REVIEW

Seed Pod Shattering in the Genus *Lotus* and its Overcoming

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Abstract: Birdsfoot trefoil (*Lotus corniculatus* L.) is a perennial species that is known for its outstanding characters as a crucial component of meadow and pasture vegetation and a highly successful fodder crop. However, its cultivation has been limited by the inability to control pod shattering. The anatomic and physiological bases of pod shattering are known and are considered to be controlled by more than one gene. This paper reviews the known causes of pod shattering and potential ways of overcoming pod indehiscence in *L. corniculatus*. Genetic transformation is possible in the genus *Lotus*; however, the useful genes determining the seed pod indehiscent character have not been identified yet. The only way of introducing pod indehiscence characters into *L. corniculatus* is by interspecific hybridization within the genus *Lotus*; embryo rescue and protoplast cultures are promising. To determine useful genotypes for crosses, investigations of pre- and post-fertilization barriers are necessary. To that end, we present here a convenient procedure for a whole-mount clearing treatment of immature seeds that leaves the cell walls of tissues intact. This is a useful technique for the study of post-fertilization barriers in *Lotus*.

Keywords: interspecific hybridization; *Lotus*; pod shattering; post-fertilization barriers; pre-fertilization barriers

Birdsfoot trefoil (*Lotus corniculatus* L.) is a perennial species known for its outstanding characters as a crucial component of meadow and pasture vegetation and a highly successful fodder crop. However, its viability in meadows and pastures has been limited by seed management, which is more demanding compared to clover-grass mixtures (Dobeš 1970), and by the lack of ability to control pod shattering. There is a high seed loss resulting from continuous flowering and the time of pod maturity during which spontaneous pod dehiscence occurs. The two pod valves twist spirally, readily ejecting the seeds into the surrounding area, which results in the loss of many seeds and a serious reduction of yield.

Birdsfoot trefoil seed management has been unsuccessfully solved by agricultural engineering (Beuselinck & McGraw 1988; Qvinfeng & Hill 1989a), through the application of biologically active compounds such as paclobutrazol (Qvinfeng & Hill 1989b), daminozide and mepiquat (White *et al.* 1978), or by breeding for a lowered shattering ability. However, there is still room for improvement in seed management. Possible yields of *L. corniculatus* could be up 400 to 600 kg/ha under optimal growing conditions and with agricultural engineering. However, during abrupt weather changes from humid to dry weather it is not unusual for the yield to be as low as 50 kg/ha.

Anatomic, genetic and physiological bases of indehiscence

Lotus corniculatus is generally known as a tetraploid (alloploid; $2n = 24$) and allogamous species with autoincompatibility (Grant 1991; Beuselinck & Grant 1995). Flowers are bisexual and largely cross-pollinated, predominately by bees. The anatomy and morphology of the pod play a role in pod shattering. Major causes of pod shattering are thought to be changes in the orientation of the cells in the pericarp, such as unequal swelling and shrinkage, and lower lignification of the mesocarp. The relative humidity at the time of harvest is also an important factor. The critical relative humidity for dehiscence is between 35% and 49%, depending on genotypes. Mos (1987) studied the anatomic structure of birdsfoot trefoil pod and its connection to shattering determining that pods with a round diameter burst earlier and more often than flattened ones (Dobeš 1970). Metcalfe *et al.* (1957), Peacock and Wilsie (1957), Phillips and Keim (1968) showed that the shattering mechanism is determined mainly by plant genotype. Shattering resistance is highly heritable and in *Lotus* it is considered to be controlled by more than one gene. Breeding to reduce shattering through recurrent selection has been unsuccessful.

Sources of indehiscence

As indehiscence is genetically determined, various sources of indehiscence may be screened within the genus *Lotus*. Such sources have been found only in species other than *L. corniculatus*. European species known to be indehiscent include *L. tetragonolobus* (*Tetragonolobus purpureus*), *L. edulis* and *L. conimbricensis* (Gershon 1961; Phillips & Keim 1968). *Lotus angustissimus* and *L. ornithopodioides* were also reported to exhibit considerable seed pod indehiscence (Phillips & Keim 1968), though these species are more distantly related to *L. corniculatus*. The only case of reduced dehiscence in the *L. corniculatus* group was found by Brecheisen (1971, in Grant 1996), who reproduced a plant of *L. japonicus* that exhibited a much lowered tendency to shatter. However, its progeny did not display this reduced shattering character. *L. uliginosus* (Grant 1996) is also reported to be indehiscent and is a member of the *L. corniculatus* group. American species are not likely to be of immediate importance for transferring the indehiscent character to birdsfoot trefoil because of their considerable difference in phenotype and chromosome number (Grant 1996) compared to European species. Natural interspecific hybridization does not occur in the genus *Lotus*.

Overcoming of pod dehiscence

Attempts to transfer the indehiscent pod trait from distantly related species via interspecific hybridization (Beuselinck *et al.* 2003), diploid bridge species, amphidiploidy, backcrossing to birdsfoot trefoil or somatic hybridization have been promising. Genetic transformation by *Agrobacterium tumefaciens* has been carried out in *Lotus*, starting with the diploid *L. japonicus* (Handberg & Stougaard 1992). Unfortunately, no gene for the seed pod indehiscence character has been found. This presents an avenue of research that should be investigated.

The complete pod shattering resistance has not been reported for any species in the *L. corniculatus* group yet. Attempts to transfer the indehiscent character by hybridization between diploid species and tetraploid *L. corniculatus* have met with little success because of differences in the chromosome number. Gershon (1961) observed that *L. uliginosus* exhibited a delay in the time until the pods dehisced, which he attributed to the absence of a gap in the mesocarp at the dorsal suture found in the pods of *L. corniculatus*. A delay of 3 to 4 days in pod dehiscence would enable the harvesting of a higher proportion of mature seed, which could have economic ramifications. Gershon (1961) tried to transfer the indehiscence pod character from *L. uliginosus* by backcrossing to *L. corniculatus*. The hybrid *L. uliginosus* × *L. corniculatus* was successfully produced, but was triploid and highly sterile. Gershon (1961) also examined a hybrid population resulting from a cross between *L. corniculatus* and an induced tetraploid of *L. uliginosus*. Fertility of the hybrids varied considerably from 0 to over 10 seeds per pod. Dehiscence in the different plants ranged from 26% to 93%. A number of generations of backcrossing and selection are necessary to overcome sterility.

As for other species, Gershon (1961) crossed *L. conimbricensis* with *L. corniculatus*, but no

seed was produced. Phillips and Keim (1968) produced hybrids of *L. corniculatus* and *L. conimbricensis* with $2n = 24$, indicating that fertilization had occurred by means of unreduced gametes of *L. conimbricensis*. The first report of interspecific somatic hybrids in the genus *Lotus* was by Wright *et al.* (1987), who attempted to transfer seed pod indehiscence from *L. conimbricensis* into *L. corniculatus*. Protoplasts from calluses of *L. conimbricensis* were fused with etiolated hypocotyl protoplasts of *L. corniculatus*, using polyethylene glycol as the fusion agent. The hybrid character of somatic hybrids was confirmed by isozyme data and chromosome analyses. Due to the sterility of pods, no information on indehiscence was gained. Similar results were obtained by Rim *et al.* (1990), who performed somatic hybridization of *L. corniculatus* and *L. conimbricensis*.

Two approaches were taken to transfer the indehiscent trait into *L. corniculatus*. First, O'Donoughue and Grant (1988) transferred the indehiscent character stepwise from *L. conimbricensis* and *L. ornithopodioides* into *L. corniculatus*, using a bridge species to help overcome the genetic distance between these diploids and *L. corniculatus*. *L. alpinus*, *L. japonicus* and *L. burtii* were used as the bridge species while the chromosome number of the resulting diploid hybrids was doubled by means of colchicine to form amphidiploids. F_1 amphidiploids *L. alpinus* × *L. conimbricensis* and *L. burtii* × *L. ornithopodioides* were obtained and the hybrid *L. alpinus* × *L. conimbricensis* was fertile with non-shattering pods. The hybrid had a tetraploid chromosome number $2n = 24$, the same as *L. corniculatus*. The indehiscent character had been successfully transferred to the amphidiploid. A final cross yielding the double hybrid (*L. alpinus* × *L. conimbricensis*) × *L. corniculatus* produced non-shattering pods, but only under growth chamber and greenhouse conditions. In the field, the majority of the pods eventually shattered. It was concluded that the indehiscent trait is controlled by more than one gene. After direct crossing the distant diploid species with tetraploid *L. corniculatus*, only sterile triploid hybrids were produced due to irregularities in the endosperm development (Jaranowski & Wojciechowska 1963).

Yang *et al.* (1990) offered a second approach, investigating the somatic hybrid from a cross between *L. corniculatus* and *L. conimbrinesis*. To overcome pod abscission, they applied gibberellic acid (GA_3) to the stigma of the flowers of the somatic hybrid. The majority of GA_3-induced pods were indehiscent. The indehiscent trait of *L. conimbricensis* was expressed in the somatic hybrid. GA_3-induced somatic hybrid pods did not coil, suggesting that structural differences in pod anatomy could account for the reduced dehiscence of somatic hybrid seed pods. The reduced seed pod dehiscence could be caused by a reduced level of seed pod lignification.

Interspecific hybridization has been carried out successfully in the genus *Lotus* using embryo culture procedures (Sharma *et al.* 1996; Galati *et al.* 2006). Of the 12 cross combinations tested, 8 were successful. Two of these, *L. alpinus* × *L. conimbricensis* and *L. burttii* × *L. ornithopodioides*, were new hybrids. The data indicate that a breeding program focused on the two new hybrids could potentially reduce shattering in *L. corniculatus* via amphidiploidy (O'Donoughue & Grant 1988).

Study of pre-fertilization and post-fertilization barriers to crossability

There are pre- and post-fertilization barriers to crossability after interspecific hybridization in the genus *Lotus*. Pre-fertilization barriers constrain the ability of pollen grains to germinate on the stigma and of pollen tubes to grow through the style. Post-fertilization barriers include defects in the endosperm and embryo development. In this study, our work was aimed at choosing useful methods for the inspection of pre-fertilization and post-fertilization barriers of crossability and the role that these barriers play in crosses between *L. corniculatus* ($2n = 4x$) and species displaying the indehiscent seed pod character such as *L. uliginosus* (diploid $2n = 2x$ and tetraploid $2n = 4x$).

Controlled pollination following castration was used with intraspecific combinations of *L. corniculatus*, *L. uliginosus* ($2n = 2x$), and *L. uliginosus* ($2n = 4x$) and the interspecific combinations resulting from the crosses of *L. corniculatus* × *L. uliginosus* ($2n = 2x$ and $4x$).

Callose staining with aniline blue was used to investigate pre-fertilization barriers. After intra- and interspecific crosses, flowers were collected in intervals of 1 h, up to 72 h after pollination. Flower maceration was performed in 1N NaOH in a water bath (60°C) for 20 to 40 min. After rinsing the flowers for 24 h under running tap

water, they were stained with aniline blue. The staining solution was prepared from 7 g K_3PO_4 dissolved in 1 l of distilled water and 1 g aniline blue. Microscopic samples were prepared by pistil excision, by mounting the specimen in glycerol on a glass slide and applying gentle pressure on a cover slide. Fluorescent signals were detected with an excitation filter of 400–455 nm and an emission filter of 475 nm (Řepková *et al.* 2006).

For the study of post-fertilization barriers, the conventional procedures of immature seed embedding in paraffin and sectioning are very time consuming. A more expeditious and convenient procedure could be a whole-mount clearing treatment of immature seeds that leaves the cell walls of tissues intact. This procedure has been routinely used in *Arabidopsis thaliana* L. (Heynh.) for the detailed study of embryo and endosperm development (Mayer *et al.* 1993; Aida *et al.* 1997; Herr 1999). The clearing procedure has also been applied to other plant species, e.g. *Glycine max* and *Phaseolus aureus* (George *et al.* 1979), *Planera aquatica* and *Cassia occidentalis* (Herr 1982), *Solanum* spp. (Stelly & Peloquin 1983), *Vicia faba* (Ramsay & Pickersgill 1986), *Linum usitatissimum* (Huyghe 1987), *Dianthus* species (Hoshino *et al.* 2000) and *Trifolium* species (Řepková *et al.* 2006), to study ovule and megagametophyte development, microsporogenesis, and embryological investigation (Herr 1982). Therefore, this clearing treatment was used in the analysis of post-fertilization barriers in *Lotus* to search for *in situ* embryo development. The investigation was focused on the identification of the most promising interspecific crosses, the maximum level of hybrid embryo development, and thus the optimal period for *in vitro* embryo cultivation.

The flowers resulting from the plants of *L. corniculatus* × *L. uliginosus* ($2n = 2x$ and $4x$) were harvested in 2, 3, 4, 7, and 9 days after pollination. Immature seeds were fixed in FAA mixture (formaldehyde, acetic acid, ethanol (5:5:90, v/v/v)) in intervals of 6 h, up to 48 h, at room temperature. After washing in 96%, 70% and 30% ethanol, a fourth wash in distilled water was conducted. Seeds were cleared in chloral hydrate (Mayer *et al.* 1993) up to 48 h at room temperature or at 4°C. Embryo development was observed with Nomarsky optics.

To study pre-fertilization barriers, 113 flowers were evaluated. The optimal time for maceration was 40 min. The growth of the pollen tubes up to the ovary was observed in the crosses of *L. corniculatus* × *L. uliginosus* ($2n = 2x$ and $4x$). No differences in pollen-tube growth rate between self- and cross-pollination were observed at the level of stigma or style, supporting a similar finding by Bader and Anderson (1962).

To investigate the role of post-fertilization barriers, the method of clearing was optimized. The optimal length of fixation was 24 h; a shorter period had a negative influence on the subsequent clearing. The full period of immature seed clearing was critical for sufficient clearing of the embryo proper. Based on our experiments, the optimal time for incubation in chloral hydrate was 48 h.

The following embryo stages were observed in reviewed time intervals after intraspecific pollination of *L. corniculatus* zygote, at the micropylar end 3 days after pollination (DAP); globular stage 6 to 7 DAP; heart stage 7 to 8 DAP. In intraspecific crosses of *L. uliginosus* ($2x$), the globular stage of the embryo at 6 to 7 DAP and the heart stage of the embryo at 7 to 8 DAP were observed. In the crosses of *L. uliginosus* ($4x$), the zygote at the micropylar end at 3 DAP and globular stage 6 to 7 DAP were observed. In the interspecific cross of *L. corniculatus* × *L. uliginosus* ($2x$), defects in embryo sacs were observed. After this interspecific cross, enlargement of immature ovules occurred, but no hybrid embryo resulted. In the interspecific cross of *L. corniculatus* × *L. uliginosus* ($4x$), only one globular stage embryo at 7 DAP was detected from 30 analyzed pollinated flowers.

CONCLUSIONS

(1) The growth of the pollen tubes up to the ovary was observed in crosses of *L. corniculatus* × *L. uliginosus* ($2n = 2x$ and $4x$). This means that no pre-fertilization barriers were detected in this combination.

(2) In crosses of *L. corniculatus* and *L. uliginosus* ($2x$, $4x$), a clearing treatment by chloral hydrate was applied to plant tissues. Clearing immature seeds and inner embryos up to the heart stage was carried out through modifications of a known procedure (Mayer *et al.* 1993). The optimal length of fixation was 24 h and the optimal length of action of chloral hydrate was 48 h. For other embryo stages, this method has not been sufficiently optimised yet.

(3) Even when the enlargement of immature seeds occurred, the post-fertilization barriers for

interspecific crosses of *L. corniculatus* × *L. uliginosus* (2*x*) included defects of the embryo sac and no hybrid embryo resulted. In the interspecific combination of *L. corniculatus* × *L. uliginosus* (4*x*), the globular stage embryo was detected.

(4) Due to the post-fertilization barriers of crossability of *L. corniculatus* × *L. uliginosus* (4*x*) cross, the embryo rescue could be used for obtaining hybrid F_1 plants.

(5) To summarize, this clearing treatment approach appears to be a valuable method in embryological investigations in a plant species and for the study of post-fertilization barriers after interspecific crosses. However, sufficient optimization is always necessary.

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