

## EVIDENCE FOR ANDROGENESIS IN THE GENUS *LOTUS* (FABACEAE)

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Attempts have been made to produce haploid plants in *L. angustissimus* L., *L. corniculatus* L., *L. emeroides* Murr., *L. halophilus* Boiss. & Sprun., 4x *L. japonicus* (Regel) Larsen and 4x *L. tenuis* Waldst. & Kit. via anther culture. Multicellular microspores were observed in anthers of *L. halophilus* cultured on potato medium. Some evidence for androgenesis in *L. corniculatus* was provided by the observation of haploid cells (dihaploid,  $2n = 12$ ) in root tips of a plant regenerated from anther-derived callus tissues. Considerable basic research is required in anther and microspore culture of *Lotus* species.

Plant regeneration of birdsfoot trefoil from callus cultures both through organogenesis (Niizeki and Grant 1971, Swanson and Tomes 1980), somatic embryogenesis (Mariotti et al., 1984; Damiani et al. 1985, 1990) and protoplast-derived calli (Ahuja et al. 1983; Niizeki and Saito 1986; Webb et al. 1987; Niizeki 1993; Vessabutr and Grant 1995) has been very successful. It has been stated that *Lotus* protoplasts show the most prolific regeneration among any forage legume protoplast system (Ahuja et al. 1983). However, attempts to culture anthers and isolated microspores of various species and interspecific hybrids have not been successful in producing androgenic plants (Niizeki and Grant 1971; Niizeki and Kita 1973; Niizeki 1977; Niizeki and Saito 1986). In none of these studies were microspore divisions leading to the formation of multicellular units and embryoids, observed in cultured anthers. Furthermore, the presence of haploid cells was not detected in anther-derived callus tissues and all regenerated plants were either tetraploid or octoploid (Niizeki and Grant 1971). A single report of spontaneous haploid parthenogenesis has been reported from a cross between a male sterile plant of *L. corniculatus* ( $2n = 24$ ) and a plant of *L. tenuis* ( $2n = 12$ ) (Negri and Veronesi 1989). The dihaploid plant was diploid ( $2n = 2x = 12$ ) and had the *L. tenuis* phenotype.

This report presents some results from anther culture studies using six tetraploid (natural (*L. angustissimus* ( $2n=24$ ), *L. corniculatus* ( $2n=24$ ), *L. emeroides* ( $2n=28$ ), *L. halophilus* ( $2n=28$ )) or synthetic (4x *L. japonicus* ( $2n=24$ ), 4x *L. tenuis* ( $2n=24$ )) *Lotus* species, two of which (4x *L. japonicus*, 4x *L. tenuis*) have not been previously cultured. These *Lotus* genotypes were used because of the theoretically greater chance of recovering haploid (dihaploid) plants from a tetraploid rather than a diploid species.

Flower buds containing anthers with microspores at the uninucleate stage were excised and surface sterilized according to a standard calcium hypochloride disinfection treatment. Anther culture was attempted on solid potato medium (Anonymous 1976) supplemented with 2 mg l<sup>-1</sup> 2,4-dichlorophenoxyacetic acid and 0.5 mg l<sup>-1</sup> kinetin (6-furfurylaminopurine); previous experiments had shown that without exogenous growth regulators, anthers shriveled and turned brown. Cultures were incubated at 25°C under continuous illumination. Only anthers with microspores at the early uninucleate stage were used. In addition, two other experiments were carried out to see the effect of

culture medium. Anthers of *L. corniculatus* genotype B259 were cultured on Murashige and Skoog's MS medium (Murashige and Skoog 1962) and anthers of genotype 84N were cultured on Kao's medium (Kao 1977) supplemented with 3% sucrose and 2 mg<sup>l</sup><sup>-1</sup> benzylaminopurine; incubation was performed in darkness at 23°C.

Microspore embryogenesis in non-callused anthers was examined after four weeks of incubation using a standard acetocarmine procedure. Callus tissues on anthers were subcultured at monthly intervals on solid MS medium supplemented with 3% sucrose, 1 mg<sup>l</sup><sup>-1</sup> kinetin and 0.1 mg<sup>l</sup><sup>-1</sup> naphthaleneacetic acid to promote organogenesis; subcultures were incubated under light at 25°C. Chromosome counts from callus tissues and regenerated plantlets were determined using standard fixation and staining schedules (Darlington and La Cour 1976).

### Results and Discussion

In cultured anthers of *L. halophilus*, microspores with up to five nuclei could be observed; cell walls had not been laid down in these microspores. Bicellular microspores showing two symmetrical cells with equally-stained nuclei were observed in the cultures at a very low frequency (ca 0.1% of the total microspore population); microspores with two asymmetrical cells with differentially-stained nuclei were more frequent (ca 2% of the total microspore population). These microspores were morphologically similar to mature pollen grains found in this species, that is, with a crescent-shaped, darkly staining generative nucleus and a round, more diffuse vegetative nucleus. Multicellular units (Fig. 1) were also observed but at a very low frequency. More advanced stages of development were not seen. However, haploid cells were not found in the callus tissues from the cultured anthers. The observations from the *L. halophilus* cultures indicate that embryoid development had been triggered. *L. halophilus* is a self-compatible species which likely produces microspores with a viable genomic constitution in the dihaploid state. One may at present only speculate as to the factors preventing the differentiation of the embryogenic microspores. Further attempts with this species appear to be warranted. In the other five species, cytological studies provided no evidence for the occurrence of embryonic divisions within the microspores or the presence of haploid cells in callus tissues.

An experiment with *L. corniculatus* genotype B259 was designed to investigate possible inhibitory effects of agar on androgenesis (Kohlenbach and Wernicke 1978). Embryoid induction could not be observed in any of the cultured anthers; profuse callus formation, however, was observed on all solid and liquid media and several callus cell lines were established. Plantlets were regenerated from these cell lines and grown to maturity under greenhouse conditions. One of the regenerants - (BFT-A9) - was derived from a culture on solid medium supplemented with 2 mg<sup>l</sup><sup>-1</sup> benzylaminopurine and 1 mg<sup>l</sup><sup>-1</sup> indoleacetic acid. When both the regenerant and the donor plant were grown under the same environmental conditions, they differed remarkably from one another. The mother plant possessed weak, prostrate stems with long internodes and light green leaves, whereas the regenerant had robust, erect stems with much shorter internodes and darker leaves. The plants presented some difference in the anthocyanin content of their stems and florets; in BFT-A9 a deep-red coloration could be observed on the stems, whereas the donor plant had entirely green stems; anthocyanins were also present on the pedicel and the calyx of the florets of the regenerant but were absent in the donor plant. Differences in the degree of pubescence were also evident; for example, few hairs were present on the calyx of the florets of the regenerant, whereas the pubescence was dense

on the calyx of the florets of the donor; the margins of most leaflets of the regenerant were ciliate, whereas, in the donor, the margins were glabrous.

An examination of squashed root-tip cells from regenerant BFT-A9 revealed a mixoploid condition; a small number of haploid ( $2n=2x=12$ ) and octoploid ( $2n=8x=48$ ) cells were interspersed with tetraploid cells. The donor plant had an uniform chromosome number of 24. This interesting finding indicates that BFT-A9 may have originated from microspore-derived callus tissues that underwent polyploidization, a common feature of cultured tissues (Bayliss 1981).

In the course of several experiments with *L. corniculatus* genotype 84N, one embryo-like structure (Fig. 2) was observed emerging through the wall of one anther. When incubated under a 16-h photoperiod, the "embryoid" rapidly turned green; a shoot was produced (Fig. 3), but all efforts to induce rooting were unsuccessful; the shoot later degenerated. Chromosome counts obtained from cells in the leaf tips showed the normal number of 24 chromosomes for birdsfoot trefoil. The origin of this plant is unclear, since it could have arisen either from somatic tissues or microspore development with subsequent chromosome doubling. This is the first report of embryogenesis from cultured anthers of *Lotus* species.

Anther culture in *Lotus* species has proven a most difficult enterprise. The most promising avenues may be with autotetraploid species that are self-compatible; the inbreeding depression brought about by total homozygosity should not be severe enough to prevent embryogenesis or differentiation of embryoids into plantlets.

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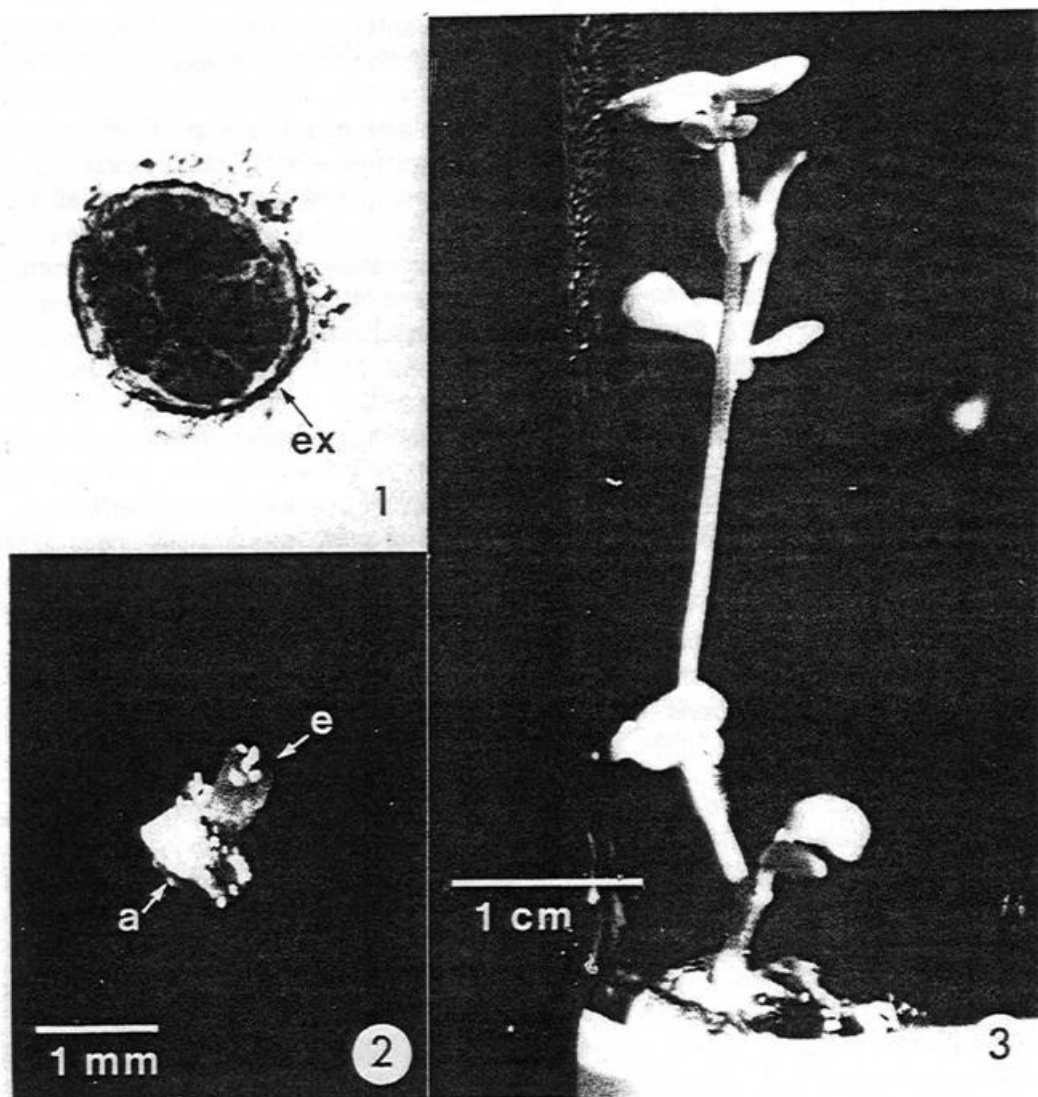


Fig. 1. Multicellular microspore from a *L. halophilus* cultured anther. Ex = exine. Fig. 2. Embryoid-like structure (e) emerging from a *L. corniculatus* (84N) anther (a) cultured on Kao's medium supplemented with 3% sucrose and 2 mg l<sup>-1</sup> benzylaminopurine. Fig. 3. Regenerated plantlet from the embryoid-like structure shown in Fig. 2.