Seed storage and germination

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1 Introduction

Legumes set seed in pods, the typical fruit structure in this broad plant taxon. In *M. truncatula*, ripe pods are compact spiky coils (Fig. 1a) that remain indehiscent. In natural conditions, fallen pods permanently retain and protect the seeds in the topsoil until pod wall softening occurs under favourably humid environmental conditions. In the laboratory, pods are stored dry and need to be mechanically broken in order to release seed.

There can be up to 12 seeds per pod (5-8 on average), depending on the growth conditions of the mother plant. As for most annual medic species, all mature *M. truncatula* seeds are protected by a hard, hydrophobic coat ("hard-seededness" character) that needs to be at least partially ruptured to allow seed imbibition and germination (Crawford *et al.*, 1989). In addition to this physical coat-imposed dormancy, *M. truncatula* seeds can display variable degrees of physiological embryonic dormancy during the 3-4 months following pod abscission.

This chapter is divided into four sections dealing respectively with seed storage and extraction from pods, seed germination, breaking embryonic dormancy, and recovery and germination of immature embryos.

More information about the reproductive development of M. truncatula is available in Chapters “Morphology, development and plant architecture of *M. truncatula*“ and “Vernalization, crossings and testing for pollen viability”, and in the literature (Benlloch et al., 2003; Moreau et al., 2006, and refs. therein). For recent information about Mt seed and pod development, see Chapter “Seed biology of *Medicago truncatula*” and Abirached-Darmency et al. (2005), Gallardo et al. (2003; 2006), Djemel et al. (2005) and Wang and Grusak (2005). Biological information about Mt seed germination is available in Chapter “Seed biology of *Medicago truncatula*” and in recent literature (Boudet et al., 2006; Bouton et al., 2005; Buitink et al., 2006; Domoney et al., 2006; Faria et al., 2005, Ricoult et al., 2006).

Laboratory procedures for seed multiplication and collection are described in Chapter “Growing *M. truncatula*: choice of substrates and growth conditions”.

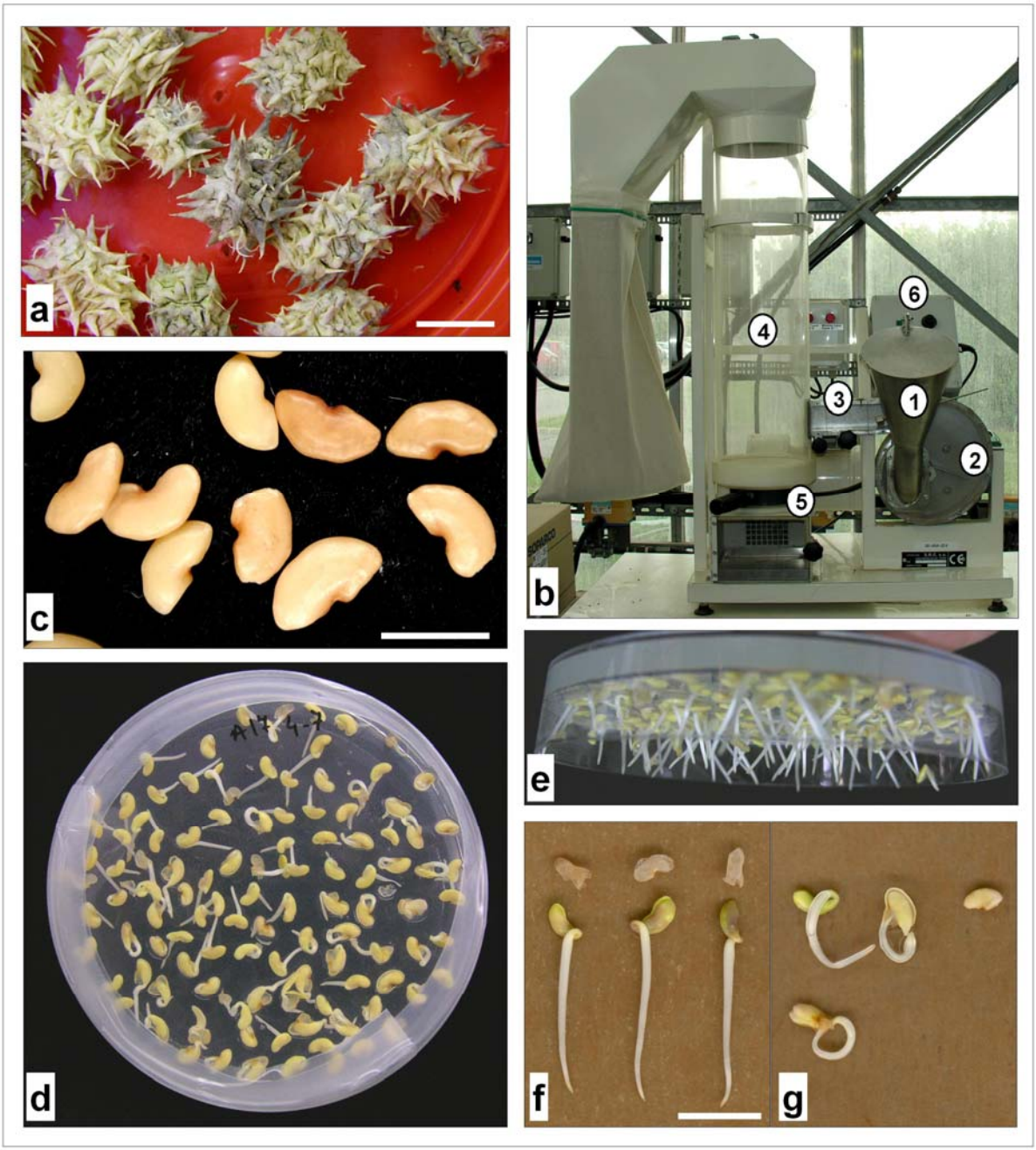

Figure 1 – *M. truncatula* seed extraction and germination

a: Mature *Medicago truncatula* pods are compact spiky coils; bar = 10 mm. **b**: Front view of the seed mill used at LIPM-Toulouse; (**1**): loading funnel; (**2**) mill chamber; (**3**): separation chamber; (**4**): blowing column; (**5**): removable sieve for harvesting extracted seed; (**6**): control panel. **c**: clean, partially scarified seeds after mechanical extraction; bar = 5 mm. **d**: top-view of inverted, sealed agar plate after 24h at 20°C; most seeds have germinated and the longer radicles are tilted due to space limitation inside the plate. **e**: side-view of inverted agar plate without lid, showing most well-germinated seeds with straight radicles. **f**: appearance of well-germinated seeds; the loose seed coat and endosperm (on top) have been manually removed from the germinating embryos; bar = 10 mm. **g**: various types of abnormal germination that generally result in seedling growth arrest (curved radicle or radicle meristem entrapped within the seed coat often leading to radicle breakage) as well as ungerminated seed; same scale as (f).

A - Seed storage and extraction from pods

Seed storage

If no mechanical scarification of the seed coat has taken place (e.g. after threshing) *M. truncatula* seeds remain impermeable and viable for many years (Crawford *et al.*, 1989). However, after threshing (or other scarification), shelf-life is drastically reduced: the colour of well scarified seeds turns to dark-brown within 6-12 months, probably due to oxidation of seed compounds, along with a dramatic decrease in seed viability. Therefore it is highly recommended to keep pods intact for long-term seed storage.

After pod harvest, allow pods to dry out at room temperature for ≥1 week under low hygrometry conditions (e.g. in a growth room with strong light influx, cf. Chapter "Growing *M. truncatula*: choice of substrates and growth conditions"). Store dry, intact pods at room temperature and low humidity, in strong paper envelopes or in screw-cap plastic vials with punched holes in the cap to allow air circulation. Seeds normally retain good viability for at least 3-5 years under such conditions. Except for germplasm conservation in genetic resource centers, specific seed storage facilities are not necessary for *M. truncatula*.

Seed extraction from pods

Due to the toughness of *M. truncatula* dried pods, extraction of seeds requires relatively harsh mechanical conditions. A number of extraction systems have been tested in various laboratories, including blenders and customized coffee grinders. We describe below three extraction methods that can be chosen according to the number of seeds required.

1. For **large-scale** extractions (>200 seeds), a commercial seed mill with rubber covered beaters or counter-beaters is the most efficient option. For example, several French laboratories routinely use a seed mill designed for small seed species and adapted to fit *M. truncatula* (SRC, Château-du-Loir, France). This apparatus comprises a sieve and a dust blower to eliminate most (large or fine) debris from extracted seed (Fig. 1b; a detailed protocol with optimized settings can be obtained from the authors). Other European labs use the "Hege 16" seed mill (Hege Maschinen, Germany) connected to a blowing column (see picture at http://www.isv.cnrs-gif.fr/embo01/manuels/index.html, Module 1). For both systems, it should be born in mind that the mill speed must be adjusted to minimize seed damage. Moreover each seed, once released from its pod, should be removed as soon as possible away from the mill chamber to minimize further coat scarification and damage to the embryo itself. Therefore, it is recommended to thresh each pod batch in a series of short (30 s) beating periods alternating with recovery of new released seeds. Using such precautions, more than 90 % of the seed can be successfully extracted from the pods, and only a few percent of seeds damaged. The level of coat scarification is generally high, and further mechanical or chemical coat scarification steps before germination (see below) may not be necessary.

2. For **mid-scale** extractions (~50 to 200 seeds), a manual method using a corrugated rubber mat is appropriate. Place up to 30 pods on the rubber mat and crush them in a circular movement using a plasterer's hawk with handle. As the pod disintegrates, the seed will fall between the corrugations and be protected from damage. However, as for the large-scale method, fractionating the extraction into repeated steps yields seed of higher quality. Virtually all the seeds in a pod batch can be extracted using this method. A very similar protocol from Doug Cook's lab is available at http://www.medicago.org/documents/Protocols/seedmill.html.

3. For **small-scale** extractions (<~50 seeds), gently crush individual pods using pliers and pull them lengthwise so that most of the seeds pop out. Using this method, the level of coat scarification is usually very low.

After extraction, seeds need to be separated from pod remnants and smaller debris. Large pod fragments and fine debris can be easily removed from the seeds (Fig 1c) either by the sieve / dust blower system integrated within a seed mill, or by manual sieving. The remaining debris, similar in size to the seeds, can be removed by differential sedimentation in a constant and adjustable air flow (e.g. using a hair dryer), or sorted by hand (straightforward in the case of small-scale extraction).

Specific material :
- seed mill (see above)
- steel pliers
- corrugated rubber mat (40 x 40 cm)
- plasterer's hawk with handle (any tool with a hard flat surface and a handle can be used), 18 x 27 cm
- set of sieves (2.5 mm sieve allowing the loose seeds through but retaining the pod fragments that still contain seeds, 0.8 mm sieve retaining the seeds and letting fine debris through)
- hair dryer

B - Germination of non-dormant seeds

M. truncatula seeds are referred to as "non-dormant" when the embryo does not display endogenous dormancy and readily germinates upon seed coat scarification. The *M. truncatula* seed coat, and especially its hydrophobic, waxy outer tegument, **must** be scarified in order to allow the penetration of water and O_2 that trigger germination: intact seeds will not germinate at all even after 1 week in normal germination conditions. Although extraction from pods scarifies the seed coat to a variable extent, a scarification step is usually performed before seed imbibition, either mechanically by abrasion or chemically with sulphuric acid. Finally, the seed outer surface needs to be sterilized prior to any experiment requiring axenic conditions.

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

1b. ***Alternative chemical scarification*** (**CAUTION**: *perform this operation in the fume hood and wear protective clothing, gloves, lab coat and safety glasses*)**:** soak seeds in a glass vial containing a few milliliters of concentrated, **anhydrous** sulphuric acid with intermittent agitation. Monitor the progress of the chemical scarification by observing the gradual appearance of small black spots on the tegument surface. When there are ~5 spots per seed on average (usually after 5-12 min), proceed to washing step 2.

2. Carefully remove **all excess** H_2SO_4 with a glass pipette and quickly resuspend the seeds in a large amount of water. Maximal removal of the H_2SO_4 is **extremely important** in order to avoid local heating upon water addition that may damage the seeds - the use of chilled water is beneficial for this step. Decant the water and rinse the seeds a further 3 times with water.

3. ***Seed sterilization****:* it is recommended to use sterile glassware and tools under a laminar flow hood. Different sources of active chloride work efficiently (sodium hypochlorite, filtered calcium hypochlorite or commercial reagent soluble tablets). Submerge scarified seeds with a 3 % active chlorine solution for 90 to 120 s. Quickly remove the bleach and extensively rinse the seed 5-6 times using sterile water. When seeds have not been previously scarified with sulphuric acid, the seed outer tegument remains hydrophobic

and seeds tend to float on the liquid; addition of a drop of detergent (e.g. Triton X-100) to the bleach solution will solve this problem.

4. ***Seed germination*:** transfer the decanted seed onto soft agar (e.g. 0.8 % (w/v) water-agar) in Petri plates. Spread uniformly up to 60 seeds per 10 cm diameter plate with the aid of sterile forceps, seal the plates with Parafilm and place them inverted in the dark at 20 °C. With seed batches of good quality, germination rates (determined by counting the number of seeds that show visible radicle protrusion) should be 80-90 % and radicles 5-10 mm long within 24-36 hours (Fig. 1d-f). Alternatively, when straight radicles are not required, the seeds can be placed in water-containing Petri dishes at 20°C in the dark. In the shallow water, the seeds germinate rapidly and the radicle growth is about 5-10 mm within 24 hours.

Additional remarks

- The duration of the H_2SO_4 treatment is a critical parameter and has to be adjusted for each batch, depending on the seed origin and coat condition: the usual time is 5-10 min, while some batches may need as short as 3 min and others up to 20-30 min treatment.
- Sterilization time is critical too (particularly for Jemalong), since over-treatment with bleach results in root meristem damage and abnormal radicle growth (similar to Fig 1g). The optimal sterilization time may vary depending on the level of contamination of seed batches and the *M. truncatula* genotype, and this should be determined before starting a large scale experiment. Germinating seeds are also very sensitive to traces of chlorine that may remain in the media or containers.
- Whereas bacterial contaminants are easily eliminated by bleach treatment, fungal contamination of some seed batches can survive it. This is probably due to the presence of spores within the seed tegument. Removing the teguments just after germination helps to reduce the occurrence of such contamination (and also ensures a perfectly synchronous expansion of cotyledons).
- Seed sterilization using 95% ethanol containing one drop of Tween 20 per 30 ml for an hour, followed by surface sterilization with 0.1% $HgCl_2$ for 15 min is not sufficient to eliminate bacterial contamination (Gao et al, 2003).
- For rapid detection of residual microbial contamination, sterilized seeds can be germinated on a rich nutrient substrate (e.g. sterile soft C medium, see Annexe; warning: germination on soft C medium is delayed by ~18h at 20°C).
- For a number of routine applications, it is not necessary to sterilize the seed. In this case, scarified seeds can be germinated on 0.8 % (w/v) water-agar or water–agarose, or even on non-sterile, water-saturated Whatman discs in Petri plates (in the latter system it is recommended to seal the plates with Parafilm to avoid radicle dessication) or in water.
- The optimal temperature for *M. truncatula* cv. Jemalong germination is 20°C. However for optimal synchronization of germination, non-dormant seeds can be stored at 4 °C for 1 day before transfer to a 20 °C incubator. Storage of germinating seed at 4 °C for up to 3 days has no noticeable effect on further growth (longer exposure to cold promotes vernalization, see Chapter “Vernalization, crossings and testing for pollen viability”).
- Probably linked to a positive role of ethylene/CO_2 on germination (see Globerson, 1978), sealing the Petri plates with Parafilm accelerates the germination rate (T50) of seeds by 20-30 % at 20°C. Higher agar concentrations slow down the germination rate (40 % slower on 1.5 % than on 0.8 % agar plates; J. Garcia and E.-P. Journet, unpublished observations)
- Seeds germinated in inverted Petri dishes have straight radicles (Fig. 1e).

Specific material

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

A similar protocol from Doug Cook's lab is available at http://www.medicago.org/documents/Protocols/seedgerm.html.

C - Breaking seed embryo dormancy

In addition to coat-imposed dormancy, fresh *M. truncatula* seeds can display physiological embryo dormancy to a variable extent, probably depending on the growth conditions of the mother plant. Such embryo dormancy appears when the pod has just matured (~1 week after pod abscission) and can last up to 3-4 months –(the so-called "after-ripening" period) when stored at room temperature in the laboratory. The germination characteristics of dormant batches is variable, often showing delayed and asynchronous germination, sometimes as low as 10-20 % after 5 days at 20 °C.

Seed dormancy is an adaptive trait that enables the seeds of many species to remain quiescent until conditions become favorable for germination (Bewley, 1997; Heggie and Halliday, 2005). The release of seed dormancy is regulated by complex interactions between environmental and genetic factors that are poorly understood. Several studies have shown that ethylene, gibberellic acid and brassinosteroids promote the germination of dormant seeds, but there is now general agreement that abscisic acid (ABA) is the primary mediator of seed dormancy (Korneef *et al.*, 2002; Gubler *et al.*, 2005). Experimentation over the past century has identified numerous physical, chemical or hormonal treatments that will reduce seed embryo dormancy.

We have tested a number of treatments on dormant *M. truncatula* seeds to break embryo dormancy (J. Garcia, D. Barker and E.-P. Journet, unpublished data). (i) The addition of 1 µM fluridone (an inhibitor of carotenoid and ABA synthesis) to the imbibition water resulted in immediate germination of seeds, strongly suggesting that *de novo* ABA synthesis is involved in *M. truncatula* embryo dormancy. Unfortunately, active concentrations of fluridone are lethal to seedlings. (ii) The total removal of the seed coat and underlying endosperm layers from imbibed seeds was also very effective in promoting germination, suggesting that these tissues might contain (or prevent the leaching of) germination inhibitors from *M. truncatula* seeds, a phenomenon already observed in a number of plant species. Unfortunately, the systematic removal of these outer seed layers would be very tedious. (iii). The addition of 1 µM benzylaminopurine (cytokinin) to imbibition water also fully broke embryo dormancy, whereas the addition of 1 µM GA3 (gibberellic acid) or 0.1 µM ACC (ethylene precursor; see also Globerson, 1978) to the imbibition water partially released dormancy. Finally, the effect of cold pre-treatment of imbibed seeds before germination at 20 °C was investigated for various temperatures and durations. It turned out that treatments at 4-8 °C for 48-72 h are sufficient to fully break dormancy and result in rapid and synchronized germination (see also Faria et al, 2005, for similar results with treatments at 4° C). The advantage of the 8 °C treatment is that the germination process starts before the end of the cold treatment, thus saving up to 12 h in the overall time needed. Therefore we recommend the following simple cold treatment protocol to break embryo dormancy.

Cold pre-treatment of imbibed seeds

Maintain inverted Petri plates with scarified and sterilized seed (see above) at 4-8 °C in the dark for 2-3 days, before transferring the inverted plates to a 20 °C incubator for germination. Most of the seeds should germinate in the first 12 h.

Remarks :

- Protruding radicles may already be visible after a 3-day treatment at 8 °C.
- Note that it is important to measure the precise temperature in the refrigerator and on cold-room shelves, since this can vary as a function of location.
- Treatment of germinating seed at 4 °C for up to 3 days has no noticeable effect on further growth and nodulation by *S. meliloti* (longer exposures to cold promote vernalization, see Chapter "Vernalization, crossings and testing for pollen viability").

D – Recovery and germination of immature embryos

Germinating immature embryos can help to shorten the generation cycles and thus save time in certain experiments (e.g. when the F1 and F2 progenies resulting from crosses are required as rapidly as possible). Alternatively, it may be essential to rescue maturing embryos if the continued survival of the mother plant becomes uncertain. According to our observations at LIPM-Toulouse, it is possible to efficiently germinate immature embryos without nutrient supply about 21 days after pollination (DAP) onwards (pods fall 30-35 DAP under standard growth conditions, see Chapter "Growing *M. truncatula*: choice of substrates and growth conditions".). This is consistent with the time needed by the developing seed to reach a good potential vigor of germination (Gallardo *et al.*, 2003). Rescue of embryos at earlier developmental stages probably requires specific culture media and conditions (Ochatt *et al.*, 2002; Gallardo *et al.*, 2006).

1. Remove a pod from the mother plant at the appropriate stage (≥ 21 DAP) : the pod color should have already turned yellow-green or whitish, and the radicle of the embryo should have lost its chlorophyll and become white.

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

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

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

Specific material

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

Appendix

Soft C (Campbell) medium

For 1 liter of C medium, add :

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

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

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