

DEVELOPMENT OF AN INTERSPECIFIC HYBRIDISATION PROTOCOL FOR *LUPINUS*

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ABSTRACT

We are developing interspecific hybridisation methods to transfer traits among the crop lupin species *L. luteus*, *L. mutabilis* and *L. albus* to *L. angustifolius*, all of which have different chromosome numbers and moderately phylogenetically separated. Investigations into pre-fertilisation barriers using SEM and fluorescence microscopy indicated that crossing was possible. Applying a hormone treatment at- and post-crossing increased pod retention but did not increase seed set. Extensive hand-pollination trials between the 4 lupin species have produced over 250 seeds from different cross combinations among these 4 species. Validation of hybrid status so far suggests that these seeds are the results of apomixes i.e. not true hybrids. The best combinations of species were *L. angustifolius* × *L. luteus* and some seeds were produced from *L. angustifolius* × *L. mutabilis*, *L. angustifolius* × *L. albus* and *L. albus* × *L. mutabilis*. We screened a range of *L. angustifolius* to determine the three best female genotypes. Pods produced by these crosses range in size from large (viable seed produced), medium (an enlarged pod with shriveled or aborted seed) and small (seeds aborted). Hybrid status of seeds from large pods is being assessed using Resistance Gene Analogue Polymorphism (3).

Immature seeds from medium-sized pods are candidates for embryo rescue. Embryo rescue protocols have been developed for 'selfed' immature seeds (14 to 21 days old). Survival of these embryos, rescued on a B5-based medium, varied with development of the embryo and osmotic potential of the culture medium. Globular-shaped embryos require a more complex culture medium than more developed embryos. If true hybrid plants can be developed using these methods, particularly for *L. angustifolius* × *L. luteus*, they will directly benefit the lupin breeding program in Western Australia by increasing seed quality and disease resistance.

KEY WORDS

interspecific hybridisation, grain legume improvement, embryo rescue, tissue culture

INTRODUCTION

Lupins, despite being a well-adapted crop in Australia on infertile, well drained soils in Mediterranean type climates, are suffering from low profit margins as a result of low grain prices in recent seasons. Improving the quality of lupin seeds (i.e. protein and oil content) can potentially increase the return to farmers, particularly if they can be sold for higher value markets including human consumption and specialised feed markets such as aquaculture.

Narrow-leafed lupin, *Lupinus angustifolius*, is the most widely produced and adapted crop but has relatively low protein (32-34%) and oil (4-6%) compared with other lupin species such as *L. luteus* (38% protein and 5% oil), *L. albus* (36% protein and 9% oil) and *L. mutabilis* or pearl lupin (42-45% protein and 16-20% oil, Clements *et al.* 2005). These species can be grown in Western Australia but are currently not as well-adapted as *L. angustifolius*.

Creating interspecific hybrids between narrow-leaf lupin and any of these three lupin species (*L. luteus*, *L. albus* or *L. mutabilis*) has the potential to create new genetic variation for lupin breeders. Increasing the seed quality of narrow-leafed lupin (protein and oil) with additional disease resistance such as brown spot from *L. luteus* is one example of the benefits that could be achieved from interspecific crossing.

There have been some previous reports of interspecific hybrids between *Lupinus* species (Kasten and Paradies *et al.* 1991; Kasten and Kunert, 1991; Przyborowski and Packa, 1997; Sawakia-Sienkiewicz and Brejda, 1999). Creating hybrids between narrow-leaf lupin ($2n = 40$) and the three species of interest (*L. luteus*, $2n = 52$; *L. albus*, $2n = 50$; and *L. mutabilis* $2n = 48$) is hampered by the different number of chromosomes and the phylogenetic distance (Crouch and Dwivedi, 2005). Many of the more successful examples have been between new-world species, which are thought to be more closely related and have the same number of chromosomes.

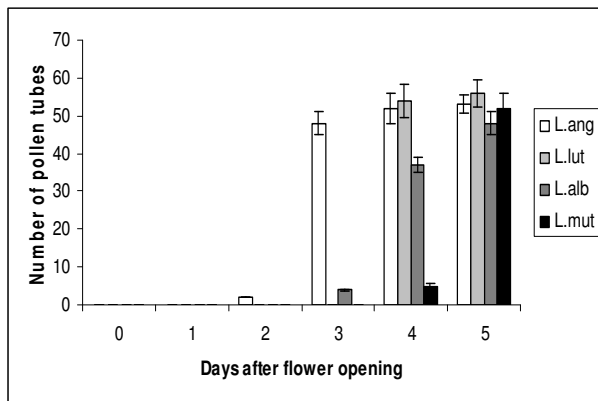


Fig. 1. Number of pollen tubes observed growing down dissected styles of *Lupinus angustifolius* cv. Coromup, *L. luteus* cv. Pootalong, *L. albus* cv. Andromeda and *L. mutabilis* breeding line P26961, 0-5 days after flower opening.

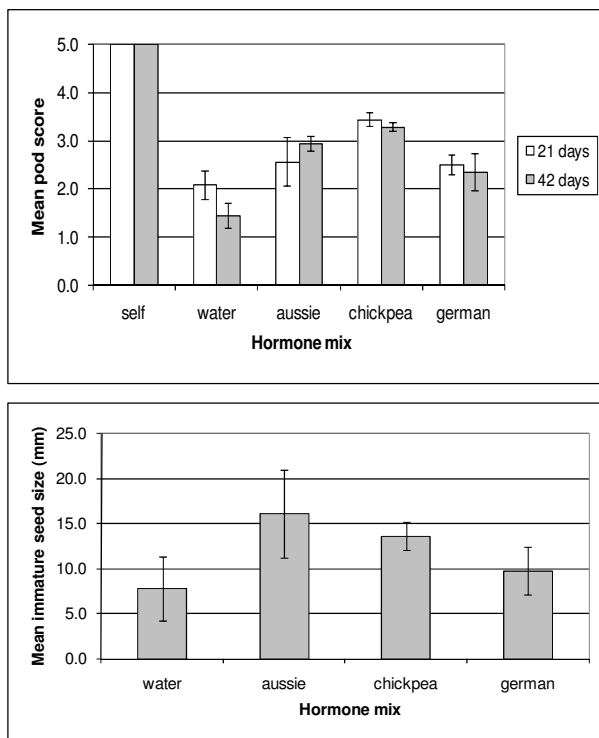


Fig. 2. Top) Average score of interspecific hybrid pod after 21 and 42 days treated with three hormone solutions (aussie, chickpea and german) compared with a control (water) and selfed pods. Bottom) average immature seed size (mm) 42 days after crossing. N.B. no embryos were observed in any of these putative hybrid seeds.

To develop interspecific hybrids information about embryo development, pollen biology, pre- and pod fertilisation barriers, crossing methods and embryo rescue techniques must be considered. Here we report on progress towards developing interspecific hybrids between these 4 species, with a particular focus on narrow-leaf lupin.

MATERIALS AND METHODS

Plants used for microscopy and embryo rescue were *Lupinus angustifolius* cv. 'Coromup', *L. luteus* cv. 'Pootalong', *L. mutabilis* cv. 'P26961' and *L. albus* cv. 'Andromeda' and were grown all year round in a phytotron at 20°C day/12°C night, with natural light.

PRE-FERTILISATION BARRIERS

Pollen of the 4 study species were germinated in pollen germination medium (Brewbaker and Kwack, 1963) using the cellophane method (Alexander and Ganeshan, 1989). Pollen tubes were allowed to grow *in vitro* for 16 hours and were stained with decolourised Aniline Blue and germination rates were determined using fluorescence microscopy (Clarke and Khan *et al.* 2004). Selfed flowers of the 4 lupin study species were also collected at 0, 1, 2, 3, 4 and 5 days after flower opening and fixed to examine *in vivo* pollen germination (Clarke and Khan *et al.* 2004). Selfed flowers/pods of *L. angustifolius* cv. Coromup were collected from main stem inflorescence 0, 1, 3, 6, 9, 12, 15, 18 and 21 DAFO. Fresh and fixed (SPURRs resin) samples studied from the flowers and pods from the main stem.

INTERSPECIFIC CROSSING AND HORMONE APPLICATION

Various hormone mixes were applied to two crosses: *L. angustifolius* cv. Coromup x *L. luteus* cv. Pootalong and *L. angustifolius* cv. Coromup x *L. luteus* cv. P28176 in glasshouse trails. Three hormone mixes, a water control and a no spray control were applied using a spray bottle at crossing, as well as one and three days after crossing. Pod development was scored 21 days after crossing and pods were harvested 42 days after crossing and immature seeds size was recorded.

Extensive field-based interspecific crossing experiments were conducted in July/August 2006 and 2007. In 2006 ninety-five different crosses were conducted between the 4 lupin species, with emphasis on the *L. angustifolius* x *L. luteus* combination. Crossing experiments in 2007 focussed on the twenty best cross combinations using results from the previous field-based and glasshouse experiments. Pod size was scored, three weeks after crossing and side branches were trimmed at this stage. Pods with mature seeds were harvested eight to ten weeks after crossing, when dry. DNA of putative hybrids and their parents were compared using resistance gene-analog polymorphism (Yan and Chen *et al.* 2003).

IMMATURE SEED AND EMBRYO RESCUE

Embryo rescue methods were developed using selfed pods ranging from 14 to 21 days after flower opening. Pods were surface sterilised in 1% bleach for 10 mins, rinsed thoroughly in sterile distilled water then embryos were dissected from immature seeds. Seeds were then transferred to culture medium and care was taken to keep the cut surface of the funiculus in contact

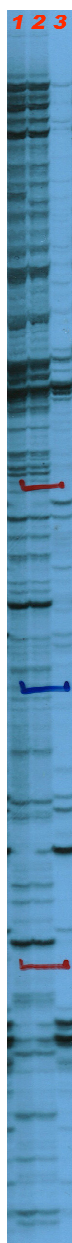


Fig. 3. Example of sequencing gel x-ray showing: (1) Female parent (*Lupinus angustifolius* cv. Mandelup); (2) Putative hybrid (*Lupinus angustifolius* Mandelup X *L. luteus* Ukraine 12); (3) Male parent (*Lupinus luteus* Ukraine 12).

L. luteus (15 ± 1) than the reciprocal cross (4 ± 1). Similarly, the cross *L. mutabilis* (maternal parent) x *L. albus* had more pollen on the style (11 ± 1) than the reciprocal (5 ± 1).

We have established that pollen viability and germinability *ex situ* is high ($> 95\%$) for all 4 species. Pre-fertilisation barriers seem to be limited to particular cross combinations, which should be minimised assuming crossing efforts focus on *L. angustifolius* as the female parent and not the reciprocal cross. This is further supported by the pod-score results from 2006

with the medium. Various culture media and embryo rescue methods were evaluated but are not reported in this study. The culture medium currently used is liquid; $1\frac{1}{2}$ times Gamborg's B5 basal medium with 750 mg L^{-1} casein hydrolysate (Kasten and Kunert, 1991), 90 g L^{-1} sucrose (Clarke and Wilson *et al.* 2007) and no plant growth regulators.

RESULTS AND DISCUSSION

PRE-FERTILISATION BARRIERS

The cellophane method was a successful method for germinating pollen of all 4 study-species *in vitro*. Pollen germination for *L. angustifolius* was $99.6\% \pm 0.2$, for *L. luteus* was $99.5\% \pm 0.5$, for *L. mutabilis* was $99.6\% \pm 0.4$ and for *L. albus* was $69.3\% \pm 1.2$. There was a lag of 2 to 4 days between flower opening and the first pollen grains recorded on the style. For *L. angustifolius*, this lag was as short as two days and the style had approximately 50 pollen cells by the third day and did not significantly increase with time (Fig. 1). There was a similar trend for the other three species although the lag between flower opening and the first pollen cells germinated on the style varied for each species. For *L. albus*, the first pollen cells were observed three days after flower opening while for *L. luteus* and *L. mutabilis* there was a lag of 4 days.

The number of pollen cells observed on the style, three days after pollination, was significantly lower for cross pollinated flowers than for selfed flowers. The direction of crossing also had a significant affect on the number of pollen cells germinating on the style. There were more pollen cells on the style for *L. angustifolius* (maternal parent) x

and 07, where pod set and size varied according to cross combination (see below).

INTERSPECIFIC HYBRIDISATION AND APPLICATION OF HORMONES

The application of hormones to the raceme at crossing did not enhance pod score or pod retention (Fig. 2a). After 21 days, all crosses had a lower average pod score and lower average pod retention than the selfed control. Crosses sprayed with the chickpea hormone solution had higher pod scores, pod retention and larger immature seed size than the control after 21 days (Fig. 2a) but similar to that of other hormone solutions. However, as there was no difference in embryo development we decided to use a water spray for field crossing experiments. Further experiments are currently underway to investigate use of hormone application further.

Results from field crossing experiments show variation between cultivars suggesting some are cultivars are better females than others (Table 1). For example the cross *L. angustifolius* x *L. luteus* produced 6 putative hybrid pods per flower crossed while the reciprocal cross produced only 2. Within this cross, the cultivar Mandelup produced the highest proportion of seeds (11%), followed by P29050 (9%) and Coromup (6%) while there were no seeds recovered when Mandelup was the maternal parent when crossed with *L. luteus* (Table 1). Similar comparisons can be made for the cross *L. angustifolius* x *L. albus* (6%) and its reciprocal (1%).

Over 100 putative hybrid seeds have been generated via this field crossing experiment. All of these seeds have been grown under glasshouse conditions and visually assessed for similarity to their parents. In every case, the seeds exhibited the same plant morphology and flowering times as their female parent and were fertile. When their DNA was assessed using RGAP analysis, 'hybrids' exhibited the same banding as the female parent (Fig. 3). This evidence suggests that either selfing is occurring via pollen transfer from non-sterile forceps in a proportion (3-5% species dependant) of crossing events or that these seeds are of apomictic or matromorphic origin. To help understand this, we are currently screening putative-hybrid seeds made by crossing heterozygous *L. angustifolius* with *L. luteus*.

EMBRYO DEVELOPMENT OF *L. ANGUSTIFOLIUS* SELFS

Embryo development of selfs was studied to provide a benchmark for 'normal' embryo development, and has been used in other crops including chickpea (Clarke and Wilson *et al.* 2006). Embryos developed to the globular stage by approximately twelve days after pollination and the endosperm was beginning to solidify (Fig. 4). By fifteen days after pollination, embryos reached the heart-shaped stage, by eighteen days they were at the cotyledonary stage and by 21 days the embryo takes up about $\frac{1}{3}$ of the seed.

Table 1. Percentage of fertile, seeds set per number of flowers crossed between *Lupinus angustifolius*, *L. luteus*, *L. albus* and *L. mutabilis*. Crosses were conducted in 2006 in tunnel houses; planting in June 2006 and harvesting in September/October 2006. NB. Totals calculated on a 'per number of flowers crossed' basis.

Female ↓ Male →	<i>L. angustifolius</i>				<i>L. luteus</i>				<i>L. mutabilis</i>				<i>L. albus</i>			Grand Total
	Mandelup	Coromup	P29050	Total	Pootalong	Ukraine#12	P28176	Total	ID8	ID13	P26961	Total	Kiev	Andromeda	Total	
<i>L. angustifolius</i>																
Mandelup	-	-	-	-	1	10	2	11	3	3	0	5	1	7	11	
Belara	-	-	-	-	0	0	0	0	4	1	0	5	0	3	4	
Coromup	-	-	-	-	5	0	1	6	6	0	0	6	3	1	6	
P29050	-	-	-	-	3	3	3	9	3	3	2	8	1	1	3	
Total	-	-	-	-	6	9	4	6	12	5	1	6	4	9	6	6
<i>L. luteus</i>																
Pootalong	2	0	0	1	-	-	-	-	0	0	0	0	0	0	0	
Wodjil	5	0	0	4	-	-	-	-	1	0	0	1	-	-	-	
Ukraine#12	1	0	0	1	-	-	-	-	1	1	6	6	-	-	-	
P28176	0	4	0	2	-	-	-	-	1	0	0	1	-	-	-	
Total	4	2	0	2	-	-	-	-	2	1	3	2	0	0	0	2
<i>L. mutabilis</i>																
P25954	0	0	0	0	0	0	-	0	-	-	-	-	0	0	0	
P26961	0	0	0	0	-	-	-	-	-	-	-	-	1	3	6	
P27808	0	0	0	0	-	-	-	-	-	-	-	-	1	0	1	
Total	0	0	0	0	0	0	0	0	-	-	-	-	2	3	5	1
<i>L. albus</i>																
Kiev	0	0	0	0	0	0	-	0	0	0	0	0	-	-	-	
Walab	1	0	1	2	-	-	-	0	0	0	3	3	-	-	-	
Andromeda	0	1	0	1	-	-	-	0	4	1	0	4	-	-	-	
Total	1	1	1	1	0	0	0	0	4	1	3	2	-	-	-	2
Grand total				1				5				3				4
																3

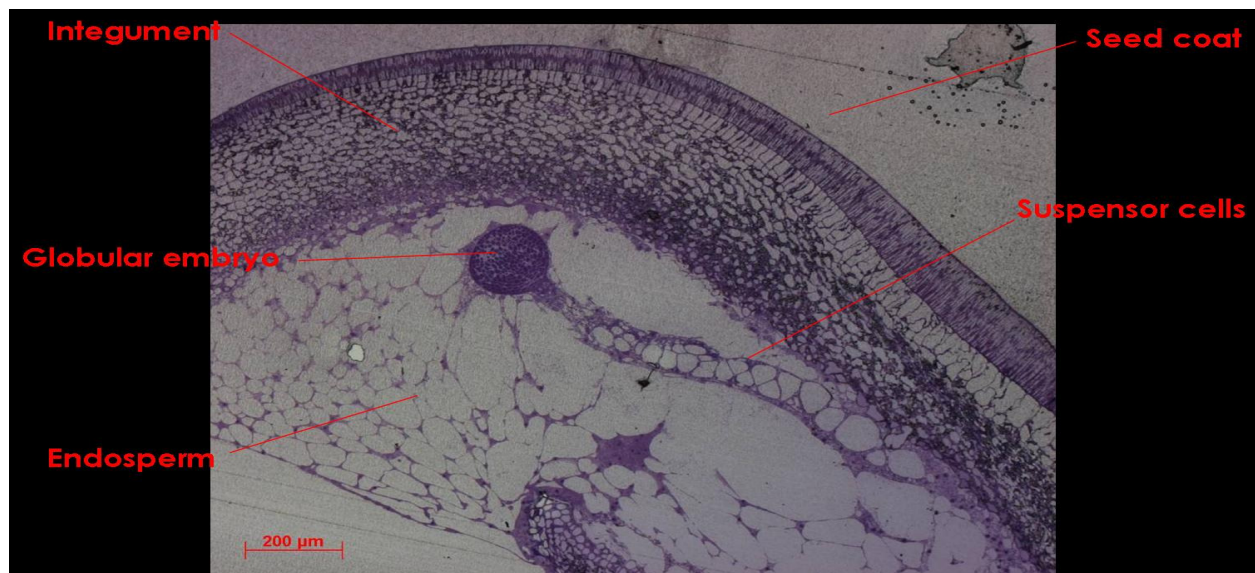


Fig. 4. Transverse section through *Lupinus angustifolius* cv. Coromup immature seed, 12 days after flower opening, fixed in SPURRs resin and stained with Toluidine Blue.

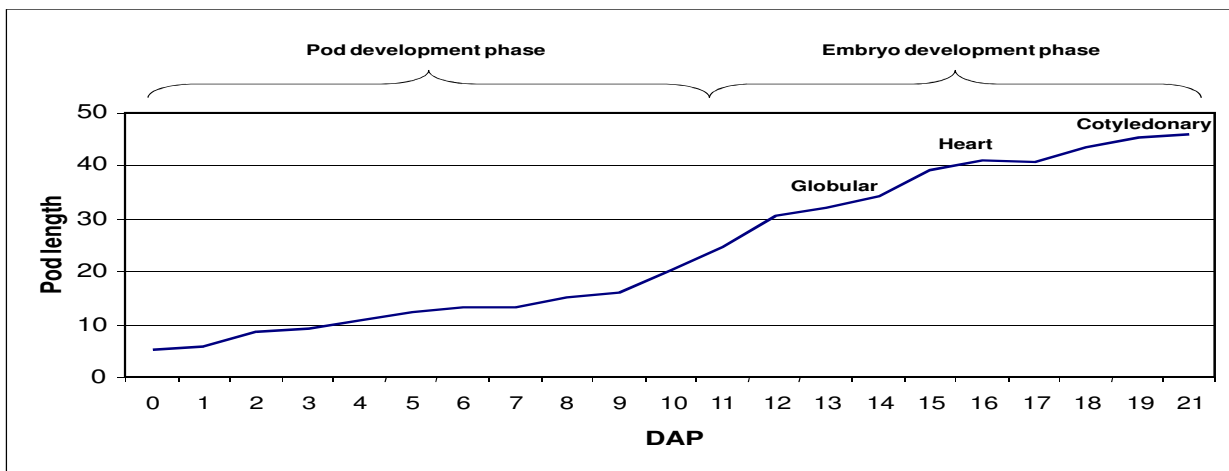


Fig. 5. Pod length and associated embryo and developmental stages of *Lupinus angustifolius* cv. Coromup for 0-21 days after pollination.

The development of *L. angustifolius* cv. Coromup seeds can be described in three general phases. The first phase, which we termed pod development, includes the first ten to eleven days after flower opening. This phase involves the extension and expansion of the pod while the immature seed remains small. The second phase that we have termed embryo development phase includes from eleven to twenty-one days after flower opening. This phase involves expansion of the immature seed and development of the embryo through globular (approximately 12 days), heart (approximately 15 days) and cotyledonary (approximately 21 days) stages (Fig. 5). The last phase of pod and embryo development is the maturation phase, where the embryo expands to fill the entire seed and eventually dehydrates and become dormant (approximately 40 days)

We propose that the best time to collecting pods to attempt embryo/ovule rescue is at 18-21 DAP. By this time, the embryo is developed enough to survive in culture (i.e. heart-cotyledonary) and it can survive on a basic culture medium. Selfed embryos collected and 'rescued' at this stage of development will grow and develop *in vitro*.

EMBRYO RESCUE OF *L. ANGUSTIFOLIUS* SELFS

Selfed *L. angustifolius* embryos were rescued in a modified system that builds on knowledge from previous research papers on lupin (Kasten *et al.* 1991; Kasten and Kunert 1991; Pryzyborowski and Packa, 1997) and chickpea embryo rescue (Clarke and Wilson *et al.* 2006). Lupin embryos rescued at the heart-shaped or cotyledonary stage (> 14 days) were able to develop into plantlets, regardless of the culture medium. Very early heart-shaped embryos performed best on a filter paper bridge in a liquid culture medium consisting of: 1½ times Gamborg's B5 basal medium with 750 mg L⁻¹ casein hydrolysate (Kasten and Kunert, 1991), 90 g L⁻¹ sucrose (Clarke and Wilson *et al.* 2006) and no plant growth regulators. Globular-shaped embryos (< 14 days) are less developed than heart or cotyledonary embryos and have a much greater need for vitamins, amino acids and carbon than differentiated embryos/tissues. Separate rescue methods are usually developed for globular- and heart-shaped embryos.

Embryo development in interspecific crosses of *Lupinus*, as with many crop species (Clarke and Wilson *et al.* 2006; Geerts and Toussaint *et al.* 2002; Schryer and Lu *et al.* 2005), is much slower than in selfed embryos. This poses a potential problem for harvesting pods for embryo rescue (i.e. when to collect hybrid embryos). From more than 2,000 crosses done in the field, less than 10% of pods formed had developed far enough to attempt embryo rescue. Of those 'rescued' the embryos were too small to be observed with a stereomicroscope.

We are currently refining the embryo rescue medium to attempt to rescue less mature embryos. We are also developing the hormone mixture further in an attempt to accelerate embryo growth and retain pods on the female for longer.

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