Vernalization, crossings and testing for pollen viability

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Introduction

This overview is focused on the main developmental features that are of practical interest for the control of floral initiation, obtaining flowering plants of adequate size and success in crossings. More detailed descriptions of the morphology, vegetative and reproductive development and plant architecture of *M. truncatula* are available in Chapter "Morphology, development and plant architecture of *Medicago truncatula*", and in the literature (Benlloch *et al.*, 2003; Moreau *et al.*, 2006, and refs. therein). Data about *M. truncatula* seed and pod development are available in Chapter "Seed biology of *Medicago truncatula*" and in Gallardo *et al.* (2003), Djemel *et al.* (2005) and Wang & Grusak (2005).

M. truncatula displays an indeterminate pattern of shoot development throughout its lifespan. Non-vernalized seedlings develop into vigorous plants that abundantly ramify and grow in a procumbent to ascendant fashion before the onset of flowering ~5-6 weeks after germination (cv Jemalong) in optimal growth chamber conditions (see Chapter "Growing *M. truncatula*: choice of substrates and growth conditions"). Each node bears one leaf and maintains the potential to generate one lateral branch from the leaf axil. For *M. truncatula*, the phyllochron, defined as the time interval between the appearances of two successive flattened leaves on an axis (Moreau et al, 2006), is 2.5 to 3 days in fast growing plants.

After floral transition, each further node develops, in addition to the leaf, 1-3 flowers born by a single stalk (Figure 1a). *M. truncatula*, as all annual medics, is an autogamous species in which anthesis (pollen release from anthers) and self-pollination occur before flower opening (cleistogamy; Crawford *et al.*, 1989). The ovary contains 10-12 ovules, all of which are usually fertilized and form viable seeds. Under favourable growth conditions most flowers successfully self-fertilize and generate pods during 2-3 weeks until the quantity of developing pods on the plant leads to abortion of newly formed flowers and to a decrease in the overall plant growth rate. Growth and successful fructification resumes after a delay that roughly corresponds to the maturation time of this first set of pods. Well-developed, non-vernalized

plants in a growth chamber can have up to 3 subsequent waves of fructification. The first of these is the most productive, and in the end pronounced senescence symptoms develop with yellowing and extensive foliage loss. If there are no space and nutrient constraints, up to 5000 seeds can be obtained from a single plant within 4-5 months.

In many plant species, time to flowering is under the control of multiple environmental cues, including photoperiod, ambient temperature and prolonged exposure of germinating seedlings to cold temperatures (vernalization). Genetic studies of vernalization requirement have shown that several regulators integrate flowering time signals and resulting signals are sent to the shoot apex meristem (for recent reviews see Heggie & Halliday, 2005; Parcy, 2005; Sung & Amasino, 2005). Annual medic species, including *M. truncatula*, differ significantly in their flowering responses to each one of the above environmental cues (Clarkson & Russell 1975; Hochman 1987; van Heerden, 1984; J. Lichtenzveig, personal observations). For some (e.g. cv. Jemalong, Fig. 2), but not all *M. truncatula* accessions, vernalization is a significant factor in reducing time to flowering when days are *long* and temperatures are *moderate* (e.g. 16h/8h and 21-25°C – see growth chamber sets below). Under greenhouse condition, with seasonal changes in day-length and temperature, the effect of vernalization on time to flowering is secondary to the photoperiodic response (e.g. cv. Cyprus is insensitive to day length and flowers 5-6 weeks after seed germination irrespective of the day length, whereas DZA315 is late to flower and highly responsive to day length).

A - Seedling vernalization

Plants with reduced growth and a shorter life cycle can be advantageous for specific applications such a crossings (easier handling) and high throughput seed amplification of numerous genetic lines in parallel, for which culture room space can be limiting and seed yields of 150-200 are sufficient. Seedling vernalization is achieved by leaving imbibed seeds on germination Petri plates at 4°C for a lengthy period of time (≥1 week), and results in plants that both show reduced vegetative growth and stem branching, and also initiate flowering earlier in their development. Alternatively, for accessions that are less responsive to vernalization, flowering time can be considerably reduced by growing plants in small soil volumes (e.g. 8 x 7 cm pots or plugs) with occasional water stress by allowing the soil to completely dry out.

For cv. Jemalong, grown in a growth chamber under the conditions described below, 1 to 2-weeks of seedling vernalization reduces the time to flowering down to 26 to 23 days, respectively (time between seed planting and anthesis of the first flower). With vernalization periods > 2 weeks, it is possible to further reduce the time to flowering down to ~20 days, but overall plant growth is poor (Figure 1b, 1c and 2a; M. Chabaud and E.-P. Journet, unpublished). **For practical purposes note that the total time to flowering including the 4°C vernalization treatment remains about 5-6 weeks, and the main advantages lie in the reduction of plant size, and the time spent in the growth room.** A significant increase in time to flowering (1-2 weeks) is observed when light intensity and growth temperature are sub-optimal.

Vernalization of *M. truncatula* also results in dramatic changes in plant development which increase with the duration of the cold treatment. In non-vernalized c.v. Jemalong plants, the first flowers on the primary stem appear on the 11^{th} – 14^{th} node, the 1^{st} node being defined as that bearing the first unifoliate leaf. After 2-weeks vernalization, growth is much less vigorous with thinner stems and smaller leaf area. Shoot ramification is also strongly reduced, and the first flowers appear on the 5^{th} – 6^{th} node (Figure 2b). A harvest of at least 15 pods, equivalent to 150 seeds, can be expected from 2-week vernalized plants.

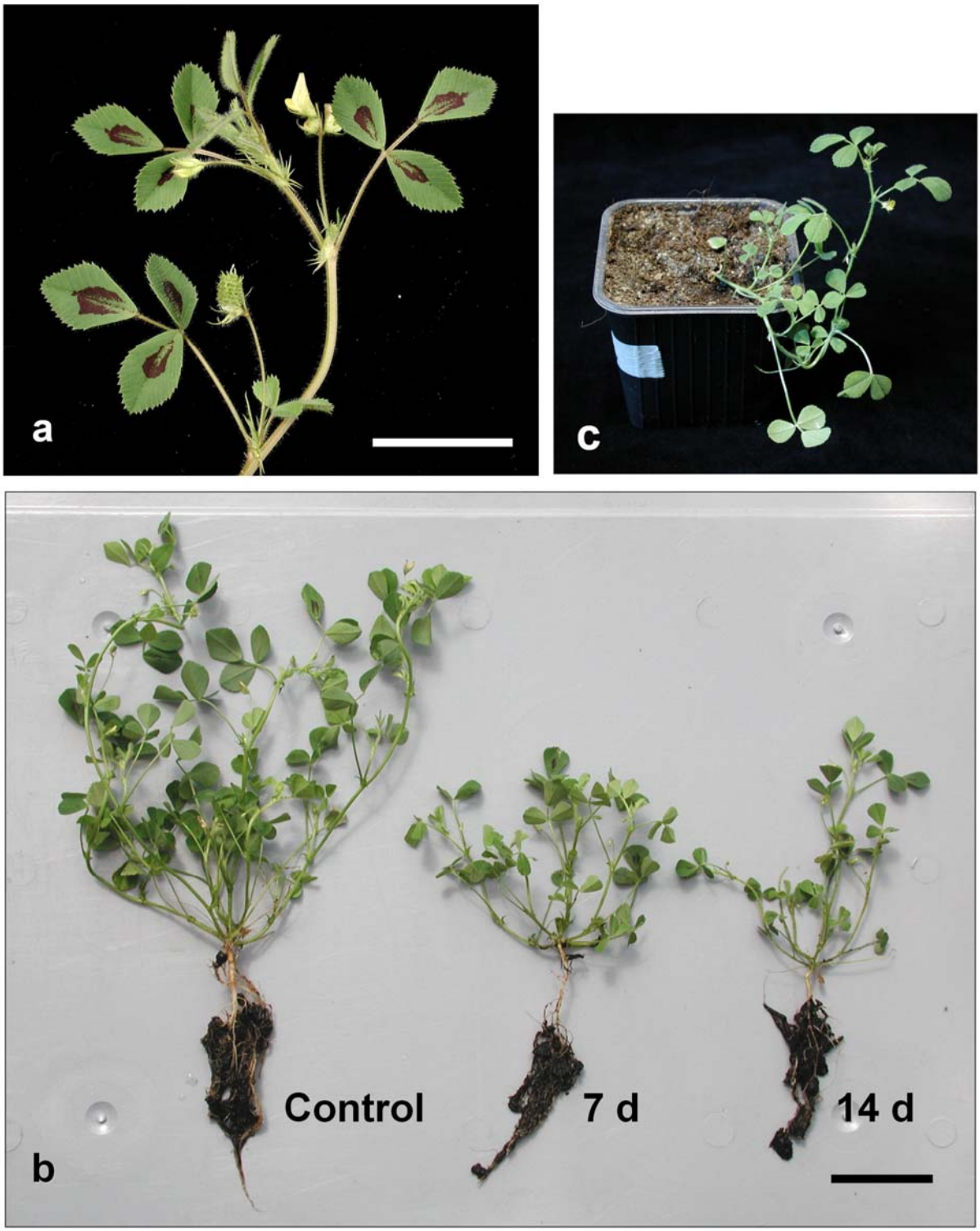

Figure 1 – Reproductive development and effect of vernalization

a: Shoot branch tip of a fast growing, non-vernalized plant after floral transition; each node bears one trifoliate leaf and 1-3 flowers attached to a single stalk. Note the gradient of developmental stages from flower bud to young pod; bar = 2,5 cm. **b**: General aspect of representative *Mt* plants 45 days after the beginning of a comparative vernalization experiment and grown in a chamber set at optimal conditions; **Control**: not vernalized; **7 d**: 7-day at 4°C; **14 d**: 14-day at 4°C. All seeds were scarified and water-imbibed at the same time.

(Figure 1, continued)
Control plants were planted immediately and vernalized plants transferred to soil just after cold treatment. Note that plants from the three treatments initiated flowering almost simultaneously (about 1 week before the picture was taken) although of course after different times in the growth chamber. Vernalization results in plants that initiate flowering earlier in their development, and show reduced vegetative growth and stem branching. Bar = 5 cm. **c**: Aspect of a 14-day vernalized plant 5 days after floral initiation (42 days total including vernalization); such plants are convenient for performing crossings.

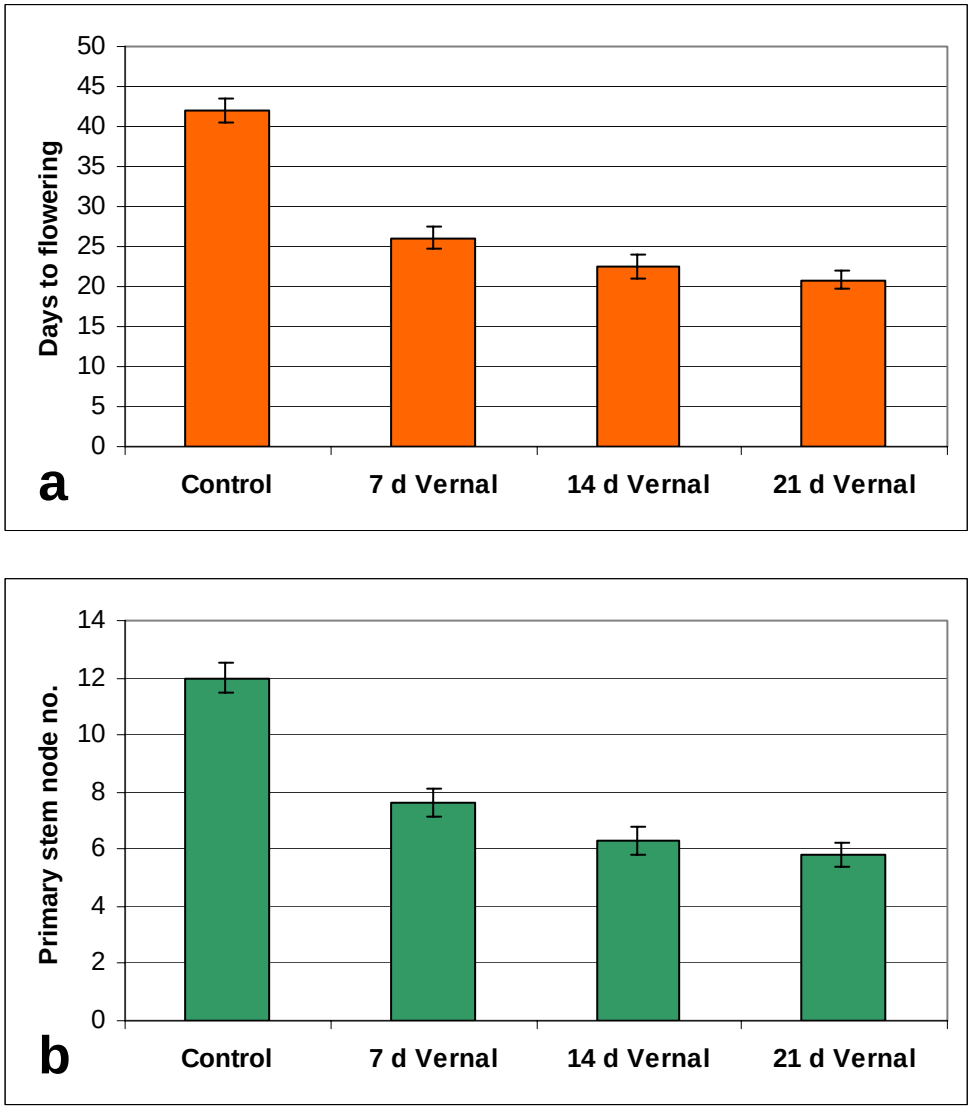

Figure 2 – Effect of the duration of vernalization on time to flowering and primary shoot node number bearing the first flower (Jemalong A17 line)

Two sets of 10 plants per condition were grown in optimal conditions in 2 similar growth chambers (250 $\mu E.m^{-2}.s^{-1}$; 16 h light at 25°C, 8h dark at 21°C). Prior vernalization at 4°C was performed on seeds imbibed on 0.8% water/agar germination Petri dishes. **a**: Days to flowering between transfer to growth room (pots) and anthesis stage of the first flower on the primary stem. **b**: Number of the primary stem node bearing the first flower. The 1st node is defined as that bearing the first unifoliate leaf. Note that following 0, 7, 14 and 21 days vernalization, flowers appeared on lateral branches 4, 2 and 2 days before and 2-3 days after the first flower on the primary stem, respectively.

B – Crossings (see video *to be added later*)

In *M. truncatula*, anthesis and self-pollination occur before flower opening (cleistogamy), a trait that complicates artificial hybridization procedures. General principles and guidelines for manual crossings in annual medics were originally established by Australian laboratories (e.g. Crawford *et al.*, 1989). The manual crossing procedure for *M. truncatula* described below, developed at LIPM-Toulouse (Thoquet *et al.*, 2002), is similar to method 3 described in Pathipanawat *et al.* (1994). Alternative steps used at ACNFP (Murdoch Univ.) are indicated where appropriate in the protocol. In order to determine the best developmental stages for *Mt* Jemalong flowers to be used in crosses, a microscopic study of pollination and fertilisation kinetics in relation to intact flower morphology was carried out (EP Journet, unpublished data). Pollen tubes growing down the style reached most of the 10-12 ovules aligned at the base of the pistil within 6-9 h post-anthesis. Thus both pollen and stigma appeared to be competent upon anthesis for immediate pollen germination and subsequent fertilization, leading to the conclusion that pollen and recipient flowers should be optimally used in crosses just after and just before anthesis, respectively (Figure 3). This was confirmed by the high success rate in our crossing experiments (see below). Similar conclusions were drawn by Pathipanawat *et al.* (1994) from interspecific crosses between several medic species.

The crossing protocol described below works efficiently for *M. truncatula* accessions other than c.v. Jemalong following the same basic principles and minor modifications (see relevant remarks).

Culture of parent plants

1. Vernalize seeds for 7-10 days at 4°C.

2. Plant seedlings into small pots (e.g. square pots 8 x 8 x 7 cm) containing a mixture of sand and compost (1:3, v/v) and transfer them to a growth chamber with high light intensity (120 to 250 $\mu E.m^{-2}.s^{-1}$; 16 h light at 25°C, 8h dark at 21°C). Water plants with *deionised* water and include complete fertilizer once every 1-2 weeks (see details in Chapter "Growing *M. truncatula*: Choice of substrates and growth conditions").

   Alternatively, grow plants in a greenhouse - ideally under a long day regime to induce early flowering of accessions responsive to photoperiod.

3. Vernalized c.v. Jemalong plants start to flower after ~ 25 days and are easy to handle due to their small size (see above). On the other hand, other accessions (e.g. DZA315 and Borung) start to flower later and can become too big. Trim older branches as needed, leaving enough young ones to maintain a good ramification potential. For plants used as females in crossing (pollen recipient), once a week systematically remove developing pods resulting from natural selfing in order to maintain full pod-forming potential. This maintenance seems less critical for pollen donors where the presence of numerous developing pods does not strongly affect pollen quality. Parent plants maintained in these conditions can be used for repeated crosses over at least 2-3 months. A single mother plant can simultaneously bear up to a dozen "crossing" pods distributed on several branches without any loss of fertility.

Remark: This protocol describes the optimal crossing procedure. Vernalization is not essential, since crosses can be performed using any healthy, flowering parent plants. However non-vernalized and therefore more voluminous plants are less practical to handle and more tedious to maintain.

Pollen sampling

1. Select and remove flowers at the optimal male stage (just past anthesis) from the male parent according to external morphological criteria: at this stage, petals are 1-2 mm longer than the calyx teeth, and a slight central depression is visible on the shaft of the standard petal (Figure 3a, 3b). Older flowers still contain some viable pollen; however they are less efficient for crossing.

2. Dissect flowers under a stereomicroscope with a cold white light source, using curved extra fine forceps and avoiding pollen dispersion caused by sudden tripping. In most appropriate flowers, where anthesis has just occurred, the released pollen appears turgescent, moist and sticky and is packed around the stigma (Figure 3c, 3d).

3. Extract whole sexual columns (pistil and stamens) with forceps and store them up to 1 h at RT inside a Petri plate, standing upwards on the edge of moist filter paper. Avoid contact between pollen and paper or water.

Choice of female flowers and crossings

1. Check that the plant chosen as female parent has formed young pods from the most recent flowers, to ensure that it is in the correct physiological conditions. Water the plant before crossing to prevent any water stress on the flowers.

2. Choose flowers on the female parent: the optimal female stage is just before anther bursting and pollen release, slightly earlier than that for male flowers (Figure 3b-d). Flowers formed on longer branches are easier to manipulate. To encourage plant resources to be diverted to the pollen recipient flower and favour pod development, the lead bud ahead of the flower and the two lateral buds or branches below the flower may be removed using a sharp scalpel (optional step).

3. Handle female flowers with care under a stereomicroscope, trying to minimize stalk twisting. Flowers can be held either between fingers or using a forceps (e.g. Fig. 4a). We propose two slightly different methods to prepare the recipient flower for cross-pollination:

a. (LIPM-Toulouse) Sever sepals and incise the standard petal longitudinally (~ 0.5 mm below its central line; Figure 3b) using the tip of a surgical blade and taking care to cut at an angle in order to avoid damage to the organs underneath. Slightly move apart the split pieces of the standard petal and the two underlying wing petals to gain visual access to the internal cavity of the flower. In case you find that anthesis has already occurred, discard the flower.

b. (ACNFP Murdoch) Carefully move sepals aside without removing them. Incise the standard petal longitudinally and then obliquely before the tip, as shown in Figure 4b. The petal tip creates a natural pouch. Gently move petal tip aside to uncover the stigma and the anthers. This method avoids accidentally slicing the stigma and provides high success rates particularly in dry environments since the petal-pouch and sepals serve as a natural protection to the stigma post-pollination.

4. Carefully remove one by one the 10 non-opened anther bags with an extra fine forceps, and visually check for the absence of released pollen on the stigma (if pollen is present discard the flower). Clean the forceps tips with paper or cloth after removing each anther bag.

5. Dab the stigma with the tip of a freshly harvested sexual column in order to saturate the sticky stigma surface with exogenous pollen (one sexual column can be used to pollinate 2 female flowers). If necessary, saturation of the stigma with pollen can be gently completed using the tip of the forceps.

6. Gently replace the pollinated pistil in its original position under the standard petal.

7. Label the internode just below the cross-pollinated flower, e.g. using a bright-colored adhesive paper tag. To prevent any mistake in subsequent identification, remove the flower bud from the next younger node. Insert the branch tip into a ~ 25-ml clear plastic vial containing 1 ml water (to maintain high humidity). Hold the branch in place with a cotton wool plug, avoiding excessive torsion or pressure on the stem (Figure 4e). Alternatively, use a tracing paper envelope to cover the pollinated flower. The flower edges are sealed with Millipore tape to allow some air diffusion (Figure 4c). Spray the branch with water to provide a humid micro-environment.

8. Keep the female plant under indirect light (risk of overheating) until the protecting vial (or envelope) is removed 48 h later. Successful crossing leads to the development of a small coiling pod that becomes visible 2-4 days after manual pollination.

9. Grey-brown pods containing hybrid seeds ripen within 4-5 weeks after crossing, and fall due to natural abscission. To avoid losing pods resulting from crosses, they should be tied at a relatively early stage to the stem - either wrapped in porous, transparent plastic sheet immobilized around the stem using staples, or covered with labeled net bags (Figure 4d).

Remarks on crossing protocol

- Performing crosses under a stereomicroscope requires some training. It is important for the manipulator to operate with forearms and wrists solidly resting on the bench. With experience, it takes ~5 min for a single cross, and 1 h for 6-8 crosses including pollen sampling and handling female plants.
- The efficiency of this method is good, with an average of 80 % cross-pollinated flowers giving pods with ≥ 5 viable seeds / pod. Three conditions appear critical for optimizing the success rate: (i) healthy parent plants (e.g. absence of thrips which feed on the pollen!); (ii) choosing the optimal developmental stage of both the pollen and the receptor pistil; (iii) saturation of the stigma surface with exogenous pollen. When these conditions are fulfilled, close to 100% crosses are successful and result in pods containing 10-12 seeds, as is the case for normal self-pollinating flowers.
- The hybrid nature of the resulting seeds can be ascertained in several ways. At LIPM Toulouse, we have extensively made use of a GUS reporter gene fusion as a genetic marker of exogenous pollen, in order to distinguish GUS-positive true F1 hybrids from GUS-negative self-progeny (Catoira et al, 2000, Journet *et al.*, 2001, Charron *et al.*, 2004). We have thus been able to verify that the frequency of residual self-pollination resulting from incomplete pollen removal is very low (less than 1 % F1 seeds). Successful hybridization may be confirmed by PCR amplification of polymorphic SSR markers, as practiced routinely at the ACNFP (Murdoch).
- This crossing protocol was initially established using several lines of the c.v. Jemalong. Significant variations in the optimal stage for crossing and in the rate of success were observed between various *M. truncatula* accessions and appear to depend mainly upon the characteristics of the maternal ecotype and the genetic distance between parental lines. For example, the flowers in accessions DZA315 or A20 reach maturity at a smaller size than in Jemalong, and stalks are also shorter in DZA315, making crosses trickier with such small flowers.

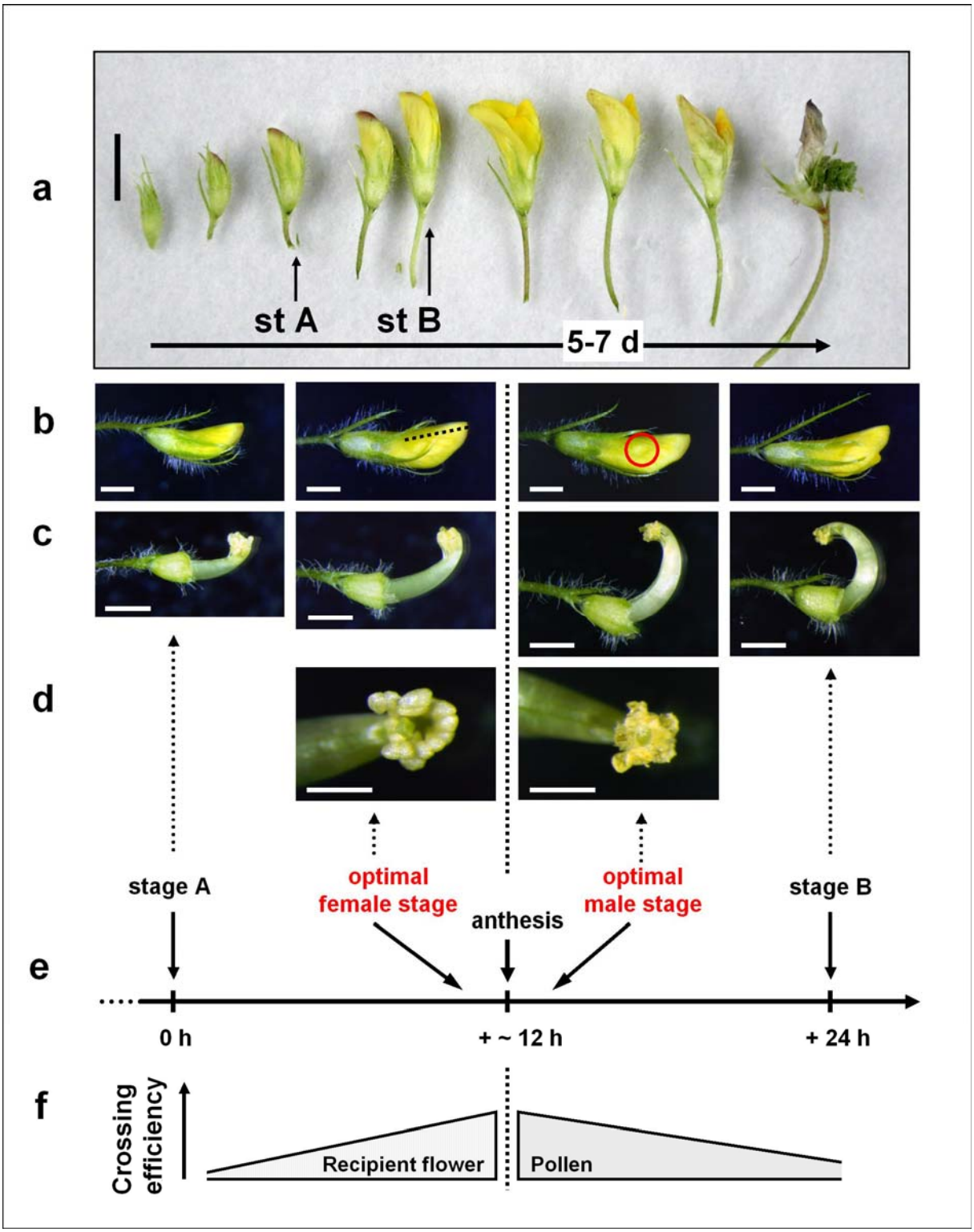

Figure 3 - Stages of flower development important for manual crossing in *M. truncatula*.

a: Series of developmental stages of non-dissected flowers of genotype Jemalong A17 covering a 5-7 day period, from the flower bud to the young coiling pod. Vertical bar = 5 mm. **Line b**: Detailed view of intact flowers between stage A and stage B. Stage A is defined as the stage when the tip of the standard petal grows past the calyx teeth. Black dotted line indicates where the standard petal is incised on flowers at the female stage optimal for cross-

(Figure 3, continued)
pollination. The black circle marks the slight lateral depression visible on flowers at the male stage optimal for pollen harvest. Bars = 2 mm. **Line c**: Same flowers as in line b, photographed after dissection to show the morphology of the staminal column surrounding the pistil. Note that petals are not trimmed in the crossing method described here. Bars = 2 mm. **Line d**: Close-up top views of the sexual column showing the intact anthers surrounding the stigma at the optimal female stage, and their collapsed aspect after pollen release ~4-h later (optimal male stage). Bars = 1 mm. **Line e**: Representative time-scale relevant to development stages shown in above lines. **Line f**: Scheme of manual crossing efficiency vs. developmental stage for the flowers used as either pollen recipients or pollen donors.

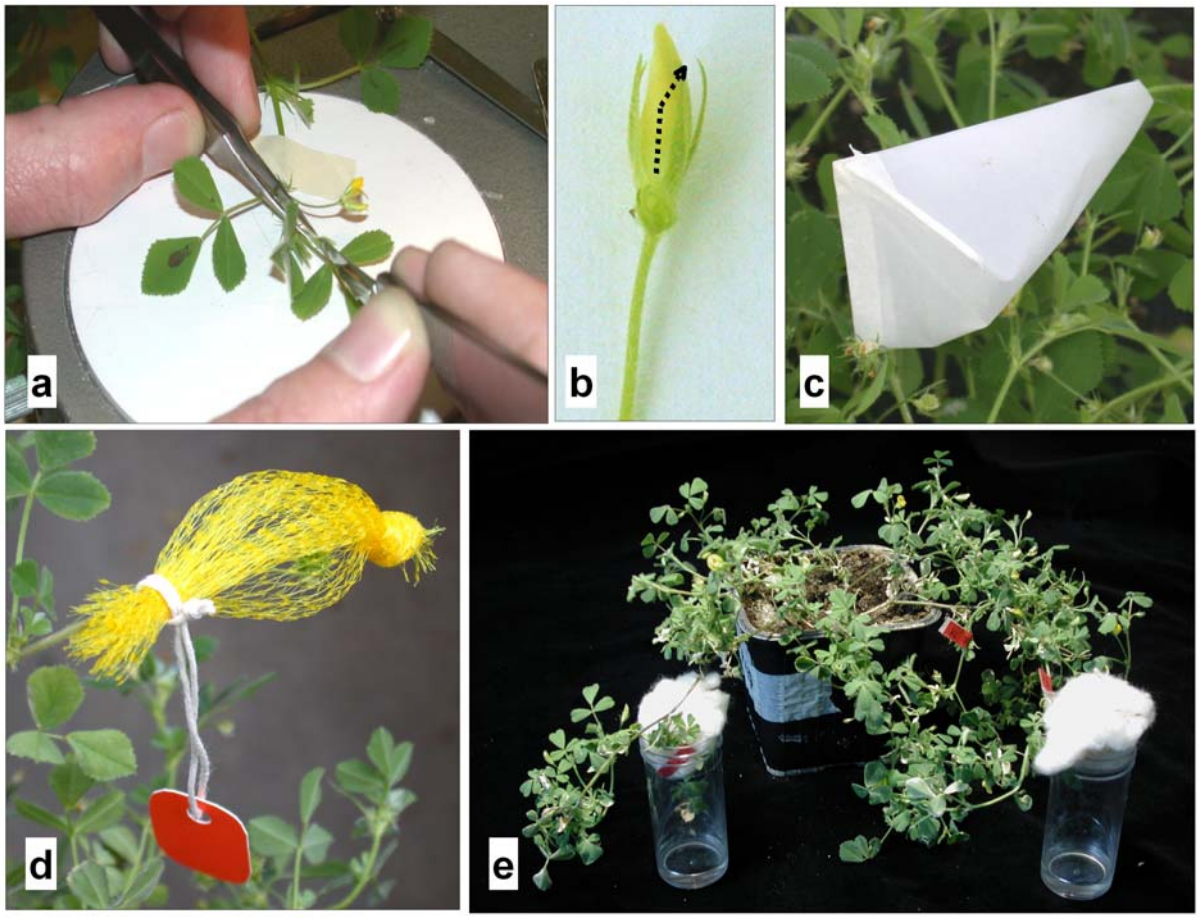

Figure 4 – Some details of the crossing procedure

a-d : Illustrations of the protocol used at ACNFP, Murdoch Univ.

a: Pollen recipient flower under stereomicroscope. **b**: Direction of incision on recipient flower (dotted line). **c**: Tracing paper envelope covering pollinated flower. **d**: Net-bag covering pod derived from cross-pollination.

e : Illustration for the LIPM-Toulouse protocol.

2.5 month-old vernalized plant bearing recently crossed flowers protected in moist vials and labeled with red tags.

Specific material

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

Other material

fine scissors, sterile water, transparent polystyrene vials (3 cm diameter, 7 cm height), cotton wool, self-adhesive labels, air-permeable transparent plastic sheet (Sealed Air / Cryovac Corp.), tracing paper (90-95g), Millipore tape.

C - Measuring pollen viability in F1 plants– (J. Lichtenzveig)

Pollen cells are sensitive to changes in the amount of chromosomal material (Griffiths *et al.*, 1996). Therefore, pollen viability is a good indicator of chromosomal aberrations. In plants, heterozygotes for chromosomal rearrangements such as deletions, inversions and translocations show significant reductions in fertility due to the presence of non-viable gametes (Griffiths *et al.*, 1996). Thus assessing viability in pollen grains from a F_1 individual might provide clues about its chromosomal configuration.

A number of histochemical stains are commonly used to assess pollen viability including fluorescein diacetate (Heslop-Harrison and Heslop-Harrison, 1970), 4',6-diamino 2-phenyl-indole (DAPI) and Alexander's stain (Alexander, 1969). Visualization of the first two dyes requires a fluorescence microscope. Alexander's stain is a reliable and rapid way to score pollen viability and requires only a light microscope (Johnson-Brousseau and McCormick, 2004). The stain contains malachite green, which stains the cellulose in pollen walls and acid fuchsine, which stains the pollen protoplasm. Aborted pollen grains lack protoplasm and thus fail to stain with acid fuchsine.

Procedure

1 - Collect flowers with mature non-dehiscent anthers, just before anthesis, in a similar stage as that of flowers used as pollen recipients in crosses (Figure 3d).

2 - Remove the anthers from the flower using the stereomicroscope.

3 - Place the anthers in a droplet of stain on a slide and shake gently to release the pollen grains into the solution.

4 - Discard the empty anthers and cover the pollen suspension with a cover slip.

5 - Assess the proportion of viable and non-viable grains from at least 100 cells. Grains that are viable will stain dark blue or purple and grains that are dead will stain pale turquoise blue under a light microscope.

Materials

- Alexander's stain – working solution (see below)
- Low magnification stereomicroscope
- Extra fine forceps for microscopy
- Scalpel
- Microscope slides and cover slips
- Hand tally counter (a differential counter is optional)

Remarks

- It is advisable to test a number of flowers per plant throughout the growing season.
- Flowers can be stored for 1-2 days at 4°C in small Petri dishes containing wet Whatman paper to keep high humidity.

Alexander's stain solutions

Stock solution (100ml)

WARNING! – **This solution constitutes a serious health hazard**.

95% ethanol	10 ml
1% malachite green in 95% ethanol	5 ml
1% acid fuchsine in H_2O	5 ml
1% Orange G in H_2O	0.5 ml
Glacial acetic acid	2 ml
Glycerol	25 ml
Phenol	5 g
H_2O	47.5 ml

The original recipe for this stain contains chloral hydrate which has been banned in many countries. Chloral hydrate and Orange G can be omitted for pollen viability assessment. Lactic acid can be used instead of glacial acetic acid.

Working solution

Dilute 1:50 in H_2O

Acknowledgements
We are grateful to David Barker for critical reading of this chapter.

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