Theor Appl Genet (1987) 75:151–156

Somatic hybridization between birdsfoot trefoil (*Lotus corniculatus* L.) and *L. conimbricensis* Willd.

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Received March 30, 1987; Accepted June 25, 1987
Communicated by P. Maliga

Summary. Somatic hybrid plants were produced by fusion of birdsfoot trefoil (*Lotus corniculatus*) cv 'Leo' and *L. conimbricensis* Willd. protoplasts. Birdsfoot trefoil etiolated hypocotyl protoplasts were inactivated with iodoacetate to inhibit cell division prior to fusion with *L. conimbricensis* suspension culture protoplasts. *L. conimbricensis* protoplasts divided to form callus which did not regenerate plants. Thus, plant regeneration from protoplast-derived callus was used to tentatively identify somatic hybrid cell lines. Plants regenerated from three cell lines exhibited additive combinations of parental isozymes of phosphoglucomutase, and *L. conimbricensis*-specific esterases indicating that they were somatic hybrids. The somatic chromosome number of one somatic hybrid was 36. The other somatic hybrid exhibited variable chromosome numbers ranging from 33 to 40. These observations approximate the expected combination of the birdsfoot trefoil (2n = 4x = 24) and *L. conimbricensis* (2n = 2x = 12) genomes. Somatic hybrid flowers were less yellow than birdsfoot trefoil flowers and had purple keel tips, a trait inherited from the white flowered *L. conimbricensis*. Somatic hybrids also had inflorescence structure that was intermediate to the parents. Fifteen somatic hybrid plants regenerated from the three callus lines were male sterile. Successul fertilization in backcrosses with birdsfoot trefoil pollen has not yet been obtained suggesting that the hybrids are also female sterile. This is the first example of somatic hybridization between these two sexually incompatible *Lotus* species.

Key words: *Lotus corniculatus* – *Lotus conimbricensis* – Somatic hybridization – Protoplast

Introduction

Birdsfoot trefoil is a perennial forage legume utilized for production of high quality hay and "bloat safe" pasture (Grant and Marten 1985). Seed pod dehiscence and seed loss reduce birdsfoot trefoil seed production. Up to 50% of the seed yield can be lost due to preharvest seed pod dehiscence (McGraw and Beuselink 1983). Indehiscent birdsfoot trefoil plants have not been observed; however, seed pod indehiscence has been observed in other *Lotus* species. A plant collection of *L. japonicus* exhibited decreased seed pod dehiscence compared with wild type *L. japonicus* and birdsfoot trefoil (Brecheisen 1971). *L. conimbricensis* has completely indehiscent seed pods (Phillips and Keim 1968). A birdsfoot trefoil cultivar expressing the seed pod indehiscence of *L. conimbricensis* would be desirable due to the potential of improved seed harvestability.

Interspecific hybridizations between birdsfoot trefoil (2n = 4x = 24) and *L. conimbricensis* (2n = 2x = 12) have met with limited success (Gershon 1961; Phillips and Keim 1968). Crosses of 2n and 4n (colchicine doubled) *L. conimbricensis* with birdsfoot trefoil failed to produce seed (Gershon 1961). In a later study, 13 putative hybrid plants were produced by pollination of 2,498 emasculated birdsfoot trefoil flowers with *L. conimbricensis* (Phillips and Keim 1968). Hybrid plants resembled birdsfoot trefoil except for pubescence, a trait presumably inherited from *L. conimbricensis*. These plants had 24 chromosomes instead of the expected 18 chromosomes and some hybrids exhibited reduced pollen fertility. Meiotic analysis of progeny from backcrosses of birdsfoot trefoil with the hybrid plants exhibiting lowest pollen fertility showed an increased frequency of univalents compared with birdsfoot trefoil. These results support the hypothesis that hybrid plants

may have been obtained by the pollination of birdsfoot trefoil with 2n pollen from *L. conimbricensis* (Phillips and Keim 1968). Further characterization of these plants has not been reported. The low frequency of putative hybrids between birdsfoot trefoil and *L. conimbricensis* reported by Phillips and Keim (1968) indicates a high degree of sexual incompatibility between the species.

Somatic hybridization is an approach to overcome the sexual incompatibility between birdsfoot trefoil and *L. conimbricensis* that offers the additional advantage of directly producing an allohexaploid hybrid. Birdsfoot trefoil protoplasts divide to form callus with prolific plant regeneration capacity making this species amenable to protoplast fusion (Ahuja et al. 1983). The objectives of this study were to construct somatic hybrids between birdsfoot trefoil and *L. conimbricensis* and to characterize somatic hybrid plants.

Materials and methods

Protoplast isolation

Birdsfoot trefoil cv 'Leo' seeds were surface sterilized for 5 min in 0.1% $HgCl_2$ and 0.1% sodium dodecyl sulfate, rinsed 3 times in sterile deionized water and germinated for 5 days in the dark at 28 °C in petri plates containing B5 agar solidified medium (Gamborg et al. 1968) without sucrose or hormones. Etiolated hypocotyls were transversely sliced into ca. 2 mm sections with a scalpel and incubated 16 h at 28 °C with rotary mixing at 50 rpm in enzyme mixture B prepared according to Ahuja et al. (1983). The enzyme was desalted using a Bio-Gel P6 column (200–400 mesh) before addition of mannitol and antibiotics and was filter sterilized before addition to hypocotyl sections.

Lotus conimbricensis Willd. seed were obtained from the USDA Northeastern Regional Plant Introduction Station, Geneva, NY. From these seed, one plant grew to maturity for seed increase. Previously, *L. conimbricensis* has been referred to as *L. coimbrensis* (Gershon 1961; Phillips and Keim 1968). *L. conimbricensis* callus cultures were initiated from axenic leaf and internode explants taken from a mature plant and plated on agar solidified UM medium (Uchimiya and Murashige 1974). Suspension cultures were initiated by inoculating 2 to 4 g callus into liquid UM medium and were subcultured weekly by 5-fold dilution into fresh liquid UM medium. Plant regeneration was not obtained from these cultures. Approximately 1 g suspension culture tissue, isolated 3 days after subculture, was digested 16 h in an enzyme mixture consisting of 0.5% Cellulysin, 0.25% Driselase, 0.25% Rhozyme HP150 and 0.125% PASE dissolved in the CPW salt solution described by Frearson et al. (1973) containing 13% mannitol (pH 5.5). The enzyme was desalted and filter sterilized as described above. Incubation conditions and duration of protoplast isolation were the same as for birdsfoot trefoil hypocotyl sections.

Protoplasts were purified by sieving through 74 μm screens to remove undigested tissue and by centrifugation for 10 min at 100 g to wash protoplasts free of the digestion solutions. Protoplasts were isolated from cells by centrifugal flotation on CPW salts containing 21% sucrose at 100 g for 10 min. Isolated protoplasts were washed 3 times in CPW salts containing 13% mannitol by centrifugation (100 g, 5 min). Protoplast yields were determined using a hemacytometer.

Protoplast fusion and culture

Birdsfoot trefoil protoplasts were treated for 20 min with 10 mM iodoacetate in W5 medium according to Medgyesy et al. (1980) immediately prior to fusion. After 3 washes by centrifugation for 5 min at 100 g in W5 medium, inactivated birdsfoot trefoil protoplasts were mixed 1 : 1 with *L. conimbricensis* protoplasts and pelleted by centrifugation at 100 g. The supernatant was removed and the polyethylene glycol, dimethyl sulfoxide fusion-inducing solution was added to the protoplasts for 5 min incubation at 24 °C followed by gentle mixing (Menczel and Wolfe 1984). The fusion mixture was diluted by adding 6 ml of 50 mM morpholinoethanesulfonate in W5 medium (pH 5.6) and incubated at 28 °C for approximately 2 h (Menczel and Wolfe 1984). Fusion mixtures were washed twice in CPW salts containing 13% mannitol and resuspended in KM8P medium (Kao and Michayluk 1975) at a plating density of 10^5 unfused *L. conimbricensis* protoplasts/ml culture medium. For each fusion experiment, some untreated protoplasts of each species were plated individually to calculate division frequencies. All procedures were performed using aseptic techniques.

Fusion frequency and iodoacetate inactivation were determined 24 h after plating. To determine protoplast fusion frequency and to adjust protoplast plating density, *L. conimbricensis* protoplasts were stained with 50 μg/ml fluorescein diacetate (Widholm 1972) in CPW salts containing 13% mannitol for 30 min at 24 °C prior to fusion. Birdsfoot trefoil protoplasts were stained by adding 15 μg/ml rhodamine isothiocyanate (Barsby et al. 1984) to the digestion enzyme mixture. Heterokaryons were identified as protoplasts that fluoresced red and green when observed using a Nikon inverted microscope Diaphot-TMD (× 100 magnification) with epifluorescence attachment TMD-EF and filter cassette B. Fusion frequency was the proportion of heterokaryons observed among unfused parental protoplasts determined in 5 microscopic fields per petri plate. Iodoacetate treated birdsfoot trefoil protoplasts were fluorescently labelled and co-cultured with *L. conimbricensis* protoplasts without fusion to determine the efficiency of inactivation of birdsfoot trefoil protoplasts.

Isolated protoplasts, fusion treatments and controls were cultured in 1.5 ml KMP8 (Kao and Michayluk 1975) medium at 10^5 protoplasts/ml in 10 × 35 mm petri dishes. Protoplast cultures were initially incubated in the dark at 28 °C for 5 days after plating. After 5 days, protoplast cultures were diluted with 0.5 ml of 3 volumes KM8P medium previously mixed with 1 volume UM liquid medium and moved to a 16 h daylength (80 μerg/m²/sm) at 28 °C. At 5 day intervals thereafter, protoplasts were diluted with 0.5 ml medium in the proportions 2 : 1, 1 : 1 and 1 : 2 of KM8P to UM medium, respectively (Ahuja et al. 1983). Division frequency was determined 5–10 days after plating by counting the number of colonies with 4 to 8 cells per total number of protoplasts in 5 microscope fields (× 100 magnification) per petri dish. After the final medium dilutions, colonies were transferred to 25 × 100 mm petri plates containing 25 ml UM agar solidified medium containing 4.5% mannitol until they were macroscopic in size.

Identification of somatic hybrids

Macroscopic colonies from four fusion experiments were transferred after 28 days culture to agar solidified plant regeneration medium containing MS salts and organic constituents (Murashige and Skoog 1962) supplemented with 0.2 mg/L 6-benzylaminopurine (Ahuja et al. 1983). Several shoots from each regenerable callus were transferred to MS medium containing sucrose but with no hormones and allowed to root. Regenerated plants were transplanted into Jiffy-7 peat pellets and

grown in a growth chamber at 22 °C with a 16 h daylength under 400 μerg/m²/s light intensity until large enough to be transplanted into pots and grown in a greenhouse. In the greenhouse plants were grown under high pressure sodium lamps (130 μerg/m²/s) with a 16 h daylength to supplement natural light.

Isozyme analyses

Isozyme analyses were performed on 31 plants regenerated from 17 callus lines from four fusion experiments, protoplast culture-derived Leo birdsfoot trefoil genotypes, and seed-derived plants of Leo birdsfoot trefoil and *L. conimbricensis*. Several different Leo samples were run on each gel for analysis of isozyme polymorphism in birdsfoot trefoil. Leaf samples for isozyme analyses were collected from plants growing in the greenhouse and growth chamber and 1 g fresh weight was extracted in 3 ml of 250 mM Tris-HCl (pH 8.5) containing 10% sucrose and 1 mM dithiothreitol. Extracts were centrifuged for 15 min at 12,000 g. Supernatant aliquots used for isozyme analyses (60 μl) were added to wells in a 1.5 mm vertical polyacrylamide slab gel. The upper stacking gel (4.5% acrylamide with a 10:2.5 acrylamide: bis ratio) and the lower resolving gel (10% acrylamide with a 28:0.7 acrylamide: bis ratio) were prepared according to Davis (1964). The running buffer in both the upper and lower reservoirs was 0.005 M Trizma base, 0.038 M glycine (pH 8.3). Electrophoresis was carried out at 20 mamps constant current per gel until bromophenol blue tracking dye migrated to the bottom of the gel. All steps were carried out at 4 °C. Esterase and phosphoglucomutase activity stains were as described by Vallejos (1983).

Chromosome number and hybrid fertility

Mitotic chromosomes of birdsfoot trefoil, *L. conimbricensis* and plants regenerated from fusion experiments were observed in root tip squashes prepared according to Giri et al. (1981) and stained in modified carbol fuchsin for 1 to 2 h (Kao 1975). Chromosomes were counted under oil emersion (×500 magnification). Pollen grains were incubated for 3 h in germination medium (Cook and Walden 1965) and scored for pollen tube elongation. Somatic hybrid flowers were backcrossed with birdsfoot trefoil pollen as described by Phillips and Keim (1968) without prior emasculation of hybrid flowers.

Results

Protoplast culture and fusion

Birdsfoot trefoil cv Leo etiolated hypocotyl protoplasts exhibited an average division frequency determined at the 4 to 8 cell stage of 16% for 5 experiments which was similar to the 20% division frequency reported by Ahuja et al. (1983). Plant regeneration was obtained from nearly all colonies resulting from birdsfoot trefoil protoplast culture. Plantlets rooted readily when moved to hormone-free medium. Division of iodoacetate-treated birdsfoot trefoil protoplasts was not observed in fusion treatments, co-cultivation controls or when inactivated protoplasts were cultured separately (data not shown). Protoplasts from *L. conimbricensis* suspension cultures formed callus. Division frequency of *L. conimbricensis* protoplasts observed in fusion treatments was on average 2%. *L. conimbricensis* callus did not regenerate plants although a variety of plant regeneration media were tested. Heterokaryon formation frequencies determined from 7 fusion experiments were from 0.4 to 7.2% using the fusion method of Menczel and Wolfe (1984).

Table 1. Frequency of plant regenerating callus lines cultured from four fusion experiments between iodoacetate-inactivated birdsfoot trefoil protoplasts and *L. conimbricensis* protoplasts

Fusion experiment no.	Total protoplasts plated[a]	No. of regenerable callus lines	
		Total	Somatic hybrids
1	660,000	15	0
2	1,880,000	13	3
3	600,000	1	0
4	590,000	3	0

[a] Equal numbers of birdsfoot trefoil and *L. conimbricensis* protoplasts were mixed and fused

Identification of somatic hybrid plants

Only somatic hybrid or cybrid callus lines were expected to regenerate plants from fusions of iodoacetate-inactivated protoplasts of the regenerating parent, birdsfoot trefoil and *L. conimbricensis* (nonregenerating) based on previously reported observations that morphogenic capacity can be dominantly inherited in somatic hybrids (Power et al. 1976, 1980). Of the colonies cultured from four fusion experiments, 32 were isolated that exhibited plant regeneration (Table 1). Plants regenerated from 3 callus lines (designated 1, 2 and 3) were tentatively identified as somatic hybrids due to root regeneration behavior. Rooting of putative somatic hybrid shoots was much slower than root regeneration of birdsfoot trefoil shoots and the shoots of the 29 other cell lines obtained from fusion treatment.

Isozyme analyses

Species-specific differences in the isozymes of phosphoglucomutase (Pgm) were observed between birdsfoot trefoil and *L. conimbricensis* (Fig. 1). Birdsfoot trefoil exhibited polymorphism in Pgm; however, the slower migrating Pgm band of *L. conimbricensis* was not observed in 14 seed and tissue culture-derived Leo birdsfoot trefoil plants (data not shown). Somatic hybrid plants from the three callus lines exhibited the Pgm isozyme bands of both parents. Pgm isozymes of somatic hybrids 1 and 2 are shown in Fig. 1. Pgm patterns observed for birdsfoot trefoil, *L. conimbricensis* and the somatic hybrids were highly reproducible between repetitions of the isozyme analysis. The somatic hybrid plants also expressed major *L. conimbricensis*-specific

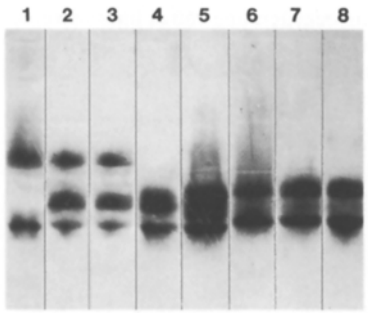

Fig. 1. Pgm patterns of (*lane 1*) *L. conimbricensis;* (*lanes 2, 3*) somatic hybrids 1 and 2, respectively; (*lane 4*) birdsfoot trefoil protoplast-derived plant; (*lanes 5–8*) birdsfoot trefoil seed-derived plants

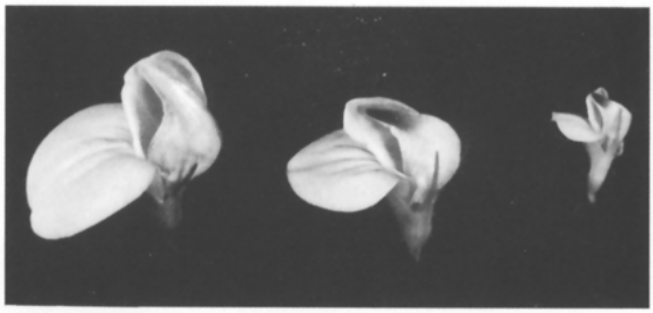

Fig. 2. Flowers of (*left to right*) birdsfoot trefoil, somatic hybrid and *L. conimbricensis* with wing petals removed to show keel tip color

esterases providing further evidence for their hybridity. However, it was difficult to consistently resolve birdsfoot trefoil specific esterases in the somatic hybrid plants (data not shown).

Somatic chromosome numbers

Chromosome numbers were determined from root tip squashes of birdsfoot trefoil, *L. conimbricensis* and somatic hybrids 1 and 2 (Table 2). Absolute chromosome numbers were difficult to determine because of the small size of *Lotus* chromosomes. Chromosome numbers reported are the most frequently observed counts for each genotype (Table 2). The somatic chromosome number observed for seed-derived and protoplast culture regenerated birdsfoot trefoil plants was 24; actual chromosome number is 2n = 4x = 24 (Dawson 1941). The somatic chromosome number determined for *L. conimbricensis* was 12 which is in agreement with the accepted chromosome number 2n = 2x = 12 (Grant 1965; Phillips and Keim 1968) (Table 2). Chromosome numbers determined from somatic hybrid plants regenerated from hybrid callus line 1 was 36 which was the expected combination of the parental genomes to give an allohexaploid (2n = 6x = 36). Chromosome numbers determined for plants regenerated from somatic hybrid cell line 2 varied from 33 to 40 chromosomes, suggesting that this somatic hybrid was unstable in chromosome number.

Table 2. Mitotic chromosome numbers from root-tip squashes of birdsfoot trefoil, *L. conimbricensis* and somatic hybrids

Genotype	Plants examined	Total cells counted	Expected chromosome no.	Observed chromosome no.
Birdsfoot trefoil	3	11	24	24
L. conimbricensis	3	20	12	12
Hybrid 1	4	9	36	36
Hybrid 2	4	63	36	33–40[a]

[a] Each plant regenerated from somatic hybrid cell line 2 exhibited similar variation in chromosome number

Somatic hybrid floral characteristics

Birdsfoot trefoil cv Leo flowers are bright yellow and generally have yellow keel tips whereas *L. conimbricensis* has small white flowers with purple keel tips (Phillips and Keim 1968). One Leo genotype observed in our base population had yellow flowers with reddish brown keel tips, which are easily distinguished from the purple keel tips of *L. conimbricensis* (Fig. 2). Furthermore, flowers with purple keel tips have not been observed in birdsfoot trefoil (W. F. Grant, personal communication). Somatic hybrid flowers were slightly smaller than birdsfoot trefoil and were light yellow with purple keel tips (Fig. 2). Interspecific hybrids between other *Lotus* species have been described that provide evidence for the mode of inheritance of flower color markers. Flower color of hybrids between diploid *Lotus* species are either intermediate to parental flower color or closer to the deeper yellow parental flower color (Grant et al. 1962). Reddish brown keel tip is transmitted dominantly in birdsfoot trefoil (Buzzell and Wilsie 1963) and in *L. japonicus* × *L. alpinus* hybrids (Grant et al. 1962). We assume that purple keel tips were also transmitted as a dominant trait in the birdsfoot trefoil × *L. conimbricensis* somatic hybrids. Therefore, the intermediate yellow flower petals with purple keel tips observed in the somatic hybrid flowers were consistent with the observed inheritance of these traits in other *Lotus* interspecific hybrids.

Somatic hybrid plants also were intermediate between the parents in inflorescence structure. The birdsfoot trefoil inflorescence is an umbel bearing about five florets on a peduncle (Grant and Marten 1985). *Lotus conimbricensis* florets emerge from the axils of leaf

Fig. 3. Inflorescence structure of (*left to right*) birdsfoot trefoil, somatic hybrid, and *L. conimbricensis*

nodes with one or two florets per node. The inflorescence of the somatic hybrid plants consisted of up to three florets terminal to a medium length peduncle (Fig. 3).

Plants regenerated from the 29 remaining callus lines produced from the four fusion experiments (Table 1) had uniformly yellow flowers and resembled birdsfoot trefoil. These plants exhibited only birdsfoot trefoil isozymes indicating that they were not hybrids. Plant regeneration was not observed from controls consisting of mixed protoplast cultures of inactivated birdsfoot trefoil and *L. conimbricensis* suggesting that these cell lines were not escapes of the iodoacetate-inactivation of birdsfoot trefoil protoplasts. It is possible that these plants were cybrids with a birdsfoot trefoil nucleus and *L. conimbricensis* cytoplasm. Iodoacetate treatment of protoplasts has been used to effect cybridization in other species (Medgyesy et al. 1980); however, the cytoplasms of these birdsfoot trefoil-like plants have not been analyzed.

Hybrid fertility

Somatic hybrid plants initiated pods without pollination which senesced and aborted within 3 to 5 days post anthesis. Pollen germination from 15 somatic hybrid plants regenerated from the three hybrid callus lines was less than 0.1% (Table 3) indicating that the somatic hybrids were male sterile. Pollen germination of birdsfoot trefoil and *L. conimbricensis* were 83% and 71%, respectively (Table 3). Anthers of somatic hybrids did not dehisce or shed pollen and the pollen clumped extensively in the germination medium. Over 600 somatic hybrid flowers were manually pollinated with birdsfoot trefoil pollen. No pods have set indicating that the somatic hybrids were also female sterile.

Table 3. Germination of pollen from flowers of birdsfoot trefoil, *L. conimbricensis* and plants regenerated from somatic hybrid cell lines

Genotype	Plants tested	Pollen germination (%)
Birdsfoot trefoil	4	83
L. conimbricensis	2	71
Hybrid 1	11	< 0.1
Hybrid 2	2	< 0.1
Hybrid 3	2	< 0.1

Discussion

Identification of somatic hybrids between birdsfoot trefoil and *L. conimbricensis* was accomplished by screening plants regenerated from fusion of iodoacetate-inactivated protoplasts of the regenerating parent, birdsfoot trefoil, and nonregenerating *L. conimbricensis*. Iodoacetate-inactivation of birdsfoot trefoil protoplasts appeared to be required for fusion hybrid recovery because no hybrids were recovered after screening over 200 plants regenerated from a fusion experiment in which birdsfoot trefoil protoplasts were not treated with iodoacetate (data not shown). In the present study, three confirmed somatic hybrids were identified among 32 plant regenerating callus lines. All plants regenerated from the somatic hybrid callus lines were sterile.

Protoplast fusion has been employed to create fertile allohexaploid somatic hybrids between other combinations of 2x and 4x species. Fertile somatic hybrids between *N. tabacum* (cms) (2n = 4x = 48) and *N. glutinosa* (2n = 2x = 24) had somatic chromosome numbers of 72 (Uchimiya 1982); however, these *Nicotiana* species are sexually compatible and therefore more closely related than birdsfoot trefoil and *L. conimbricensis*. Chromosome rearrangements and/or aneuploidy preexisting in the *L. conimbricensis* suspension culture may have been transmitted to the somatic hybrids causing abnormal meiosis that resulted in hybrid sterility. Aneuploidy in somatic hybrid 2 was suggested by the range of from 33 to 40 chromosomes detected in the hybrid (Table 2). In future experiments, mesophyll *L. conimbricensis* protoplasts will be used in fusions to minimize chromosomal variation in the *L. conimbricensis* donor protoplasts because aneuploidy often causes sterility in somatic hybrids (Harms 1983). However, we cannot rule out other causes of somatic hybrid sterility such as homeologous pairing between the two genomes during meiosis that would produce unbalanced gametes, differences in developmental timing of sporogenesis between the parents that were not resolved in the somatic hybrids, or possibly deleterious alien nuclear-cytoplasmic interactions (Harms 1983). Furthermore, combi-

nations of these putative mechanisms may be affecting somatic hybrid sterility.

This is the first report of somatic hybridization between birdsfoot trefoil and *L. conimbricensis* to produce this potentially valuable hybrid. Future research will be focused on determination of the mechanism of hybrid sterility to elucidate a strategy for the production of fertile somatic hybrids as a means to transfer of seed pod indehiscence of *L. conimbricensis* to birdsfoot trefoil germplasm.

Acknowledgements. This research was supported by a grant from the Minnesota Crop Improvement Agency, Minnesota Agricultural Experiment Station project No. 0302-4813-56 and University of Minnesota Graduate School grant number 0749-5246-47. The authors thank B. Will and J. Yang for technical assistance. Minnesota Agricultural Experiment Station Journal Series, Paper No. 15,326.

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