Improving Seed Production of Lotus

by Graeme Sandral, Daniel Real and Liz Nutt

March 2010

RIRDC Publication No 10/014
RIRDC Project No. PRJ-000506
Foreword

The aim of this project was to develop information and/or technologies that would allow a viable seed industry to be established for perennial Lotus in Australia. Currently no commercial seed production of Lotus is undertaken in Australia and to develop this new industry several important considerations needed to be addressed, including:

- Improved seed yields at Australian latitudes. *Lotus corniculatus* is a long day plant requiring 14.5 hours of daylight or more for adequate flower production and in Australia these light requirements are not satisfied adequately
- Reduced seed losses during and just prior to harvesting due to pod shattering
- Better adapted species, in particular those that are drought tolerant, to ensure continued demand for seed.

The area of adaptation for new *Lotus corniculatus* varieties in the Murray Darling Basin alone covers some 8.2 m hectares, providing for the first time a high quality forage legume for livestock over summer in areas where the soil and climatic conditions exclude the use of Lucerne and White clover. This can only be fully achieved if a seed crop industry can be established within the economic constraints of commercial seed production systems in Australia. This project was focused on overcoming the technical constraints of *Lotus corniculatus* seed production.

Seed growers will be able to grow new seed crops of *Lotus corniculatus* using harvesting techniques that are different to those currently in use for Lucerne and White clover.

Farmers in areas where Lucerne or White clover has failed due to low soil pH, waterlogging or summer drought, will for the first time have a perennial legume suitable for sowing on farm.

The project has been successful in developing harvesting methodologies for *L. corniculatus* that can be used in Australia to achieve yields comparable to those achieved overseas. The project has also helped generate the first self compatible cultivar of *L. corniculatus* which will be commercialised for use in Australia.

This report is an addition to RIRDC’s diverse range of over 1900 research publications and forms part of our Pasture Seeds R&D program, which aims to: Improve the overall productivity and returns from producing and marketing quality Australian pasture seeds and Improve the technical and economic information available to suppliers and users of pasture seed.

Most of RIRDC’s publications are available for viewing, free downloading or purchasing online at [www.rirdc.gov.au](http://www.rirdc.gov.au). Purchases can also be made by phoning 1300 634 313.

**Peter O’Brien**
Managing Director
Rural Industries Research and Development Corporation
About the Authors

Mr Graeme Sandral is a Pasture Plant Ecologist with NSW DPI and has over 20 years experience in plant collecting, germplasm evaluation, breeding and selection as well as farming systems development. This project was developed by Mr Sandral and aimed to generate knowledge and or plants of Lotus that could help established as a new seed industry in Australia.

Dr Daniel Real is a Plant Breeder currently working in the Future Farming System CRC. He has formal plant breeding training combined with 17 years experience in breeding both annual and perennial legumes, culminating in the release of eight forage varieties.

Liz Nutt was the Research Assistant employed on this project. She has over 13 years of involvement in pasture research most of which has focused on plant improvement. Her skills in this area were invaluable to the outcome of this project.

Acknowledgments

We would like to acknowledge the help and insights provided by, Jonathan Warden (Research Officer) and Mike Blair (Research Station Manager). Also we would like to thank the farmers that participated in this research activity, particularly the Snell and Eastcott families at Waroona WA for providing land.

We would also like to thank NSW Department of Primary Industries, Department of Agriculture and Food Western Australia, the Salinity CRC, the Future Farming Industries CRC and The University of Western Australia.

Abbreviations

CRC – Commonwealth Research Centre
NSW DPI – New South Wales Department of Primarily Industries
RIRDC – Rural Industries Research and Development Corporation
R&D – Research and Development
EMS – ethylmethanesulfonate
PBR – Plant Breeders Rights
AWI – Australian Wool Innovation
LC07AS – A new selfing line of Lotus corniculatus developed as part of this project.
M1 – First generation mutants
M2 – Second generation mutants
## Contents

**Foreword** ................................................................................................................................................ ii

**About the Authors** ................................................................................................................................ iv

**Acknowledgments** ................................................................................................................................. iv

**Abbreviations** ........................................................................................................................................ iv

**Executive Summary** ............................................................................................................................ vii

**Introduction** ........................................................................................................................................... 1

  - Breeding self compatible *Lotus corniculatus* ......................................................................................... 2
  - Harvesting *Lotus corniculatus* ............................................................................................................. 3
  - Harvesting methods ................................................................................................................................ 6
  - Harvest 2006 ........................................................................................................................................... 7
  - Harvest 2007 ........................................................................................................................................... 7
  - Harvest 2008 ........................................................................................................................................... 9
  - Mutation breeding ................................................................................................................................... 10
    - Plant Materials .................................................................................................................................... 10
    - Optimization of EMS concentration .................................................................................................... 10
    - Development of the mutant populations (M1 and M2) ...................................................................... 10
  - Breeding self compatible *Lotus corniculatus* ......................................................................................... 11
  - Harvest 2005/06 ..................................................................................................................................... 13
  - Harvest 2007 ........................................................................................................................................... 14
  - Harvest 2008 ........................................................................................................................................... 16
  - Mutation breeding ................................................................................................................................... 17
    - Optimization of EMS concentration .................................................................................................... 17
    - Development of the mutant populations (M1 and M2) ...................................................................... 17
  - Breeding self compatible *Lotus corniculatus* ......................................................................................... 18

**References** ............................................................................................................................................ 23
Tables

Table 1  Number of seeds required to achieve 150 viable plants after EMS treatment ........................................ 11
Table 2  Treatments applied to seeds from the M1 harvest and original seeds of Gifu and LC041 .................. 11
Table 3  Seed yield (kg/ha) before and after harvesting and percentage of seed in the harvested material ....... 15
Table 4  Comparison of desiccant treatment conducted in 2007 and 2008 are presented (kg/ha) and percentages in parenthesis ..................................................................................... 17
Table 5  Number of plants showing chlorotic symptoms per species and EMS treatment ............................ 18
Table 6  Weight of seeds harvested in bulk for the plants showing chlorotic symptoms and normal plants for each species ........................................................................................................... 18
Table 7  Crosses that produced selfing progenies planted in 2007 ................................................................... 19
Table 8  Number of individual plants sown in 2007 from a single plant from a cross that produced selfers and number of selfing plants produced in 2008 ......................................................................... 20

Figures

Figure 1  Cutting treatment on 31 August 2005 (left) and 11th October 2005 (right) ............................. 7
Figure 2  Lotus corniculatus seed crop before harvesting in 2007 .......................................................... 8
Figure 3  Harvesting the cut and swath treatment (left), sprayed treatment (middle) and direct harvest treatment (right) in January 2006 ................................................................. 8
Figure 4  Lotus corniculatus stand before harvesting 4 February 2008 ................................................. 9
Figure 5  The three spraying time treatments on the day of harvest .......................................................... 9
Figure 6  Shows the flowering intensity of L. corniculatus recorded over 2004 and 2005 ...................... 13
Figure 7  Shows the Number of flowers/m² for each cutting time (September, October) and the un-cut control ................................................................. 14
Figure 8  Number of open flowers (inflorescences) in a first year L. corniculatus seed crop ........ 15
Figure 9  Number of yellow inflorescences, green, dried and shattered pods in January and February 2008 .................................................................................................................. 16
Figure 10 Germination counts for L. corniculatus and L. japonicus after being treated with EMS ... 17
Figure 11 Twelve F₁ crosses between BL1 and LC041 evaluated with the molecular marker TMO744 ....... 19
Executive Summary

What the report is about

This project focused on generating knowledge and technologies that would allow the development of a new seed industry for *Lotus corniculatus* in Australia. The three main areas of research included the development of seed harvesting methods for *L. corniculatus*, the development of a new cultivar less dependent on insect pollination and the development of non-shattering pod types that would reduce seed losses prior to and during harvesting operations. The project also had close linkages to an AWI project (EC344) which primarily focused on breeding and selecting *Lotus corniculatus* for Mediterranean Climates of Australia. The impact of the AWI research on the RIRDC project is, that it provides for the first time, well adapted varieties of *Lotus corniculatus* that will generate demand for seed.

Who is the report targeted at?

This research is targeted at seed growers that will undertake seed production of new *Lotus corniculatus* varieties using knowledge generated from this project. The research also targets farmer in areas receiving more than 600 mm of a.a.r. that have experienced Lucerne or White clover failure due to low pH, winter waterlogging or summer drought.

Background

This project was initiated from three core problems which included; 1) the limited range of perennial legume seed crops available to seed growers, 2) the absence of perennial legumes from large part of the landscape covering 600 to 900 mm of a.a.r. and 3) the need to reduce soil water recharge for salinity management in the mid-catchment regions of Australia.

To reduce soil water recharge, deep rooted perennial species are required to increase on-site water use and reduce soil water discharge. However large areas of Australia’s mid-catchment (600 to 900 mm a.a.r.) have acid soils or are waterlogged in winter or have lengthy summer droughts, which limit the use of perennial species such as Lucerne and White clover. Consequently large parts of this landscape do not have viable perennial legume options. To overcome this problem, new deep-rooted varieties of *L. corniculatus* were developed.

To be successful these new varieties need to be part of a viable seed industry, which means ensuring seed price is within acceptable limits for farmers. The major constraints with perennial Lotus species include low seed production and even lower harvested yields caused by pod shattering prior to and during seed harvesting operations.

Screening for naturally occurring non-shattering types identify no natural variation for this trait in *L. corniculatus* hence other methods were pursued. This project took two approaches to reduce and eliminate seed losses. The first approach looked to refine harvesting technologies and is a low risk strategy with lower returns. The second approach which was to develop non-shattering types by altering genes was a higher risk strategy with potentially higher returns. The other aspect perused in this project was to increase seed yields by developing self compatible types of *Lotus corniculatus*.

New and better adapted *L. corniculatus* varieties were also developed in AWI EC344 to increase demand for seed. These new varieties have a conservative area of adaptation covering some 8.2 m hectares in the Murray Darling Basin (600 to 900 mm a.a.r.) and are capable of providing high quality forage for livestock over summer where Lucerne and/or White clover fail to establish or persist. This potential can only be achieved if seed yields are increased and excessive pod shattering is managed through harvesting methodologies or overcome through gene manipulation.
Aims/objectives

Project Aim: To increase harvested seed yield in *Lotus corniculatus* so a viable seed industry can be sustained in Australia.

Objective 1: To examine seed harvesting technologies in the USA and New Zealand for *L. corniculatus* and *L. pedunculatus*, and refine these procedures for harvesting in Australia. Outcome 1: To ensure the current limitations on seed harvesting of *L. corniculatus* are reduced so viable seed industries can exist in Australia.

Objective 2: To overcome the problem of pod shattering, closely related diploid species of *Lotus corniculatus* were mutated to examine the potential for reduced or nil pod shattering. Outcome 2: To produce a small selection of perennial diploid Lotus species that have a substantially reduced potential for pod shattering.

Objective 3: To reduce the reliance of *Lotus corniculatus* on insect pollinators and to increase seed yields. Outcome 3: To produce a self compatible *Lotus corniculatus* cultivar with improved seed production.

Methods used

Harvesting technologies gathered from seed growers of *Lotus corniculatus* in the US, New Zealand and Uruguay were examined and adapted for Australian conditions. From this research, management practices were identified that minimises seed loss from harvesting operations. This approach partly overcomes seed losses at harvest through better timing and operational practices.

The mutagenic chemical EMS (C\textsubscript{6}H\textsubscript{8}O\textsubscript{3}S) was used to randomly disrupt gene segments to eliminate the genes responsible for pod shattering. The approach involved mutating diploid *Lotus japonicus* and a selfing tetraploid of *Lotus corniculatus*. The potential mutants of these species were then screened for non-shattering phenotypes.

In addition to the above, hand crossing between *Lotus corniculatus* selections was undertaken to examine the inheritance of self compatibility, a very rare trait found in this species. These crosses produced a self compatible well adapted cultivar with improved seed production. This work was also undertaken with assistance from project AWI EC344.

Results/key findings

The findings of this research include:

1) Harvesting practises: For seed harvesting of *Lotus corniculatus* several important management practises need to be considered including; monitoring of peak flowering and podding, the application of a desiccant such a paraquat at 70 percent pod maturity or at 35 days after peak flowering, harvesting with a conventional header 48 hours after the desiccant application with header concave settings at 2 mm and drum speed at 1200 rpm using a very low fan speed setting for air flow.

Using the approach outlined above similar seed production yields were achieved to those in the USA, Canada and Uruguay (100 kg/ha) where viable birdsfoot trefoil (*Lotus corniculatus*) seed industries exist.

2) Non-shattering pods: The mutation breeding with *Lotus japonics* and the self compatible *Lotus corniculatus* was not able to induce non-shattering mutants or mutants with improved agronomic characteristics. Many mutants were developed, however, these generally had chimeric plant parts (albino parts), and most of these had either very poor vigour, lack of flowering or poor seed production.
Research in non-shattering also examined a range of other Lotus species for natural mutants that produced non-shattering phenotypes. After the examination of approximately 3000 accessions making up some 60,000 plants, 13 natural mutants of *Lotus arenarius* were found. This project however was focused on perennial legumes and it was found that these selections were long lived annuals.

3) Self compatible cultivar: A self compatible genotype of *Lotus corniculatus* was identified and both the AWI project (EC344) and RIRDC project (DAN 209A) examined the heritability of the self compatibility trait. The details of these studies can not be released however this research has lead to the selection of a self compatible cultivar of *Lotus corniculatus*. The advantages this cultivar include; it does not require pollinators in the seed production process and it has significantly higher seed yields.

**Summary**

The project has been successful in developing harvesting methodologies for *L. corniculatus* that can be used in Australia to achieve yields comparable to those achieved overseas. The project has also helped generate the first self compatible cultivar of *L. corniculatus* which will be commercialised for use in Australia.

**Implications for relevant stakeholders:**

*Lotus corniculatus* varieties developed in AWI EC344 and the self compatible cultivar jointly developed with this project have a conservative area of adaptation of 8.2 m ha. This includes areas receiving more than 600 mm of a.a.r that have (i) hash summers which result in the failure of White clover, (ii) acid soils that result in the failure of Lucerne and (iii) winter waterlogging that also results in the failure of Lucerne. *Lotus corniculatus* can bring substantial benefits to these areas including (a) year round legume production for areas that have not previously sustain perennial legumes (b) more effective control of soil water recharge via improved water use (c) greater carrying capacity and/or reduced hand feeding (d) protection against bloat (e) reduced methane output from ruminants and (f) the development of a new seed industry in Australia. Note (d) and (e) are a result of tannins found naturally in Lotus corniculatus.

A major limitation to these benefits being achieved is the establishment of a viable Australian seed industry. Research undertaken in this project will allow for the first time a viable seed industry to be established in Australia. As a consequence seed growers will benefit from the emergence of a new seed crop industry and RIRDC will benefit from the share it has in the first self compatible *L. corniculatus* cultivar selected for Mediterranean climates. This cultivar has higher seed production than other out-crossing types.

**Recommendations**

1) The findings of harvesting methods should be included in the commercialization brief being developed for the three *L. corniculatus* varieties that will be released from project AWI EC344 and the cultivar that will be jointly released by DAN209A and AWI EC344. This approach will ensure that seed harvesting techniques are made available to seed companies and seed growers.

2) RIRDC should support the commercialisation of the new self compatible *L. corniculatus* cultivar.
Introduction

Background

There are very few perennial forage legumes in commerce in Australia. For example the areas and species registered for certified seed production in 2006 include; 15,593 ha of *Medicago sativa* L. (lucerne); 4605 ha of *Trifolium repens* L. (white clover), 99 ha of *Trifolium pratense* L. (red clover) and 32 ha of *Trifolium fragiferum* L. (strawberry clover) (Australian_Seed_Authority, 2007; OECD (Organization for Economic Cooperation and Development), AOSCA (Association of Official Seed Certifying Agencies) and DSCS (Domestic Seed Certification Schemes for Australia).

Other perennial forage legumes sown in Australia such as *Hedysarum coronarium* L. (Sulla) and *Cytisus proliferus* L. (Tagasaste) were either not certified for seed production in 2006 or the area was too small and was reported within “other legumes” which had less than 40 ha of certified seed production.

Related to the lack of perennial legume choice described above are landscape problems including a distinct lack of perennial legumes suitable for mid-catchment regions of Australia that are too waterlogged or acid for Lucerne and too summer dry for White clover. Moreover these landscapes in the mid-catchment regions of Australia contribute significantly to soil water recharge and saline discharge. To reduce these problems new perennial legumes are required that target these landscapes. The opportunity therefore is to develop new perennial legume seed crops that are adapted to these regions which can be harvested using conventional harvesting equipment.

About *Lotus corniculatus* and its reproductive development

*Lotus corniculatus* (birdsfoot trefoil) is a perennial forage legume suited to medium to high rainfall areas (mid-catchment regions), which tolerates acid soils and winter waterlogging. With improvements to its summer drought tolerance and seed production this species could be commercially viable in Australia as a seed crop and forage plant for areas too summer dry for White clover and too acid or waterlogged for Lucerne.

*Lotus corniculatus* is also an indeterminate long-day plant that flowers best in locations with photoperiods longer than 14.5 hours on the summer solstice (McKee, 1963). This photoperiod is found at latitudes higher than 37° (Baker and Baker, 2008) and these locations include; Oregon in the USA (42°), British Columbia in Canada (51°), parts of Europe and New Zealand. The campos region of South America (Southern Brazil, Uruguay and Eastern Argentina) is one of the few exceptions where *Lotus corniculatus* is planted at latitudes from 28° to 36° S (Blumenthal and McGraw, 1999).

In Australia, commercial applications of *Lotus corniculatus* is limited to pasture demonstrations with only cultivar Grasslands Goldie commercially available (Ayres et al., 2006a). Cultivar Grasslands Goldie was bred in New Zealand in latitudes from 37° to 45° S from 1973 to 1976 (Charlton et al., 1978; Miller, 1993; Scott and Charlton, 1983; Widdup et al., 1987) and consequently can not be established as a seed crop in Australia.

Research conducted by Ayres et al. (2006a; 2006b) studied the potential of Grassland Goldie in NSW, Australia. It was found to be agronomically promising for low fertility acidic soils on tablelands and slopes where the average annual rainfall is 650-1000 mm. For example, at Glen Innes in northern NSW (29°44’07”), the total annual rainfall is 849 mm with a distribution of 36%, 19%, 19% and 26% during summer, autumn, winter and spring respectively. However, due to its poor flowering at lower latitudes; the seed production needed to form a seed bank for successful recruitment is not achieved, and this impacted on the overall persistence of *Lotus corniculatus* swards. This research highlighted the need to breed a cultivar for lower latitudes with improved flowering and seed production.
The breeding program of NSW DPI located at Glen Innes, is releasing cultivar “Phoenix (Lotus corniculatus) for the northern upper catchment of the Murray-Darling Basin in NSW Northern Tablelands and North-West slopes (Ayres et al., 2008). This cultivar is expected to be adapted to regions with summer dominant high rainfall (Ayres et al., 2006a).

In Western Australia, *Lotus corniculatus* was bred since 2002/3 to produce 4 varieties for high and medium rainfall Mediterranean environments. These regions have a winter dominant rainfall pattern and a dry period with almost no effective rain for two to five months.

At least two varieties in total from the NSW and WA breeding programs will be available in the market place over the coming years, and the success of these varieties will depend on having a profitable seed industry that can deliver enough seed at a competitive price. Indeed seed production is regarded as the most difficult challenge to widespread dissemination of *Lotus corniculatus* (Beuselinck, 1997). The major constraint in seed production is the large seed losses that occur from seed pod shattering prior to and during seed harvesting operations (Seaney and Henson 1970) as well as the poor seed production which occurs at latitudes relevant to Australia.

The AWI project (EC344) based in WA primarily focused on breeding for increased flower production and seed set, a more determinant flowering pattern, summer survival in a Mediterranean climate and fodder production. The selected lines have also retained there capacity to perform in acid and/or waterlogged soils. The release of these well adapted lines is also likely to impact on the demand for seed.

This RIRDC project (DAN209A) primarily focused on improving seed harvested yields from *Lotus corniculatus*. Initial screening looked for naturally occurring non-shatter pod types and found no natural variation for this trait in *Lotus corniculatus*, hence other methods were pursued. Two approaches were taken which looked to reduce and eliminate seed losses. The first approach aimed at reducing seed losses looked to develop and refine harvesting methodologies. The second approach aimed at eliminating seed losses was to develop non-shattering types by altering genes and was seen as a higher risk strategy with much higher returns. In addition to these activities the development of a self compatible cultivar of *Lotus corniculatus* was undertaken to increase seed yield and produce a more determinant flowering pattern. Outlined below are the research areas undertaken.

**Mutation breeding**

Mutation breeding (the second approach described above in results/key findings) is a common tool used to improve specific traits in many cultivated plants. There are many ways mutants are induced including treatment with, X-rays, ethylmethanesulfonate (EMS) or ethylamine.

EMS is one technique which has been used extensively to generate mutations. EMS is a chemical mutagen that induces point mutations by reacting with DNA to cause transitional changes in nucleotide sequences (Kanra and Brunner 1970). This mutation method was used to examine the possibility of inducing non-shattering phenotypes of diploid *Lotus corniculatus*.

**Breeding self compatible *Lotus corniculatus***

*Lotus corniculatus* is a cross-pollinated species that requires insect pollinators to produce seeds. A good seed crop requires at least 3 bee-hives per hectare to fully pollinate the crop. In Australia relying on naturalised and native pollinators is likely to severely limit seed production. Hence the project examined self compatible forms of *Lotus corniculatus* to reduce potential pollination constraints. Very few genotypes of *Lotus corniculatus* are naturally self-pollinating and one of them used in this project is line LC041. This autogamous character is useful to reduce the need for pollinators in large-scale seed production.
All of the self compatible types of *Lotus corniculatus* were shown to be poorly adapted to Mediterranean conditions; therefore hand-crossing was performed with some of the well adapted breeding lines and the progeny was tested for the self compatibility trait.

**Harvesting *Lotus corniculatus***

The cutting height for harvesting is usually around 10 cm, therefore an even and derby free seedbed is required. The seeds (850,000 seeds/kg) are small and require accurate soil depth placement at sowing. Seedling growth is slow hence a weed free seed bed is critical. Grass weed control presents no problem and Select® at high rates is the preferred option. Broadleaf weed control is problematic but can be achieved with acceptable levels of crop damage. Nutrients should not be a limiting factor when growing *Lotus corniculatus* and seed should be inoculated with the appropriate strain of *rhizobium*.

**Flowering and pollination**

In the USA, the best latitudes reported for flowering and seed production are from 40°N to 50°N (Beuselinck, 1997). In Australia, only parts of Tasmania fall within this zone. To improve flowering and seed production at latitudes between 28 and 32 degrees individual plants from early flowering accessions were selected and utilised in the AWI breeding and selection project (EC344).

Out-crossing genotypes of *Lotus corniculatus* (i.e. the vast majority which require pollinators) flower indeterminately from early spring to autumn. Concentrated flushes occur in December and February; however this is affected by grazing, moisture, day length and plant genetics. For a given cultivar and location the main objective for seed production is achieving a synchronized flush of flowers. This synchronized flowering can be achieved to some extent by grazing or cutting the seed crop in early spring to get a uniform recovery of the crop, which is often followed by a uniform flowering.

Flowering is also dependant on the moisture being available after defoliation of the seed crop. Dry conditions after the last defoliation are likely to reduce recovery, flowering and seed set. At the other extreme high levels of soil moisture are likely to ensure the crop remains in a vegetative state for longer and this will result in significant amounts of biomass accumulated prior to harvesting, which often causes problems and additional seed losses at harvest.

**Podding**

One of the main problems in the seed production of *Lotus corniculatus* is that the pods shatter when they become dry. Indeterminate flowering (a large spread of flowering time) results in pods being at varying levels of maturity, from green through to dry shattered pod. The critical point for good seed production is to synchronized flowering and harvest at the right moment to maximize the harvesting of mature pods, and good quality seed.

The optimum time of harvesting is crucial and in *L. corniculatus* seed pod colour is an indicator of seed maturity. The colour of immature pods are dark green, they progress to green-purple to light green and more mature pods are green-white, light golden brown to dark brown (Fairey and Smith, 1999). For a single pod, light green is the earliest that a pod can be harvested with adequate seed germination, however its not until pods turn green-white that the seeds will reach maximum weight (Winch and MacDonald, 1960). The progressive development of pods start as waxy, deep green and they change colour to purple approximately 1 week after full bloom. Then, they change to dark green (about day 15) and to light green between 22 to 24 days after full bloom. Pods require 3 or 4 more days (days 25 to 28) to turn light brown and another 4 days (29 to 32) to turn dark brown to black (Anderson 1955). Carambula (1980) reported a purple colour in the first week after pollination, dark green at day 15, light green at 22 to 24 days, light brown at 28 days and dark brown at 32 days.
Since all pods do not mature at the same time, an optimum proportion of green pods, mature pods and shatter pods is required to maximise seed harvested yield. For example, in Uruguay the best harvesting time is when there are 19% of green pods and 81% brown pods (Costa and Panizza, 1997).

Alternative methods can be used to predict optimal harvesting time. For example Fairey and Smith, (1999) found that 35 days after peak flowering, the seed crop can be harvested. In western Oregon, Garcia-Diaz and Steiner (2000b) harvested their crops 27, 26 and 31 days (1994, 1995, and 1996 respectively) after the time of peak flowering, with corresponding 11, 9 and 35% of seed lost due to shattering. If days after peak flowering is not used then the proportion of mature pods can be used as an estimate of optimal harvest time. According to Anderson, (1955) and Winch and MacDonald (1960), seed yield should be optimum if 75 to 85% of the pods are mature (brown) at harvesting time. Supporting this, Carambula (1981) recommends starting harvesting when there are 60 to 70% of mature pods and 10% of shattered pods.

Whichever approach is used to determine optimal harvest time close attention needs to be given to the rate of development as the time taken for pods to change from green to mature and finally to shatter is dependant on temperature and relative humidity. For example, a bulk sample of cultivar Empire dehisced when relative humidity dropped to 40% (Metcalfe et. al. 1957). In Western Australia high temperatures and low relative humidity are experienced over summer and shattering can occur over just a few days. Consequently the window for harvesting high seed yields in WA is quite narrow.
Objectives

Project Aim: To reduce seed losses from *Lotus corniculatus* during harvesting so a viable seed industry can be sustained in the long term.

Objective 1: To examine seed harvesting technologies in the USA (contact person Paul Beuselinck) and New Zealand (contact person Warren Williams) for *L. corniculatus* and *L. pedunculatus*, and refine these procedures for harvesting in Australia. Outcome 1: To ensure the current limitations on seed harvesting of *L. corniculatus* are reduced so viable seed industries can exist.

Objective 2: To overcome the problem of pod shattering it is proposed that perennial diploid Lotus species (2n = 2x = 12) will be collected from Genetic Resource Centre around the world and mutants of these will be chemically induced to examine the potential to reduce or eliminate gene expression for pod shattering. Outcome 2: To produce a small selection of perennial diploid Lotus species that has a substantially reduced potential for pod shattering.
Methodology

Harvesting methods

There are 3 main harvesting methods that were investigated and these include: (a) green direct heading, (b) cutting, swathing and dry harvesting and (c) dry harvesting after herbicide desiccation.

(a) Green direct headed

Advantages:
- Only mature pods are threshed and usually good quality seed is obtained.
- Rainfall events prior to the harvesting dry rapidly in comparison with the swath method.

Disadvantages:
- Green foliage is difficult to pass through the header and usually seed losses are very high.
- Header speed is much slower than other methods.
- Seed losses over 50% can be experienced (Carambula 1981).
- Harvested seed is collected in the header box together with green leaves which needs to be dried quickly on drying racks to avoid fermentation and seed death.
- The requirement for drying racks limits the application of this method.

(b) Cut, swathed and dry harvested

Advantages:
- This method requires cutting and swathing before harvesting and despite this it is usually cheaper than green direct heading.
- Yields are usually higher as pods tend to ripen more evenly making more seed available after 2 days of drying in the swath.

Disadvantages:
- While cutting and during the drying period, shattering will occur. However, if cutting when plants are slightly damp, shattering can be minimised.
- Rainfall events after cutting can be very detrimental and may germinate seed in the swath.
- This method also results in some undeveloped seed being harvested which reduces quality. This problem can be address to some extent by seed processing, however this adds an additional operation and cost.
- On some occasions, strong winds can blown the dry swath material resulting in significant reductions in yield.

(c) Herbicide desiccation and direct heading

Advantages:
- Material is only handled once unlike the cut and swath method.
- Standing dry material dries easily after rain.
• The header operations are the fastest of the three methods and consequently this method is usually the cheapest of the three methods.

Disadvantages:
• The window of opportunity for harvesting is much more critical when using this method and shattering can be a very serious problem if harvesting is not undertaken at the appropriate time.

Harvest 2006

The harvesting methodology studies started in late spring 2004 when 1ha of *L. corniculatus* was sown at Waroona, Western Australia in an area suitable for flood irrigation. Due to the late sowing, the first year crop was not harvested, however, the production of flowers and seeds were monitored during the early summer of 2005. In May 2005, the dry matter production on the experimental site was 7.5 t/ha. Subsequent to this assessment the site was grazed to a height of 5 cm. In August 2005, a split-plot experiment was established with main plot (date of last defoliation: August or October), sub-plot (maturity level: medium or high) and sub-sub-plot (Harvesting method: direct harvest, cut and swath or sprayed with desiccant). Each sub-sub plot was of 10m x 2m which allows for the width of the experimental harvester (eg. 1.6 m cut). Over the summer period (05/06), flowering was monitored.

Figure 1 Cutting treatment on 31 August 2005 (left) and 11th October 2005 (right)

The two main treatments (last cut in August or October) as well as an un-cut area next to the trial produced almost the same peak of flowering which occurred in the last week of November in this second year seed crop. Peak ripening occurred in mid January 2006 some 40 to 45 days after peak flowering. As there were no flowering time differences between the two cutting times and the un-cut control; it was decided to include a larger area of the paddock for testing the three harvesting methods instead of using the sub-sub-plots of the original split-split-plot design. Consequently each of the three harvesting treatments (cut and swath, direct harvesting and desiccant spray) was applied to 40m long strips and 3 replicates.

Harvest 2007

A second test area was established on 4th August 2006, (*L. corniculatus* cv. San Gabriel) over 0.4 ha sown at 10 kg/ha on Owen Eascott’s Farm, Waroona, WA. Prior to sowing glyphosate was applied on the 1st June and 21st July to control weeds and fertilizer (300kg/ha) was spread prior to sowing (3.68 N, 11.23 P, 5.88 K, 5.29 S, 13.97 Ca, 0.09 Cu, 0.07 Zn and 0.01 Mo). Lorsban insecticide (500ml/ha) was sprayed on 6th October and 20th October to control grasshoppers and the herbicide Spinnaker (70 g/ha) was sprayed on 11th October to control Toad Rush. In early 2007, a sub-set of the best treatments of the 2006 harvest were applied. The number of flowers was monitored weekly and peak flowering identified.
The experimental design was a randomized complete block design with 3 replicates and 3 treatments which included; cut and swath, a desiccant spray (3 L/ha Reglone + 200mm wetting agent) followed by direct heading and direct green harvesting. Each treatment and replicate was applied to a 70m strip 1.5 m wide. The seed yield before machine harvesting was estimated by sampling 5 quadrats of 50cm x 50cm.

A photograph of the seed crop before treatments were imposed is presented in Figure 2 and harvesting of the different treatments in Figure 3.

Figure 2 *Lotus corniculatus* seed crop before harvesting in 2007

The results of the harvest in March 2007, allowed us to select the treatments to be imposed in early 2008 for the next harvesting process.

Figure 3 Harvesting the cut and swath treatment (left), sprayed treatment (middle) and direct harvest treatment (right) in January 2006
Harvest 2008

In early-December 2007, the *Lotus corniculatus* area was grazed by sheep with almost no green leaves remaining after grazing. The seed crop was able to recover very well and on the 4th February was as shown in Figure 4. The number of flowers was monitored weekly and the peak of flowering identified. Thirty days after the peak of flowering was the target day for harvesting.

Figure 4 *Lotus corniculatus* stand before harvesting 4 February 2008

In February 2008, a randomized complete block design of 3 treatments and 3 replicates was set up over the same area harvested in 2007. The treatments (Figure 5) were to harvest 48, 72 and 96 hours after spraying a desiccant (Reglone @ 3 L/ha). The hypothesis was that 72 hours was the optimum time, 48 hours would be still too green for harvesting and after 96 hours most of the seed would have shattered. Each treatment and replicate was a strip of 2.5m wide by 22 m long. The harvesting was in the centre of each plot with a cutting width of 1.6 m.

Figure 5 The three spraying time treatments on the day of harvest
**Mutation breeding**

The study examining mutation breeding was conducted over three years at the University of Western Australia Field Station (Shenton Park). It involved three stages, the first to optimize the EMS concentration required to generate the maximum number of point mutations without inducing plant sterility. In the second stage, the M1 stage, EMS was applied at optimal concentrations to seeds and plants were monitored and seed harvested off the mutated plants. Finally, the M2 stage was to grow out the progeny of the mutated plants and monitor them for non-shattering mutants.

**Plant Materials**

Two seed sources were selected for treatment with EMS, an autogamous tetraploid *L. corniculatus* (LC041) and the diploid model legume, *Lotus japonicus* cultivar Gifu.

**Optimization of EMS concentration**

The EMS mutagenesis of the seeds from LC041 and Gifu, were performed using batches of 500 seeds. The seeds were scarified using a compressed air pot scarifier, surface sterilized in commercial bleach (~5% NaOCl) for 2 minutes and rinsed 8 times with sterile deionised water. Seeds were then soaked in the deionised water for 3 hours, to imbibe the seeds, which were then soaked in 0.2%, 0.4%, 0.8% and 1.6% EMS solutions, whilst on a shaking table set at 30 r.p.m. for 16 hours.

After the EMS treatment, the seeds were rinsed in continuous running water for 2 hours to remove any residual EMS solution. Seeds were then placed into Petri dishes, lined with a double layer of wet Whatman filter paper, and placed in germination cabinets at 15 ºC and allowed to germinate. Germination counts were done after four days, and a final count was completed after seven days.

**Development of the mutant populations (M1 and M2)**

Based on the results of the preceding analysis, EMS concentrations of 0.2%, 0.3% and 0.4% were selected for *L. japonicus* and 0.2% to 1.2% for *L. corniculatus*, to produce the mutant populations. The number of seeds to achieve 150 viable plants was calculated based on the germination rates of the preceding analysis (Table 1). The batches of seed were counted out and then mutated in the appropriate EMS concentrations using the procedure outlined above. Following the mutation treatment the seeds were placed into Petri dishes, and then sown into 40 cell seedling trays, with 3 seeds per cell. They were grown out in a naturally lit screen-house and after 3 weeks they were thinned to 1 seedling per cell. After a further 3 weeks seedlings were transferred to 2.2 L plastic pots. Three seedlings were placed in each pot and were grown to full maturity within their treatments in the screen-house. They were monitored for symptoms of mutation, such as chlorosis of the leaves and stem, which were identified and marked. The plants were allowed to set seed and then monitored to identify non-shattering plants.
For each species and EMS treatment, two bulk samples were harvested. The first bulk sample consisted of the plants not showing chlorotic symptoms, and the second consisted of a bulked harvest of all plants with chlorotic symptoms.

In 2006, the M1 generation seeds were utilized from plants showing chlorotic symptoms to evaluate them in the field nurseries. These seeds were imbibed and sown out into 40 cell seedling trays. A total of 720 Gifu seedlings were grown out, consisting of 240 seedlings from each treatment of 0.2%, 0.3% and 0.4% EMS. A total of 600 seeds of LC041 were grown from the bulked harvest of treatments with 1.0% and 1.2% EMS. These seedlings were grown in the screen-house for six weeks and then transplanted into the field as single spaced plants at 75cm spacing. The plants were monitored individually to identify plants with possible mutations. When the plants flowered, pods were tagged and shattering assessed.

In 2007, another treatment with EMS following the same protocol as in 2006, was applied to seeds harvested from the M1 generation and original seeds (Table 2).

### Table 2 Treatments applied to seeds from the M1 harvest and original seeds of Gifu and LC041

<table>
<thead>
<tr>
<th>Seed Origin</th>
<th>Species</th>
<th>Accession</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2% Seed from non marked plants</td>
<td><em>Lotus japonicus</em></td>
<td>Gifu</td>
<td>0.2% EMS</td>
</tr>
<tr>
<td>Original seed no previous treatment</td>
<td><em>Lotus japonicus</em></td>
<td>Gifu</td>
<td>0.2% EMS</td>
</tr>
<tr>
<td>0.3% Seed from non marked plants</td>
<td><em>Lotus japonicus</em></td>
<td>Gifu</td>
<td>0.3% EMS</td>
</tr>
<tr>
<td>0.3% Seed Harvested from marked plants</td>
<td><em>Lotus japonicus</em></td>
<td>Gifu</td>
<td>0.3% EMS</td>
</tr>
<tr>
<td>Original seed no previous treatment</td>
<td><em>Lotus japonicus</em></td>
<td>Gifu</td>
<td>0.3% EMS</td>
</tr>
<tr>
<td>1.0% Seed from non marked plants</td>
<td><em>Lotus corniculatus</em></td>
<td>LC041</td>
<td>1.0% EMS</td>
</tr>
<tr>
<td>1.0% Seed from marked plants</td>
<td><em>Lotus corniculatus</em></td>
<td>LC041</td>
<td>1.0% EMS</td>
</tr>
<tr>
<td>Original seed no previous treatment</td>
<td><em>Lotus corniculatus</em></td>
<td>LC041</td>
<td>1.0% EMS</td>
</tr>
<tr>
<td>1.2% Seed from non marked plants</td>
<td><em>Lotus corniculatus</em></td>
<td>LC041</td>
<td>1.2% EMS</td>
</tr>
<tr>
<td>Original seed no previous treatment</td>
<td><em>Lotus corniculatus</em></td>
<td>LC041</td>
<td>1.2% EMS</td>
</tr>
</tbody>
</table>

A total of 200 seeds for each of the 10 treatments were transplanted to a spaced plant nursery and plants were monitored to detect any mutations, specially non-shattering phenotypes.

**Breeding self compatible *Lotus corniculatus***

Thirty clones of each of three cross-pollinated elite breeding lines (BL1, BL2 and BL3) and the self-pollinated LC041 were produced in 2004. In 2004/5, 90 inflorescences of each of the cross-pollinated accessions were emasculated and subsequently pollinated with LC041. Successful crosses were harvested.
In 2005, a random sample of 100 F1 seeds were planted per cross in an insect-proof screen-house, together with the parent plants. These accessions of *L. corniculatus* have no easily measurable phenotypic markers that can be used to identify a positive cross, therefore molecular studies were conducted with 15 microsatellite markers (SSR), 10 from lupin and 5 from *Lotus japonicus* genome sequencing project (website http://www.kazusa.or.jp/lotus/). To identify positive cross-pollination SSR markers were evaluated on the four parent plants to determine the best polymorphic primer to use in the screening of F1 plants. The molecular markers identified seven, four and three F1 positive crosses from the 12 plants per cross.

In 2006, 1156 hand-crosses were made where each of the 14 selected F1’s were backcrossed to each of their respecting parents, selfed and sib-mating within each cross and among crosses. Seeds from these crosses were sown and the selfing plants were harvested. In 2007, seeds from selfing plants were planted and again only the selfing plants were harvested.
Results

Harvest 2005/06

The production of flowers, pods and seeds were monitored during the early summer of 2005 and are presented in Figure 6.

Figure 6 Shows the flowering intensity of *L. corniculatus* recorded over 2004 and 2005

In the 2004 trial none of the 2 flowering peaks (30 and 35 inflorescences/m²) were considered adequate for harvest, and accordingly no harvest was undertaken in 2005.

In May 2005, the dry matter production on the experimental site was 7.5 t/ha. The dry matter produced on the site from May (when grazed for the first time) to the end of August was 3.5 t/ha. The accumulated dry matter production from May to mid-October was 5.5 t/ha and the re-growth of the cut area from the end of August to mid-November was 2.7 t/ha, while the average dry matter production was 64 kg DM/ha/day.

In 2005 the number of flowers of *L. corniculatus* was monitored for the two main treatments and the un-cut control (Figure 7).
The three treatments (cut in August, October and un-cut) produced almost the same peak of flowering which occurred in the last week of November in this second year seed crop. Peak ripening occurred in early January 2006 some 40 days after peak flowering. As there were no flowering time differences between the two cutting times and the uncut control; it was decided to include a larger area of the paddock for testing the three harvesting methods. None of the treatments were able to set good quality seed due to a large attach of insects (*Nezara viridula*) that feed on forming seed. Insect control was not possible on this farm due to insecticide application restrictions relevant to beef production.

**Harvest 2007**

In 2007 the peak of flowering occurred in mid-February in this first year seed crop (Figure 8) and the peak ripening occurred in mid March, some 35 to 40 days after peak flowering. The flowering to ripening period for 2007 was similar but not the same as that recorded in 2006.
The potential seed production is based on the following assumptions: 300 inflorescences/m\(^2\) \(\times\) 5 pods/inflorescence \(\times\) 20 seeds/pod = 30,000 seeds/m\(^2\). Therefore 30,000 seeds/m\(^2\) \(\times\) 1.2g/1000seeds \(\times\) 10000m\(^2\)/ha = 360 kg/ha.

This projection was then compared with machine harvested results and is presented in Table 3.

**Table 3 Seed yield (kg/ha) before and after harvesting and percentage of seed in the harvested material**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Seed Yield measured before header harvesting (kg/ha)</th>
<th>Machine harvested Seed Yield (kg/ha)</th>
<th>Kg/ha of seed lost and in parenthesis the percent lost (compared the proceeding two columns)</th>
<th>Kg/ha of seed lost when compare with 452 kg/ha and in parenthesis the percent lost</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cut and Swath</td>
<td>91</td>
<td>82</td>
<td>9 (10%)</td>
<td>370 (82%)</td>
</tr>
<tr>
<td>Direct Harvesting</td>
<td>452</td>
<td>113</td>
<td>339 (75%)</td>
<td>339 (75%)</td>
</tr>
<tr>
<td>Desiccant spray</td>
<td>244</td>
<td>80</td>
<td>164 (67%)</td>
<td>372 (82%)</td>
</tr>
<tr>
<td>Mean</td>
<td>262</td>
<td>92</td>
<td>170 (65%)</td>
<td>360 (80%)</td>
</tr>
<tr>
<td>l.s.d.</td>
<td>183</td>
<td>16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The seed yield before harvest for the direct harvested treatment (452 kg/ha) is used as the estimate of potential seed yield. The previous estimate of the potential seed yield (e.g. using inflorescences/m\(^2\) \(\times\) pods/inflorescence \(\times\) seeds/pod = seeds/m\(^2\) and seeds/m\(^2\) \(\times\) 1000 seed weight (g) \(\times\) 10000m\(^2\)/ha = Potential seed yield (kg/ha)) was 360 kg/ha and was an under estimate. This is explained by the possibility that the inflorescence counts did not capture peak flowering, hence 452 kg/ha is used as the potential seed yield.

Results indicate mayor seed losses occur using the cut and swath treatment and the desiccant treatment (82%). The seed yield of the cut and swath treatment had large seed yield losses in the cutting and swathing operation (prior to harvest) however once seed was in the swath very small losses occurred. The desiccant treatment had large losses in the heading operation and in the final
analysis both systems had similar harvested seed yields. Losses were due to the pods dropping to the ground and or pod shattering.

The highest seed yield was obtained by the direct green harvest treatment. However, the main problem with this treatment is the handling of the green material with seeds collected in the header-box which need to be dried before the seed is killed by over-heated via fermentation of green material. If the harvesting area is large this method is not considered suitable.

**Harvest 2008**

The yellow inflorescences, green, dried and shattered pods were monitored during January and February 2008 (Figure 9). The peak of yellow inflorescences production was in mid to late January 2008. Thirty days after the peak of flowering was the target day for harvesting.

![Graph showing number of yellow inflorescences, green, dried and shattered pods in January and February 2008](image)

**Figure 9 Number of yellow inflorescences, green, dried and shattered pods in January and February 2008**

Analysis of 2008 results showed that there was no significant differences (P= 0.05) for forage yield or seed yield among the areas assigned to the different treatments.

After the spraying treatments were imposed (spraying at 48, 72 or 96 hours before harvest), no significant differences for forage yield were recorded between spray treatment with an average forage yield of 2926 kg/ha. Spraying however at any of the intervals described above did reduce dry matter yield by 2085 kg/ha due primarily to leaf drop, and some flowers and seed loss. The seed yield followed a similar trend to that recorded for forage yield. For example the seeds yield was reduced from 187 kg/ha to 95 kg/ha (49.1% reduction) when desiccation was compare to no desiccation. The timing of desiccation in this example had no significant impact on seed yield. The seed yield means for the 48, 72 and 96 hours were 37, 37 and 36 kg/ha of cleaned seed respectively.

These results indicate that only 38% was harvested from the available seed at time of harvesting and desiccation resulted in a decline of 20% compared with the untreated stand.
A comparison of the results obtained in 2007 and 2008 for the desiccant treatments are presented in Table 4. The results are almost the same in both years, with only 18% and 20% of the available seed harvested. Based on these result 100 to 200 kg/ha of harvested seed is achievable in *Lotus corniculatus*.

**Table 4** Comparison of desiccant treatment conducted in 2007 and 2008 are presented (kg/ha) and percentages in parenthesis

<table>
<thead>
<tr>
<th>Harvest</th>
<th>Seed yield before spraying the desiccant</th>
<th>Seed yield after spraying the desiccant</th>
<th>Clean seed after harvesting</th>
</tr>
</thead>
<tbody>
<tr>
<td>2007</td>
<td>452 (100%)</td>
<td>244 (54%)</td>
<td>80 (18%)</td>
</tr>
<tr>
<td>2008</td>
<td>187 (100%)</td>
<td>95 (51%)</td>
<td>37 (20%)</td>
</tr>
</tbody>
</table>

**Mutation breeding**

**Optimization of EMS concentration**

The germination counts for the two species and doses of EMS are presented in Figure 10.

![Figure 10 Germination counts for *L. corniculatus* and *L. japonicus* after being treated with EMS](image)

The germination was reduced more at high concentrations of EMS in *L. japonicus* than in *L. corniculatus*. A 50% reduction in germination was archived with 0.3% and 0.6% EMS for *L. japonicus* and *L. corniculatus* respectively.

**Development of the mutant populations (M1 and M2)**

Based on the results of the preceding analysis of germination, EMS concentrations of 0.2%, 0.3% and 0.4% were selected for *L. japonicus* and from 0.2% to 1.2% for *L. corniculatus*. The number of plants showing chlorotic symptoms per species and EMS treatment is presented in Table 5.
Table 5 Number of plants showing chlorotic symptoms per species and EMS treatment

<table>
<thead>
<tr>
<th>% EMS Solution</th>
<th>Total number of plants</th>
<th>Plants showing chlorosis</th>
<th>% of plants showing chlorosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMS 0.2% Gifu</td>
<td>150</td>
<td>5</td>
<td>3.33</td>
</tr>
<tr>
<td>EMS 0.3% Gifu</td>
<td>150</td>
<td>13</td>
<td>8.67</td>
</tr>
<tr>
<td>EMS 0.4% Gifu</td>
<td>150</td>
<td>22</td>
<td>14.67</td>
</tr>
<tr>
<td>EMS 0.2% LC041</td>
<td>150</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>EMS 0.4% LC041</td>
<td>150</td>
<td>1</td>
<td>0.67</td>
</tr>
<tr>
<td>EMS 0.8% LC041</td>
<td>150</td>
<td>3</td>
<td>2.00</td>
</tr>
<tr>
<td>EMS 1.0% LC041</td>
<td>150</td>
<td>8</td>
<td>5.33</td>
</tr>
<tr>
<td>EMS 1.2% LC041</td>
<td>150</td>
<td>12</td>
<td>8.00</td>
</tr>
</tbody>
</table>

For both species the higher doses of EMS was able to produce higher percentages of chlorotic plants (Table 5) and lower seed weight (Table 6) as an indication of more severe mutations.

The total weight of seeds harvested in bulk for the plants showing chlorotic symptoms and normal plants for each species is presented in Table 6.

Table 6 Weight of seeds harvested in bulk for the plants showing chlorotic symptoms and normal plants for each species

<table>
<thead>
<tr>
<th>Genus species</th>
<th>Treatment</th>
<th>Seed weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lotus japonicus</em></td>
<td>EMS 0.2% (bulk)</td>
<td>45.02</td>
</tr>
<tr>
<td><em>Lotus japonicus</em></td>
<td>EMS 0.3% (bulk)</td>
<td>6.76</td>
</tr>
<tr>
<td><em>Lotus japonicus</em></td>
<td>EMS 0.4% (bulk)</td>
<td>4.4</td>
</tr>
<tr>
<td><em>Lotus japonicus</em></td>
<td>EMS 0.2% (Chlorosis)</td>
<td>0.04</td>
</tr>
<tr>
<td><em>Lotus japonicus</em></td>
<td>EMS 0.3% (Chlorosis)</td>
<td>0.09</td>
</tr>
<tr>
<td><em>Lotus japonicus</em></td>
<td>EMS 0.4% (Chlorosis)</td>
<td>2.53</td>
</tr>
<tr>
<td><em>Lotus corniculatus</em></td>
<td>EMS 0.2% (bulk)</td>
<td>181.68</td>
</tr>
<tr>
<td><em>Lotus corniculatus</em></td>
<td>EMS 0.4% (bulk)</td>
<td>209.4</td>
</tr>
<tr>
<td><em>Lotus corniculatus</em></td>
<td>EMS 0.6% (bulk)</td>
<td>62.88</td>
</tr>
<tr>
<td><em>Lotus corniculatus</em></td>
<td>EMS 0.8% (bulk)</td>
<td>38.89</td>
</tr>
<tr>
<td><em>Lotus corniculatus</em></td>
<td>EMS 1.0% (bulk)</td>
<td>10.94</td>
</tr>
<tr>
<td><em>Lotus corniculatus</em></td>
<td>EMS 1.2% (bulk)</td>
<td>31.52</td>
</tr>
<tr>
<td><em>Lotus corniculatus</em></td>
<td>EMS 1.0% (Chlorosis)</td>
<td>6.37</td>
</tr>
<tr>
<td><em>Lotus corniculatus</em></td>
<td>EMS 1.2% (Chlorosis)</td>
<td>8.17</td>
</tr>
</tbody>
</table>

From the two spaced plant nurseries of 1320 plants in 2006 and 2000 plants in 2007, there were no plants identified as having delayed shattering or no shattering. Also, none of the plants had useful mutated characteristics. Only two plants out of the 3320 spaced plants had improved plant vigour, but they did not set seed.

**Breeding self compatible Lotus corniculatus**

In 2005, the 300 F1 hybrids and parents BL1, BL2 and BL3 did not produce seed inside the insect free screen-house while the selfing parent (LC041) did. This indicates that the autogamy character for self-pollination is a recessive trait. Interestingly the hand-crosses conducted in 2004/5 between the cross-pollinated mother plants and the pollen donor LC041, only had 27% of the inflorescences that produced pods and their seeds were only 40% of the normal seed weight. This indicates that there is some incompatibility between the two types of *L. corniculatus*.

None of the 10 lupin SSR’s were successful discriminators among parent plants and only three out of the five primers from *L. japonicus* successfully discriminated all four parents. Primer TMO744 was selected for further screening of the crosses because it produced the most different banding pattern for
each of the 4 parent plants (Figure 11). From the 12 plants tested in each of the 3 crosses we identified at least 67%, 50%, and 100% successful crosses from BL1, BL2 and BL3 respectively. Twelve F1’s between BL1 and LC041 are presented in Figure 11.

![Figure 11 Twelve F1 crosses between BL1 and LC041 evaluated with the molecular marker TMO744](image)

From the 1156 hand-pollinations conducted in 2006, only 328 were successful, corresponding to 40 crosses. All seeds from all crosses were planted in 2007 and only 7 crosses were able to produce some selfer individuals (Table 7). Seeds from each individual selfing plant were harvested in 2007.

<table>
<thead>
<tr>
<th>Mother plant</th>
<th>Pollen donor</th>
<th>Cross Code</th>
<th>Total No. crosses</th>
<th>% Success</th>
<th>Total number of plants</th>
<th>% Selfing plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-7</td>
<td>1-3</td>
<td>104</td>
<td>10</td>
<td>50</td>
<td>5</td>
<td>20</td>
</tr>
<tr>
<td>1-7</td>
<td>LC041</td>
<td>110</td>
<td>66</td>
<td>73</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>1-11</td>
<td>2-8</td>
<td>92</td>
<td>20</td>
<td>15</td>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>3-2</td>
<td>3-2</td>
<td>113</td>
<td>16</td>
<td>63</td>
<td>44</td>
<td>11</td>
</tr>
<tr>
<td>3-2</td>
<td>3-8</td>
<td>63</td>
<td>13</td>
<td>8</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>3-2</td>
<td>LC041</td>
<td>64</td>
<td>59</td>
<td>27</td>
<td>20</td>
<td>75</td>
</tr>
<tr>
<td>3-8</td>
<td>LC041</td>
<td>121</td>
<td>14</td>
<td>14</td>
<td>5</td>
<td>100</td>
</tr>
</tbody>
</table>

Backcrossing to the selfing parent, selfing or sib-mating were the only type of crossings that produced self pollinated individuals. Progenies derived from the parent BL2 did not produce any selfing progeny. If the selfing character was regulated by just 1 recessive gene, the proportion of selfing plants in the F2 generation, Back-cross 1 generation to the selfing parent and to the non-selfing parent would be 25%, 50% and 0% respectively. Our results were 17%, 59% and 0%, however the number of progenies per cross was not enough to have good data to study proportions accurately.
Seeds from a random selfer plant from each of the seven crosses were planted again in late 2007 and progenies allowed to set seed in an insect-proof screen-house (Table 8).

**Table 8 Number of individual plants sown in 2007 from a single plant from a cross that produced selfers and number of selfing plants produced in 2008**

<table>
<thead>
<tr>
<th>Cross Code</th>
<th>Number of individuals planted</th>
<th>Number of selfing plants</th>
</tr>
</thead>
<tbody>
<tr>
<td>63-1</td>
<td>182</td>
<td>0</td>
</tr>
<tr>
<td>64-5</td>
<td>140</td>
<td>0</td>
</tr>
<tr>
<td>92-1</td>
<td>47</td>
<td>0</td>
</tr>
<tr>
<td>104-1</td>
<td>72</td>
<td>0</td>
</tr>
<tr>
<td>110-1</td>
<td>216</td>
<td>216</td>
</tr>
<tr>
<td>113-5</td>
<td>182</td>
<td>0</td>
</tr>
<tr>
<td>121-3</td>
<td>127</td>
<td>38</td>
</tr>
</tbody>
</table>

Again, if the selfing character is regulated by a single recessive gene, all progenies from the selfing plant should be selfers. This was only true for cross 110, but not for the other 6 crosses. Therefore, the inheritance of the selfing character is not well understood yet and further research is needed.

The 216 plants from plant 110.1 from cross 110 were very uniform and strong selfers. Seeds from these 216 plants were bulked harvested and are the basis of the new selfing cultivar.
Implications

The aim of this research was to establish all the necessary components for a new and viable seed industry of *Lotus corniculatus*. The following have been put in place and include: a new self fertilising variety of *Lotus corniculatus* adapted to Australian conditions, and harvesting methodologies that will ensure adequate seed capture via conventional header harvesting. The implications for these developments are discussed below.

1) Harvesting methods have been established that allow seed yields in the order of 100 kg/ha to be captured from non-Australian varieties of *L. corniculatus*. These yields are comparable to those achieved overseas in viable seed industries of this species and should allow the establishment of a *L. corniculatus* seed industry in Australia. All the Australian bred varieties and in particular the selfer will have harvested seed yields much higher than those testing and reported here. Small scale testing indicates that seed yields of the Australian varieties will be 2 to 4 times higher than the varieties used in this study.

The requirements for seed crop production include: (a) a well adapted cultivar (see point 2 below), (b) irrigation (c) a well prepared seed bed which includes a fine and level seed bed free of stones and sticks, (d) adequate nutrition (e) an evenly grazed stand up to mid spring, (f) a weed and insect free stand, (g) bees placed on site at the commencement of flowering, if using out-crossing types (h) monitoring of peak flowering and podding (i) a desiccant applied at 70 percent pod maturity or 35 days after peak flowering (j) harvesting with a conventional header 48 hours after the desiccant application (k) header settings include a concave settings at 2 mm and drum speed at 1200 rpm with a very low wind speed setting.

These findings (above) will be made available to seed companies and seed growers via commercialisation briefs that are currently being developed for the new Australian varieties of *L. corniculatus*.

2) Development of the first selfing *L. corniculatus* variety ensures that pollinators are not required for seed crop production. Preliminary results suggest seed yield of this variety is significantly higher than that achieved by out-crossing varieties. It should also be highlighted that all the harvest methodology testing in point 1 above was undertaken using non-Australian cross-pollinated varieties of *Lotus corniculatus*, hence it is anticipated that the self fertilising variety and the other three Australian cross pollinated varieties will have higher seed yields than those achieved in the harvesting methodology study. The new Australian varieties are both adapted to Australian conditions and have high seed production which will facilitate a greater probability of establishing a viable seed industry.

The selfing variety (code LC07AS) will be commercialized in the Future Farming Systems CRC provided approval is provided by RIRDC. The cultivar is jointly owned by RIRDC, AWI, FFI CRC and NSW DPI.

3) Non-shattering selections of *Lotus arenarius* (annual form) have been house at the SARDI Genetic Resource Centre should future circumstance warrant their development.

4) Mutation studies undertaken to induce non-shattering phenotypes were unsuccessful despite significant effort. This approach was indentified as high risk, high return in the initial project proposal and unfortunately was not successful.
Recommendations

1) *Lotus corniculatus* line LC07AS be commercialised through the Future Farming Systems CRC. This line will be the first selfing cultivar of *Lotus corniculatus* developed exclusively for seed production in Mediterranean climates of Australia.

The commercialisation considerations include; seed increase, Plant Breeders Rights (PBR), selection of a commercial partner(s) and equity sharing arrangements.

2) Development of a commercialisation brief for LC07AS which includes, amongst other things, the knowledge generated in this project on seed production and harvesting of *Lotus corniculatus*. 
References


Australian Seed Authority. 2007. Areas registered in Australia for certification under OECD, AOSCA and domestic seed certification schemes to 24 January 2007.


http://www.orchidculture.com/COD/daylength.html#35N.


Currently there is no commercial seed production of Lotus in Australia. This project aimed to develop information and technologies that would allow a viable seed industry to be established for perennial Lotus in Australia.

The project has been successful in developing harvesting methodologies for *L. corniculatus* that can be used in Australia to achieve yields comparable to those achieved overseas. The project has also helped generate the first self compatible cultivar of *L. corniculatus* which will be commercialised for use in Australia.

RIRDC is a partnership between government and industry to invest in R&D for more productive and sustainable rural industries. We invest in new and emerging rural industries, a suite of established rural industries and national rural issues.

Most of the information we produce can be downloaded for free or purchased from our website <www.rirdc.gov.au>.

RIRDC books can also be purchased by phoning 1300 634 313 for a local call fee.

Improving Seed Production of Lotus
by Graeme Sandral, Daniel Real and Liz Nutt
Publication No. 10/014

Cover photo: Cutting experiment, Waroona, WA

Most RIRDC publications can be viewed and purchased at our website: