitrogen source depends on the experiment

- Add the stock solutions to 10 liters of de-ionized water
- Adjust to the appropriate pH (6 or 7) with KOH 2M