Rhizobial inoculation and nodulation of *Medicago truncatula*

E-P Journet[1], F. de Carvalho-Niebel[1], A. Andriankaja[1], T. Huguet[2] and D.G. Barker[1]

[1]Laboratoire des Interactions Plantes-Microorganismes (LIPM), CNRS-INRA, BP52627, 31326 Castanet-Tolosan Cedex, FRANCE.

contact : barker@toulouse.inra.fr

[2]Laboratoire Symbioses et Pathologies des Plantes, INP-ENSAT, BP 32607 Auzeville, 31326 Castanet Tolosan cedex, FRANCE.

contact : thierry.huguet@ensat.fr

Introduction

The endosymbiotic interaction between legumes such as *M. truncatula* and soil bacteria known as rhizobia involves the associated root internalisation (infection) of the microsymbiont and the induction and development of nodules, specialised nitrogen-fixing root organs. Nodulation is initiated when legume roots come into contact with free-living rhizobia, and *M. truncatula* nodulation assays usually involve inoculating the entire root system (previously grown in the absence of the microsymbiont) with a non-limiting inoculum of the appropriate *Sinorhizobium meliloti* strain. In wild-type *M. truncatula* genotypes, this results in a synchronous, transient wave of infection/nodulation of the regions of the root system susceptible to rhizobial infection - i.e. the developing root hair zone located behind the growing root tip Under these conditions further nodulation is suppressed for a certain period of time (in general at least 1 week) by the so-called systemic autoregulation mechanism (reviewed in Caetano-Anolles and Gresshoff, 1991). Therefore, the total number of nodules formed depends upon the size and degree of ramification of the root system at the time of inoculation. In addition, since nodulation is negatively regulated by the presence of combined nitrogen in the growth medium/substrate, nodulation assays must be performed in N-free or low N conditions (in general ≤ 1 mM NH_4NO_3). Nodulation is of course also dependent upon other physiological factors, but in general good plant growth results in efficient nodulation. Here we describe protocols for the nodulation of *M. truncatula* under a variety of plant growth conditions, including pot growth, aeroponic growth, growth in plastic/paper pouches and in test-tubes, as well as the nodulation of the hairy roots of composite plants following *A. rhizogenes* transformation (Chapter "*Agrobacterium rhizogenes*-mediated root transformation").°

## *I. Inoculation/nodulation of plants growing in pots/trays*

M. truncatula can be grown in well-drained pots or trays containing soil and/or other solid substrates (sand, perlite, vermiculite etc. see chapter "Growing *Medicago truncatula*: choice of substrates and growth conditions"). For reliable nodulation, it is advisable to pre-sterilise the substrates. A relatively large volume (~ 200 ml/L of substrate) of a diluted rhizobial suspension (e.g. $OD_{600} = 10^{-3}$) is poured onto the substrate surface of the pot/tray to initiate infection and subsequent nodulation.

II. Inoculation/nodulation of plants growing in aeroponic containers (caissons)

1. Decontaminated seeds are germinated as usual (see chapter "Seed storage and germination") and seedlings are transferred to an aeroponic container and grown as described in chapter "Growing *Medicago truncatula*: choice of substrates and growth conditions".

2. For rapid inoculations (within the first 4-5 days of growth and before the emergence of lateral roots), seedlings can be grown directly on nitrogen-free medium. Even seedlings inoculated immediately after transfer to the container nodulate efficiently on the primary root. (see Figure **1A**).

3. For delayed inoculations, grow plants to the desired size in nitrogen-rich medium (see chapter "Growing *Medicago truncatula*: choice of substrates and growth conditions"). Typical culture times vary between 1-3 weeks.
2-3 days before inoculation, replace the complete medium in the container by nitrogen-free medium so that plants will be partially N-starved at the moment of inoculation.

4. For inoculation, switch off the mist-generator and add to the container medium $1/10^3$ v/v of a fresh culture of *S. meliloti* grown in TY/Ca^{2+} medium [5g/l Bacto-tryptone, 3g/l Yeast extract, 6mM $CaCl_2$ (added after autoclaving), pH = 7.2] to early stationary phase (approx. 1.0 OD_{600}), and then washed in the N-free plant growth medium. This corresponds to a final concentration of ~10^6 bacteria/ml. Agitate the tank several times to disperse the inoculum in the growth medium and re-start the motor after the cover has been replaced.

Comments:

- Nodulation is rapid, efficient and synchronous in this system, with nodules first visible as small bumps 3 days post-inoculation, and nitrogen fixation starting 2-3 days later.
- Rhizobial cultures should be **fresh**. Storage of bacteria at 0-4°C before inoculation can delay the kinetics of nodulation by at least 24 h.
- In our experience, rhizobial contamination of aeroponic cultures prior to inoculation can easily occur when adequate precautions are not taken. The nitrogen-rich growth medium will generally inhibit infection/nodulation if there is such a contamination - however as soon as the nitrogen is removed, infection will initiate. Thus, premature nodulation is a strong indication of prior rhizobial contamination. It is therefore important to use thoroughly disinfected aeroponic containers (see chapter "Growing *Medicago truncatula*: choice of substrates and growth conditions") and to carry out all initial manipulations wherever possible under quasi-sterile conditions. In addition, if other aeroponic containers present in the same growth room have already been inoculated, beware of possible airborne rhizobial contamination – for this reason it is recommended to avoid opening tanks inside the growth room.
- Aeroponic-grown seedlings can also be used for the analysis of early Nod factor responses. After growth for 2-3 days in nitrogen-free medium, seedlings can be "pulled out" from the container lid with the help of forceps and transferred to tubes containing Nod factor solutions. At this stage seedling are about 8-9 cm long and without laterals. Nod factor treatment of these seedling roots results in strong and homogeneous NF-induced responses such as epidermal *ENOD* gene activation. Seedling roots can then be directly analysed or frozen in liquid nitrogen and stored at -80°C for later analysis.
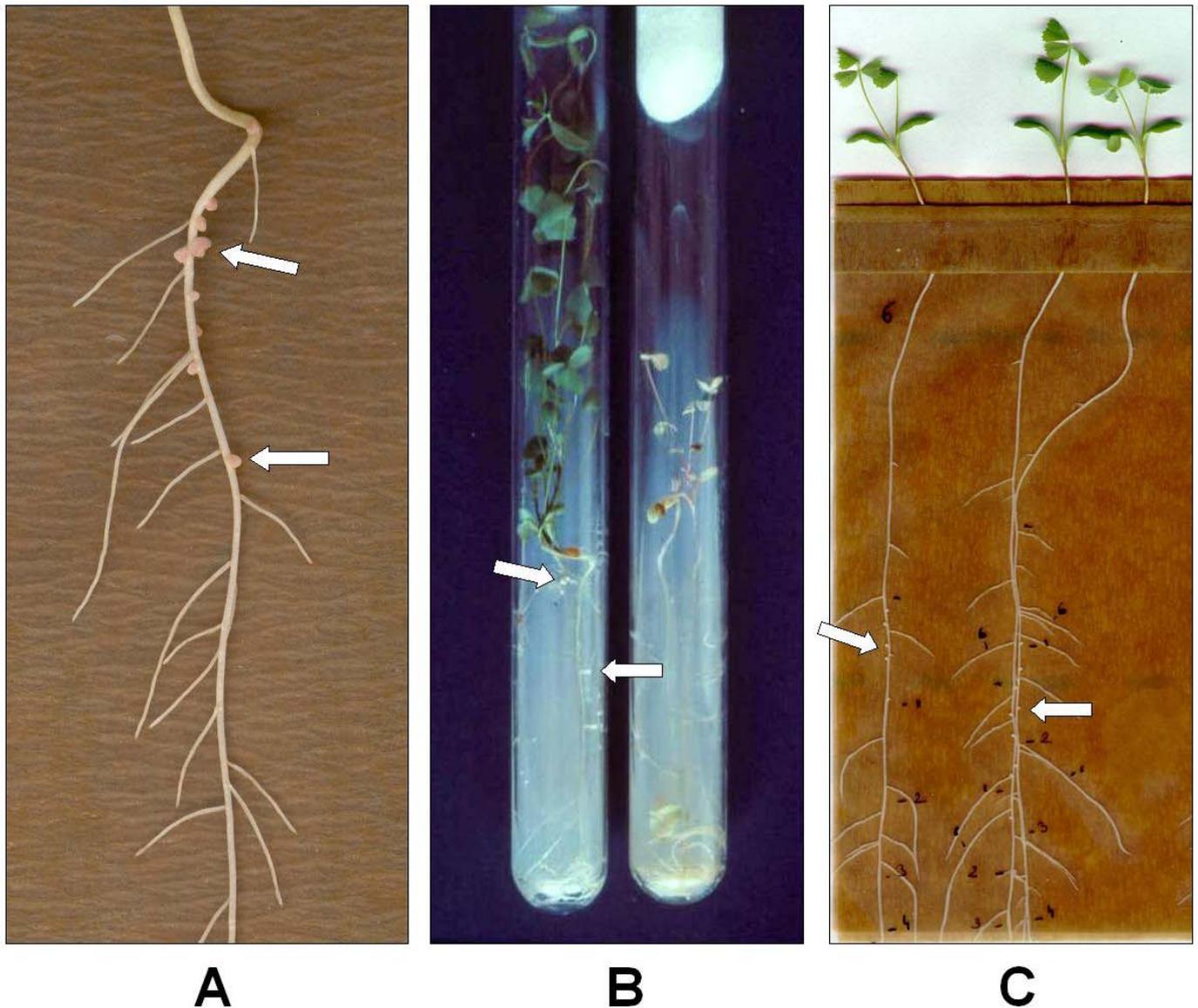
Figure 1. Nodulation of *M. truncatula* A17 seedlings grown in aeroponic chambers (A), tubes (B) and plastic/paper pouches (C).

A. Nodules (arrows) are clearly visible on the upper part of the main root 10 days after rhizobial inoculation of seedlings growing in an aeroponic container (see section ***II.2***). In this experiment the seedling was inoculated immediately after transfer to the container - note that there is no nodulation yet on the lateral roots because of the autoregulation mechanism. For clarity, the plant has been extracted from the container and photographed on pouch paper.

B. Nodules (arrows) are discernable on the root system of the plant growing on an agar support in the left-hand tube 45 days after inoculation. The control root on the right has not been inoculated, and the effect of nitrogen-starvation is very clear.

C. Nodules (arrows) are visible on the primary roots of pouch-grown plants 10 days after rhizobial inoculation. Again, as in **A**, the seedlings were inoculated prior to lateral root development, and therefore no nodulation is yet visible on the laterals. The pouches have been marked to indicate root growth following inoculation (see comments to section ***III***)

III. Inoculation/nodulation of plants in growth pouches

1. Grow *M. truncatula* seedlings (5-6 per pouch) in paper/plastic pouches (as described in Chapter "Growing *Medicago truncatula*: choice of substrates and growth conditions") in Fahraeus medium containing up to 1mM NH_4NO_3. Plants can be inoculated at any stage of growth, but of course more nodules will be formed upon inoculation of well-developed root systems with multiple laterals (after approx. 7-10 days of pouch growth)

2. For inoculation, grow the appropriate *S. meliloti* strain in 5 ml TY/Ca^{2+} medium to early stationary phase (approx. OD_{600} = 1.0). Alternatively, freshly-grown bacteria on agar TY/Ca^{2+} plates can be used. After centrifugation, the bacterial pellet is re-suspended in sterile water to $1-5 \times 10^{-3}$ OD_{600} ($\sim 1-5.10^6$ bacteria/ml).

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. 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.

Comments:

- In the pouch system, infection threads are formed in root hairs from around 36-48 h post-inoculation, and developing nodule primordia and young nodules on wild-type genotypes become visible through the transparent pouch cover from 4-5 days post-inoculation onwards. In general young emerging laterals are the most efficiently nodulated.

- 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. Excessively **high** cell suspension **densities** (> 0.1 OD_{600}) may even trigger defence responses resulting in poor nodulation.

- It is important not to add more than a total of 1 ml inoculum to each pouch, since *M. truncatula* roots do **not** appreciate excessive liquid in the pouch (see Chapter "Growing *Medicago truncatula*: choice of substrates and growth conditions").

- The spatio-temporal pattern of nodulation can be precisely followed by marking (ideally a permanent ink marker) on the transparent cover-sheet the position of growing root tips at regular intervals (e.g. every 24 h; see Figure **1C**). Be careful not to press on the plastic too close to the root tip!

- An alternative method to study early symbiotic responses/stages (after rhizobial inoculation or Nod factor treatment) is to grow plants on pouch paper laid on Fahraeus agar plates (8-10 plants/plate) (see Sauviac *et al.,* 2005).

Note that M. truncatula plants growing directly on agar plates often nodulate poorly (see below).

IV. Inoculation/nodulation of plants in tubes

1. Disinfected seeds are germinated as usual (chapter "Seed storage and germination") and grown vertically on 0.7% agar in the dark in Petri dishes.

2. Prepare agar slants by pouring nitrogen-free Fahraeus medium (15 g/l agar) into a glass tube, and allowing it to solidify in an "inclined" position. Each tube is closed with a polyethylene or cotton plug.

3. When the radicle length is about 5 mm, a single seedling is introduced into each tube and placed on the top of the agar slant. The tubes are then placed in racks in an upright position in a growth chamber (22°C, 16 h/8 h), and grown for 48 h.

4. Prepare the bacterial suspension as described in section ***III.2.***

5. Inoculate each tube with 0.3 ml of bacterial suspension. Check that the liquid contacts both sides of the entire root.

6. Inoculated tubes are then returned to the growth chamber (22°C, 16 h/8 h).

7. Depending on the plant line and the bacterial strain, nodulation should be clearly visible 5-8 days after inoculation (see Figure **1B**).

Comments:

- As mentioned above *M. truncatula* is **very sensitive to agar !** It is essential to check several brands and qualities of agar before starting experiments in order to assure adequate nodulation.

- The delay of 48 h before inoculation is to allow the root system to develop sufficiently for good nodulation and to allow the seedling to anchor to the agar surface.

- The size of the tube and the quantity of medium must be adapted to the final size of the plant at the end of the experiment. The duration of the experiment obviously depends on its objective, whether it is to determine nodulation kinetics, to carry out ARA measurements or to perform dry weight measurements. However, duration must be compatible with the capacity of the tube in order that the latter is not a limiting factor for the growth of the plant. For example, tubes of 22cm x 2cm filled with 20 ml of medium are convenient for up to 8 weeks of growth.

- Bacterial density is not critical. However, excessively high bacterial densities and/or inoculation volumes should be avoided (see also comments in section ***III***).

V. Inoculation/nodulation of transformed roots of composite M. truncatula plants

Approximately 3 weeks after *A. rhizogenes* inoculation (see Chapter "*Agrobacterium rhizogenes*-mediated root transformation"), transformed roots are sufficiently well developed for direct experimental studies. At this stage, transgenic roots are generally selected either on the basis of antibiotic-resistance (e.g. kanamycin; see Boisson-Dernier, Andriankaja *et al*., 2005) and/or constitutive expression of the fluorescent marker protein DsRED (Limpens *et al.* 2004).

1. Composite plants bearing transformed roots are transferred to a pouch-paper support laid on Fahraeus agar plates without either nitrogen or antibiotic selection. This constitutes an efficient system for studying nodulation, Nod factor responses, mycorrhization and nematode infection (see Boisson-Dernier, Andriankaja *et al*., 2005). After transfer, transgenic root position can be marked on the transparent petri dish cover in order to follow root growth (in general there is a positive correlation between root growth and the efficiency of symbiotic responses). In the case of fluorescence selection, the positions of transformed roots should be labelled with a non-fluorescent marker.

2. Approximately 5 days after transfer of plants to pouch paper-covered plates, root systems are inoculated with *S. meliloti* by global application with a 5-10 ml suspension of 5×10^5 bacteria/ml (Vernoud *et al.,* 1999). Roots partially immersed in the bacterial suspension are incubated in a horizontal position for 1 h. The excess bacterial suspension is then poured off and plates are incubated vertically for 3-7 days Bacterial infection and the first nodules are observed respectively 3 and 5-6 days post-inoculation.

Comment:

- If transgenic roots were selected only on the basis of antibiotic (Km)-resistance, it is important to cut off the non-transformed roots that showed growth arrest on Km-containing medium before transferring to antibiotic-free paper support /Fahraeus agar plates. In the case of fluorescent roots, cutting off the non-fluorescent roots is optional.

References:

Boisson-Dernier A. Andriankaja A., Chabaud M., Niebel A., Journet E.-P., Barker D.G. and de Carvalho-Neibel F. (2005). *MtENOD11* gene activation during rhizobial infection and mycorrhizal arbuscule development requires a common AT-rich-containing regulatory sequence. *Mol. Plant-Microbe Interact.* **18**:1269-1276.

Caetano-Anollés, G. and Gresshoff, P.M. (1991). Alfalfa controls nodulation during the onset of *Rhizobium*-induced cortical cell division. *Plant Physiol.* **95**:366-373.

Limpens, E., Ramos, J., Franken, C., Raz, V., Compaan, B., Franssen, H., Bisseling T. and Geurts R. (2004). RNA interference in *Agrobacterium rhizogenes*-transformed roots of *Arabidopsis* and *Medicago truncatula*. *J. Exp. Bot*. **55**:983-992**.**

Sauviac L., Niebel A., Boisson-Dernier A., Barker D.G. and de Carvalho-Niebel F. (2005). Transcript enrichment of Nod factor-elicited early nodulin genes in purified root hair fractions of the model legume *Medicago truncatula*. *J. Exp. Bot.* **56**:2507-2513.

Vernoud V., Journet, E-P. and Barker, D.G. (1999). *MtENOD20*, a Nod factor-inducible molecular marker for root cortical cell activation. *Mol. Plant-Microbe Interact.* **12** :604-614.