Cuttings and grafts

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Introduction

This chapter deals with various manipulations that involve sectioning of vegetative parts followed by reconstitution of a whole plant - rooting of seedling shoots, propagation by cuttings and grafting. Protocols for establishing split-roots (divided root systems growing in separate compartments) for both plantlets and well-developed plants will be presented in the next issue of this chapter.

Whereas cuttings are useful for the clonal propagation of a given plant genotype, grafts and split-root systems are useful tools in studies on systemic, long-distance signalling within the plant.

A - Rooting of seedling / plantlet shoots

A variety of experiments in *M. truncatula* biology (e.g. the recovery of mutant genotypes with interesting root phenotypes) require the regeneration of whole plants from seedling / plantlet shoots sectioned at the base of hypocotyls. To do this, sectioned young shoots can be transferred to vials containing water and returned to standard light/temperature/hygrometry growth conditions. Vigorous adventitious roots will appear on the hypocotyls within 3 days (Fig. 1a-c). Note that newly-formed roots elongate much faster in tap water or in dilute nutrient solution compared to deionized water.

Alternatively, shoots can be directly planted into water-soaked soil and grown in moderate light conditions under a transparent cover maintaining high hygrometry (see Chapter "Growing *M. truncatula*: choice of substrates and growth conditions"). Stem growth should resume within 1 week, indicating that an efficient adventitious root system has been formed.

Note that new (lateral) roots will form even faster when the upper part (> 1 cm) of the primary root has been left on the seedling (Fig. 1d).

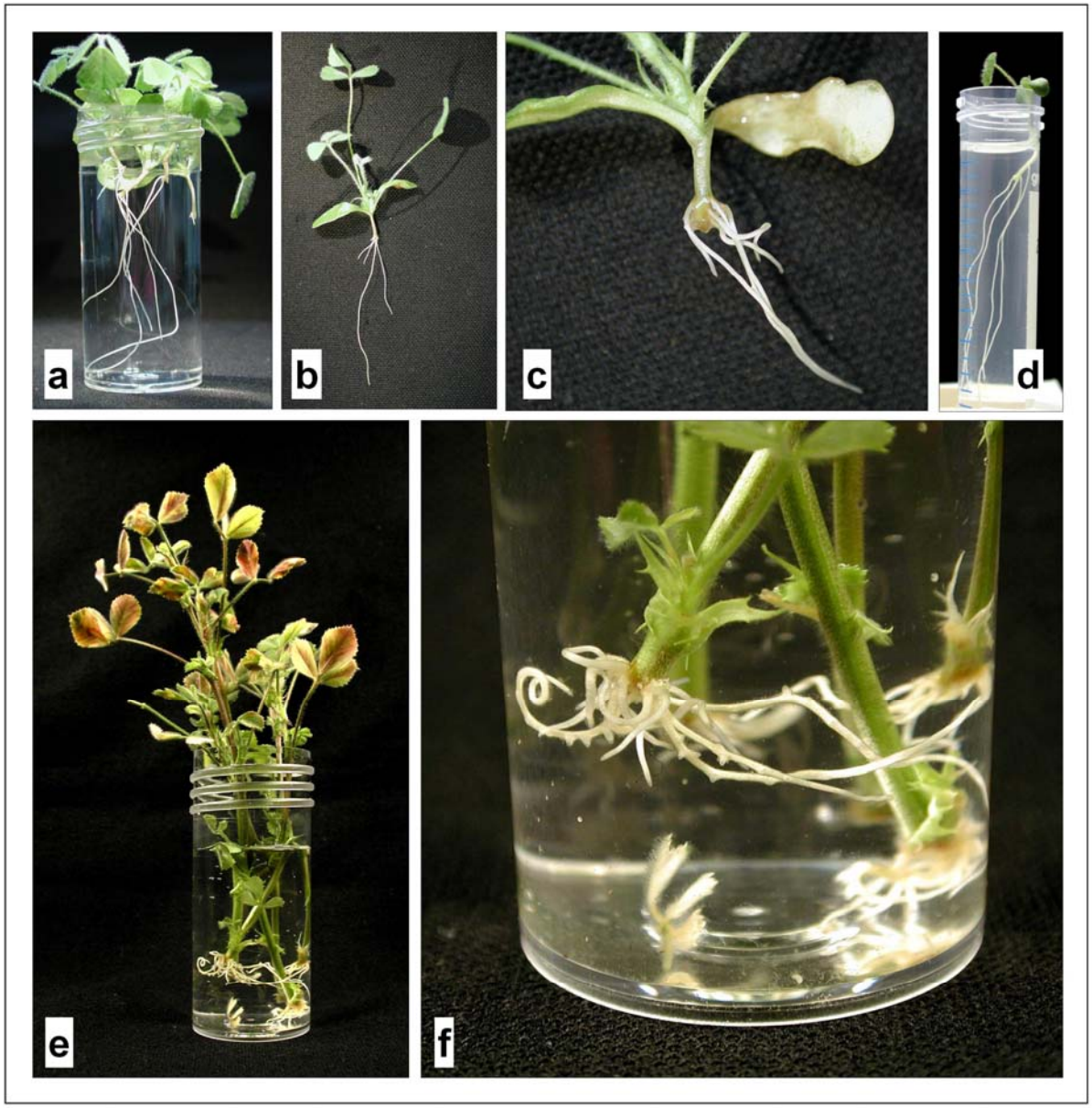

Figure 1 – Rooting of seedling / plantlet shoots and of stem cuttings in water

a: Thirteen day-old plantlet shoots showing adventitious roots formed from the base of hypocotyls after a 5-day incubation in tap water. **b** and **c**: Close-up views of a single rooted plantlet and of the basal region of the hypocotyl showing a cluster of adventitious roots, respectively. **d**: Eight day-old seedling showing well developed lateral roots after sectioning within the upper region of the primary root followed by a 5-day incubation in tap water. **e**: Overall aspect of branch tip cuttings after 14 days in tap water; the older leaves show characteristic senescence symptoms due to the lengthy rooting period. **f**: Magnified view of (e) showing the numerous adventitious roots clustered at the bottom end of the cuttings; 4 out of 5 cuttings have generated roots in this experiment.

B – Plant propagation by cuttings

Plant propagation by cuttings also involves the generation of adventitious roots, this time from the basal end of stem fragments. Although this organogenetic process is slower in *M. truncatula* stems as compared to hypocotyls, *M. truncatula* genotypes can be easily propagated by making cuttings from a single plant.
Several variants of the method with similar efficiencies can be used depending on whether the substrate is liquid (water) or solid (soil, water-soaked perlite, sterile agar). Two protocols (rooting in soil or agar) are described in detail below, along with comments about rooting in water. A cuttings protocol from the ISV CNRS (Gif/Yvette, France) including use of water-soaked perlite is available at http://www.isv.cnrs-gif.fr/embo01/manuels/index.html, Module 1).

Rooting in soil

Rooting in soil is our preferred routine method for non-axenic *M. truncatula* cuttings since it appears slightly more efficient in hour hands than rooting in water. Two options for rooting in soil are presented here:

- rooting in "Jiffy" peat bags in a horizontal position (LIPM-Toulouse): the small size of Jiffys allows early detection of well-rooted cuttings. Early tests had indicated that a horizontal position was positive for rooting efficiency.
- rooting in a standard vertical position in a pot of solid substrate (ACNFP-Murdoch).

Common steps are described in the first protocol and differing steps in the second protocol are indicated afterwards.

Rooting in "Jiffy" peat bags laid in a horizontal position

1. It is recommended to start from healthy and actively growing plants in order to increase the rooting success to ≥ 80 %. Choose young, vigorous branches from the donor plant in order to generate branch sections with at least 4 nodes bearing opened leaves (older portions of the plants do not root as readily). Section the stem in the middle of the internode.

2. Immediately transfer the cut branch tips to a beaker of water in order to avoid dehydration and cavitation of the xylem.

3. Trim the stem at right angles with a sharp (new) razor blade just below (1-3 mm) the oldest node, as nodes appear to be the preferred site for adventitious root emergence. Make a clean cut, as a messy cut will encourage disease. Pull off the leaves from the nodes that will be below the surface of the rooting medium. Sometimes it is helpful to further reduce the amount of leaves, leaving only those not fully formed at the apical meristem. Leave the stalk of the lower leaf to anchor the cutting upon planting into the substrate. Remove any flower or axillary branch.

4. The application of commercial rooting hormones containing auxin (e.g. 0.4 mg/ml Indole-3-Butyric Acid, mixed with talcum powder) helps to stimulate rooting. Pour a small amount of the rooting hormone into a clean Eppendorf tube to prevent contamination of the entire rooting hormone stock. Dip the base of the stem, including the node area, into the rooting powder. The stem should be dry when dipped. Commercial rooting products often include a fungicide, which is probably useful given the damp conditions required for rooting success. Tap off excess powder, since too much hormone can inhibit rooting.

5. Use Jiffys saturated with sterile water. Poke a hole with a tweezers in the peat before inserting the cutting 2-3 cm deep in order to avoid loss of the rooting hormone. Press the bag sideways to ensure a good contact between cutting and substrate.

6. Lay the Jiffys on their sides (with the cuttings in a horizontal position) in a transparent plastic box (35x23 cm,14 cm high, which can take up to 15 cuttings) and place the closed box in a growth chamber under indirect light (~100 $\mu E.m^{-2}.s^{-1}$; 16 h / 8 h) to 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 peat bags.

9. Jiffys with rooted cuttings can be planted vertically at this stage into larger pots, and no longer need air hygrometry control.

Rooting in pots in a standard vertical position (steps differing from the above protocol)

- Step 3: cut the stem at a 45^{o} angle below the lower leaf node.
- Step 5, suitable rooting media: autoclaved, water soaked perlite : vermiculite (2:1) or any porous, easily drained medium in a clean container.
- Step 6: cover container and cutting with a transparent plastic film to maintain high humidity
- Step 7: adjust water level as required using half-strength nutrient solution (liquid fertilizer). Check the rooting medium every few days to make sure it remains moist.
- Step 8: when you notice new growth (after 2 to 3 weeks), there is a good chance that the roots are developing.

Remarks

- Propagation of flowering branches yields plants that maintain flowering and resume flower bud formation as soon as the cutting has recovered normal growth rate.
- Jemalong cuttings incubated in tap water alone (no solid substrate) in test tubes will form adventitious roots within 8-15 days (Fig. 1e-f). The cuttings with ≥ 5 mm roots can be transfered to soil or to an aeroponic chamber (refer to Chapter "Growing *M. truncatula*: choice of substrates and growth conditions"). Shoot growth will resume within a few days. The addition of 5mM $CaCl_2$ to the water stimulates rooting and cuttings can be inserted through holes in floating polystyrene discs (communicated by Simon Ellwood, ACNFP Murdoch).

Specific material

Sterile razor blade or scalpel

Jiffies (Jiffy-7 peat pellets, Jiffy International AS, Norway) or small pots with pasteurised potting mix

Tweezers

Rooting hormone

Rooting in agar (axenic conditions)

This protocol is suitable for the maintenance and propagation of clonal material grown on agar media in axenic conditions (see also Chapter "*Agrobacterium tumefaciens*-mediated transformation and *in vitro* plant regeneration of *M. truncatula*"). Plantlets grown *in vitro* on

SHb10 agar medium (Chabaud *et al.*, 1996) turn yellow and die after 3-4 months. For optimal maintenance, clonal propagation should be performed 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 but is not pushed 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.

C - Grafts between shoot and root system

Grafting provides a simple and powerful approach to generate chimeric plants from different genotypes, and thus to assess the cell / organ autonomy of gene action. The technique of grafting has been widely used in a variety of plant species (including *Arabidopsis*) and has greatly contributed to the understanding of processes such as shoot branching control, flowering, and disease resistance (e.g. Foo et al, 2005; Bainbridge *et al.*, 2006; Chen *et al.*, 2006). In addition to classical scion/rootstock grafts, more complex graft combinations can be performed such as interstock grafts, Y grafts and two-rootstock grafts (e.g. Foo et al, 2001).

In legumes, grafting has been used extensively to study the effects of shoots on autoregulation of root nodulation, in species such as soybean (Delves *et al.*, 1986, 1987; Sheng and Harper, 1997) and more recently in the model legumes *Lotus japonicus* (Nishimura *et al.*, 2002; Oka-Kira *et al.*, 2005) and *Medicago truncatula* (Penmetsa *et al.*, 2003).

The following grafting protocol is adapted for reciprocal root / shoot grafting of young *M. truncatula* plantlets in axenic conditions. It has been used with success in several studies (e.g. Tirichine *et al.*, 2000; Ané *et al.*, 2002; Penmetsa *et al.*, 2003, with a slight modification, see below). Similar protocols including modifications in the shaping and sealing of the graft junction have been described recently (Lohar and VandenBosch, 2005; Prayitno *et al.*, 2006).

Indications for grafting mature plants (> 1 month old) grown in pots are also provided below.

Grafts on plantlets

1. Grow plantlets for ~3-4 days in sterile growth pouches (cf Chapter "Growing *M. truncatula*: choice of substrates and growth conditions") until roots are ~8 cm long (Fig. 2a).

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 desiccation.

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 within the lower end of the chlorophyllous zone using a fresh and clean razor blade (this operation must not crush the sectioned tissues). Transfer the resulting scions (aerial parts) and rootstocks to sterile water in Petri dishes until needed.

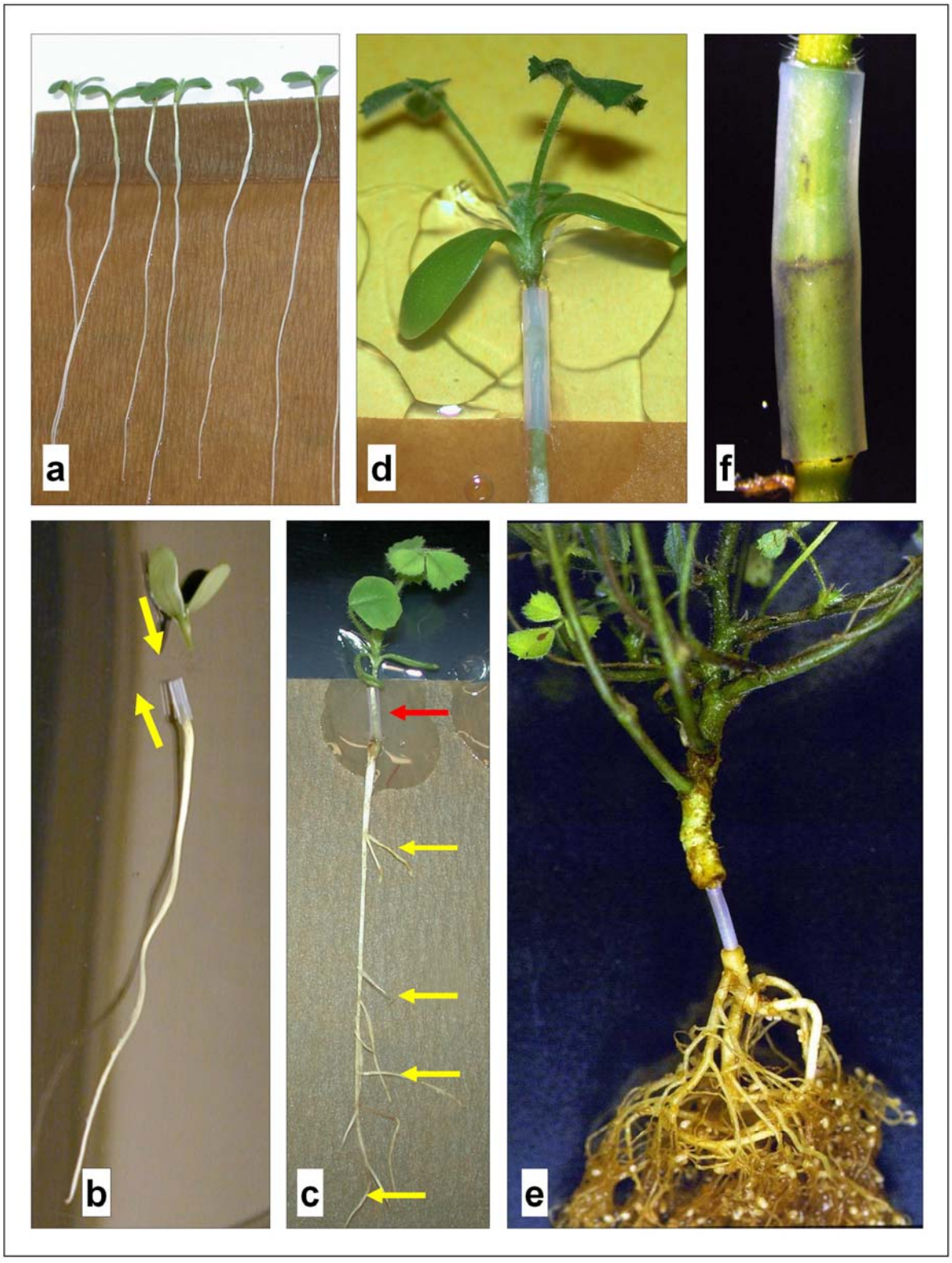

Figure 2 – Grafts between shoot and root systems
a: Plantlets after 4 days of growth in sterile pouches ready to be used as starting material for grafting. **b**: Illustration corresponding to step 5 of the grafting protocol, showing the upper end of the rootstock (hypocotyl tissue) already capped with a tubing segment (0.9 mm internal diameter) and ready for insertion (arrows) of the scion hypocotyl. **c**: Grafted plantlet on a

(Figure 2, continued)
paper / agar overlay 18 days after grafting; red arrow indicates the tubing with the graft junction visible inside, immobilized on the paper by a low-melting agarose plug; yellow arrows point to lateral roots formed after grafting, which indicates that the graft has been successful. **d**: Detailed view of the graft region embedded in agarose. **e**: Chimeric grafted plant (rootstock : WT genotype; scion : *sunn-2* supernodulator genotype) uprooted after 7 weeks of culture in soil; the supernodulating root phenotype indicates that root nodule number is under shoot control (see Penmetsa *et al.*, 2003); note that the plant has managed to develop well despite performing the graft at the plantlet stage using narrow tubing (S. Cros-Arteil, unpublished data). **f**: Close-up of a successful graft performed on mature plants; the sealed graft junction remains visible through the tubing (2.5 mm internal diameter). Pictures b), c) and d) were taken by Ralph Pawlowiez and Fabienne Vailleau.

5. With care successively insert a rootstock 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** (Fig. 2b, 2d). 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 or to paper / agar overlays in square plates (12x12 or 24x24 cm; cf Chapter "Growing *M. truncatula*: choice of substrates and growth conditions"). 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(Fig. 2c-d). 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 $\mu E.m^{-2}.s^{-1}$; 16h 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 as they emerge from the tubing lower end, and should be regularly cut off in order to promote rootstock growth.

Remarks

- Growing seedlings in pouches yields plantlets with longer hypocotyls by comparison with those grown on agar plates and easier to manipulate for grafting.
- 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.
- Newcomers should be aware that **grafting in *M. truncatula* is not straightforward**. However, with some practice, 40 to 60 % of the grafts should grow after a lag phase.
- For nodulation studies, inoculation with *S. meliloti* can be performed a few days after root growth has resumed (Fig. 2e).
- Penmetsa *et al.* (2003) have used a very similar protocol in which the graft junction was sealed by application of 1.5% (w/v) purified water agar (Sigma) in the absence of capillary tubing (Cindy Smith, Stanford University, California).

Specific material (sterilized)

- transparent, stiff polyethylene tubing, 0.8-0.9 mm internal diameter.
- square plates (24x24 cm) containing 200 ml Fahräeus medium with 15 g/l agar.
- for paper / agar plate overlays, plain "38# seed germination paper" from Anchor Paper Company (www.anchorpaper.com; see Chapter "Growing *M. truncatula*: choice of substrates and growth conditions")
- 15 g/l sterile low-melting agarose in water

Grafts on mature plants

Grafting mature plants (> 1 month old) grown in pots is easier than with plantlets, owing to their larger size and better mechanical resistance. The principle of operation is basically the same as above. However, note that it is difficult to perform in axenic conditions, that grafting within the first internode may be easier and cleaner than within the hypocotyls, and that a plastic tubing with larger diameter is required (Fig. 2f).

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