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Evidence for the existence of 2n gametes in *Lotus tenuis* Wald. et Kit. ($2n=2x=12$): their relevance in evolution and breeding of *Lotus corniculatus* L. ($2n=4x=24$)

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Summary. Crosses between male sterile *L. corniculatus* ($2n=4x=24$) and *L. tenuis* ($2n=2x=12$) plants were performed in order to verify the presence of 2n gametes in *L. tenuis*. All but one of the plants from these crosses had $2n=4x=24$ and the *L. corniculatus* phenotype; this plant had $2n=2x=12$ and the *L. tenuis* phenotype. The plants also showed good quantity of pollen at tripping, good pollen fertility and good percentage of seed setting in the backcross to *L. corniculatus*. On the whole, both cytological and morphological observations, showing that all but one of the plants from *L. corniculatus* × *L. tenuis* were normal tetraploids, suggest the existence of diploandrous gametes in *L. tenuis*. On the other hand, haploid parthenogenesis probably gave origin to the dihaploid plant $2n=2x=12$.

Key words: *Lotus corniculatus* – *Lotus tenuis* – 2n gametes – Evolution – Breeding

Introduction

Lotus tenuis Wald. et Kit. (narrow-leaf birdsfoot trefoil) and *Lotus corniculatus* L. (birdsfoot trefoil) are forage legumes of great importance in sowing pastures, not only in continental, but also in sub-mediterranean areas (Veronesi et al. 1984; Grant and Marten 1985; Veronesi and Negri 1985; Arcioni et al. 1985; Negri 1987).

The origin of the above-mentioned species and the taxonomic relationships between them have stimulated a considerable degree of interest and scientific work. Dawson (1941), in his basic work, suggested that *L. corniculatus* is an autotetraploid from *L. tenuis*. This was based upon chromosome number (*L. tenuis* $2n=2x=12$, *L. corniculatus* $2n=4x=24$), morphological characters and tetrasomic inheritance of cyanogenesis in birdsfoot trefoil.

In particular, tetrasomic inheritance, also found in *L. corniculatus* for pubescence, clorophyll deficiency, leaf colour, flower colour, corolla striping, keel tip colour and self-incompatibility (Poostchi and Mac Donald 1961; Buzzel and Wilsie 1963; Bubar and Miri 1965) would suggest an autopolyploid origin. Wernsman et al. (1964), observing meiotic behaviour of *L. corniculatus*, of induced autotetraploids from *L. tenuis*, of their hybrids and of backcrosses of these hybrids to parental species, also found a high degree of homology between *L. tenuis* and *L. corniculatus* chromosomes and gave further support to Dawson's hypothesis. Larsen (1954), considering the close resemblance of *L. alpinus* and *L. corniculatus*, suggested that *L. alpinus* instead of *L. tenuis* was the diploid parent of the autotetraploid *L. corniculatus*.

On the other hand, other studies on the meiotic behaviour of *L. corniculatus*, the phenolic content of several *Lotus* species, their karyotypes and meiotic behaviour, the production of interspecific hybrids and their meiotic behaviour (Harney and Grant 1965; Somaroo and Grant 1971a, b; 1972a, b, c; Chengh and Grant 1973) have tended to support an allopolyploid origin, as already postulated by Stebbins (1950). In particular, Somaroo and Grant (1972a, b) suggested that the most important species in the evolution of *L. corniculatus* were *L. japonicus* and *L. alpinus*. More recently, Ross (1984), on the basis of tannin production, self-incompatibility, phenolic content and *Rhizobium* specificity of several *Lotus* species, virtually excluded the fact that *L. corniculatus* arose through autopolyploidy from *L. tenuis* or *L. alpinus*. He suggested that *L. corniculatus* evolved through interspecific hybridization of *L. alpinus* and/or, less probably, *L. tenuis*, as the female parent, and *L. uliginosus* as the male parent, followed by chromosome doubling of the hybrid. He also suggested that other species of the *L. corniculatus* groups could have contributed to the evolution of the species. Further support of the hypothesis that *L. corniculatus* arose through an allopolyploidization process is given by the disomic inheritance of tannins (Dalrymple et al. 1984).

As to whether auto- or allopolyploidy has played a role in the evolution of *L. corniculatus*, it is probable that this species also arose through a sexual polyploidization process involving 2n gametes. In fact, sexual polyploidization, conferring heterotic responses and wider adaptation, is a much more advantageous process than somatic doubling (Mendiburu and Peloquin 1976). Besides, there is a rapidly expanding detection of 2n gametes in several, non-related species (Satina and Blakeslee 1935; Rhoades and Dempsey 1966; Bingham 1969; Bringhurst and Gill 1970; De Wet and Harlan 1970; Quinn et al. 1974; Myers et al. 1984; Veronesi et al. 1986), which suggests their evolutionary importance. Nevertheless, no report on the presence of 2n gametes exists in diploid *Lotus* species related to *L. corniculatus*.

Assessment of their presence could be very interesting not only to evolutionist botanists, but also to plant breeders, firstly because 2n gametes offer the plant breeder the possibility of maximizing heterozygosity by breeding at diploid level and then restoring the polyploid condition through the union of 2n gametes from hybrid parents, in such a way producing a highly heterozygous and vigorous hybrid (Chase 1963; Bingham 1980). Secondly, because 2n gametes permit unidirectional genetic introgression from wild diploids to the cultivated tetraploids (Bingham 1968; Stanford et al. 1972; Barnes et al. 1977; Den Nijs and Peloquin 1977), a phenomenon also important in the maintenance of genetic structure and variability of natural tetraploid populations (Demalry 1982). This study was conducted with the aim of ascertaining the presence of 2n gametes in *L. tenuis* Wald. et Kit., a probable diploid parent of *L. corniculatus*, which also presents an active winter growth character worthy of being transferred into *L. corniculatus* accessions (Negri and Veronesi 1986).

Materials and methods

In 1983, while a *L. corniculatus* germplasm collection was being evaluated, three male sterile plants (J19, J1 and F20) were detected. These plants, repeatedly evaluated for pollen presence at tripping and percentage of fertility at selfing during the summers of 1983, 1984, 1985 and 1987, never produced pollen or selfed seed. Their chromosome number was always $2n=4x=24$ (Negri 1987; Negri et al. 1989).

The detection of tetraploid male sterile plants suggested the opportunity of easily verifying the compatibility of the *L. corniculatus* × *L. tenuis* cross, and the eventual presence of 2n gametes in *L. tenuis*. Since the male steriles never produced selfed seed, in the *L. corniculatus* (male sterile) × *L. tenuis* cross all the seed collected from *L. corniculatus* should have come from the cross. Subsequent observations of morphology, fertility and chromosome number of the plants obtained from this cross should have given information on their ploidy level. If coming from the union of normal gametes ($n=12$ for *L. corniculatus* and $n=6$ for *L. tenuis*), they should have been triploid and probably shown limited fertility. On the contrary, if coming from the union of normal *L. corniculatus* gametes and 2n *L. tenuis* gametes, they should have been tetraploid and shown normal fertility.

In order to verify the above mentioned hypothesis, in September 1984, the male sterile plants were cloned and the clones were grown in the greenhouse during autumn and winter 1984. In March 1985, 16 ramets for each male sterile clone were transplanted in alternate rows in the experimental field with the same number of randomly taken plants of *L. tenuis* from a living germplasm collection. Each set of 16 ramets for each clone was enclosed under an isolation cage and small honeybee cages were introduced when plants reached full bloom stage (June). Legumes were collected on male sterile plants at maturity. Small quantities of seed were collected: 0.012 g on male sterile J1, 13.4 g on J19 and 0.060 g on F20. A small part of the seed collected in each of the three isolation cages was immediately scarified and germinated in petri dishes under optimal conditions.

From each cross, the following numbers of plants were observed: 13 from J19 × *L. tenuis*, 7 from J1 × *L. tenuis* and 4 from F20 × *L. tenuis*. In order to determine their ploidy level, they were grown in the greenhouse during autumn and winter and then cloned to have a sufficient number of root tips per plant. The following procedure was utilized to determine chromosome number of each: small root tips, 4–8 mm long, were detached, treated in 8-hydroxyquinoline for 3 h and then fixed in 3:1 ethanol-acetic acid overnight. Root tips were then stained using the Feulgen-technique. Countings were made on at least ten metaphases per plant which had well-spiralized and separate chromosomes.

On each genotype the following estimates of fertility were also taken: (1) Quantity of pollen at tripping, determined by scoring each floret of at least three inflorescences from 0 = no visible pollen to 5 = maximum amount of pollen. (2) Pollen fertility, determined by evaluating the presence of acetocarmine-stainable pollen. Four flowers were randomly selected to make a slide and at least 1000 pollen grains were classified for fertility. Well-stained, plump, symmetrical pollen grains were considered to be fertile; poorly stained, shrunken, irregular ones were considered to be sterile. Percentage of pollen fertility was then calculated. (3) Percentage of seed setting in the back-cross to *L. corniculatus*. A minimum of six inflorescences for a total of at least 24 flowers per plant, was crossed by hand, utilizing a pollen pool obtained from five *L. corniculatus* genotypes with abundant pollen production and a pollen fertility percentage above 85%. Percentage of seed setting was calculated as ratio between seeds obtained and flowers pollinated.

Results and discussion

L. tenuis is confirmed to be related to *L. corniculatus* since the cross between these two species appears to be possible under conditions similar to natural ones. However, this study did not permit the amount of genetic flow to be quantified. The different seed set observed in genotypes J19, J1 and F20 was probably due to different incompatibility alleles operating in the cross with *L. tenuis* genotypes.

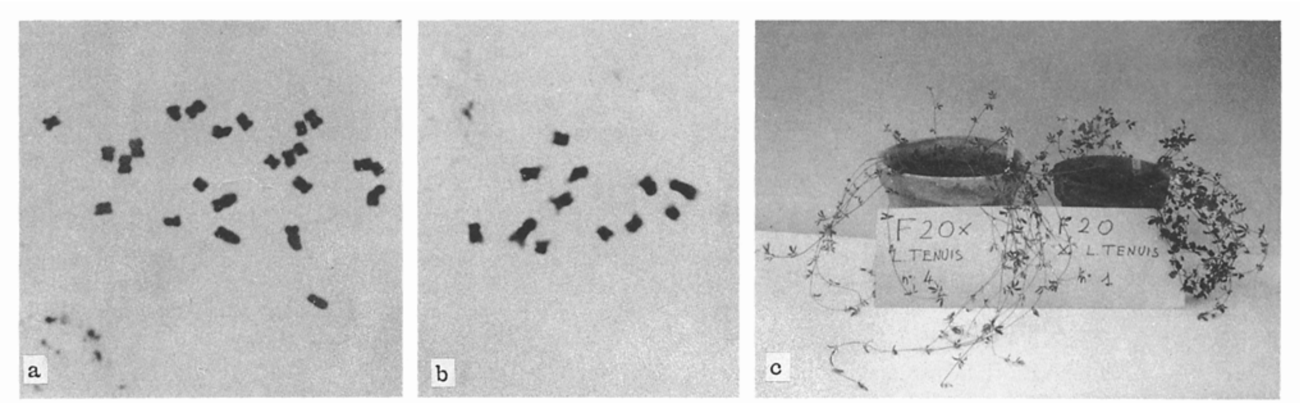

Fig. 1. **a** Mitotic metaphase of plant J1 × *L. tenuis* no. 6 ($2n = 4x = 24$); **b** Mitotic metaphase of plant F20 × *L. tenuis* no. 4 ($2n = 2x = 12$); **c** Di-haploid plant F20 × *L. tenuis* no. 4 on the left and normal tetraploid plant F20 × *L. tenuis* no. 1 on the right; note the *L. tenuis* phenotype of the former

Table 1. Fertility estimates of plant obtained from *L. corniculatus* (4x) × *L. tenuis* (2x) crosses: average quantity of pollen at tripping (0 = no pollen, 5 = maximum amount of pollen), percentage of fertile pollen, and total number of pollinated flowers, total number of seeds obtained, average seed setting per pollinated flower, maximum seed setting per pollinated flower in backcrosses to *L. corniculatus*

Plant		Average quantity of pollen at tripping (0–5)	Percentage of fertile pollen	Total no. of pollinated flowers	Total no. of seed obtained	Average seed setting per pollinated flower	Maximum seed setting per pollinated flower
J19 × *L tenuis* no.	1	5.0	67.1	74	92	1.24	5.40
	2	5.0	82.4	31	349	11.26	23.67
	3	3.5	77.6	113	654	5.79	11.25
	4	3.0	61.3	*	*	*	*
	5	3.5	82.7	44	346	8.65	10.80
	6	3.9	89.2	57	465	8.16	21.00
	7	3.9	76.0	54	0	0.00	*
	8	0.0	*	24	76	3.17	11.50
	9	4.5	97.2	65	409	6.29	15.30
	10	4.0	70.1	41	165	4.02	9.00
	11	2.9	82.1	53	487	9.19	18.00
	12	4.0	67.5	43	217	5.05	8.30
	13	4.3	98.3	46	78	1.69	7.00
J1 × *L tenuis*	1	3.0	64.2	31	31	1.00	5.75
	2	3.9	97.0	49	6	0.12	2.00
	3	4.1	47.3	*	*	*	*
	4	3.7	96.8	43	0	0.00	0.00
	5	*	*	*	*	*	*
	6	3.4	97.1	50	182	3.64	13.50
	7	3.5	37.5	28	2	0.07	1.00
F20 × *L tenuis*	1	5.0	93.4	48	45	0.94	6.00
	2	4.6	*	*	*	*	*
	3	*	86.9	46	90	1.96	12.66
	4	4.0	98.2	49	6	0.12	1.00

* not recorded

All but 1 of the 24 plants from *L. corniculatus* 4x × *L. tenuis* 2x were tetraploid (Fig. 1a). This plant, F20 × *L. tenuis* no. 4, had a somatic number of chromosomes $2n = 2x = 12$ (Fig. 1b). The lack of triploids in examined materials shows the existence of an effective triploid block also in *L. corniculatus*. As already pointed out in several species (Brink and Cooper 1947; Hanneman and Peloquin 1968; Von Wangeheim et al. 1960; Johnston et al. 1980), it probably operates in interploid crosses, eliminating almost all triploid embryos due to endosperm imbalance.

As far as morphological characters of the examined plants are concerned, they were very vigorous and resembled the tetraploid *L. corniculatus*, except for F20 × *L.*

tenuis no. 4, which had linear-lanceolate leaflets and longer internodes and looked like the diploid *L. tenuis* (Fig. 1 c).

At least one fertility estimate was made for each plant (Table 1), with the exception of J1 × *L. tenuis* no. 5, which died early on.

The average quantity of pollen at tripping ranged from 0.0 to 5.0 but, in general, was rather abundant; the average score was higher than 3.0 in 18 out of 22 plants.

Percentage of fertile pollen was generally high, ranging from 37.5 to 98.3%; 19 out of 20 plants showed values higher than 60.0% and 12 higher than 80.0%.

Finally, average percentage of seed setting in backcrosses to *L. corniculatus* ranged from 0.07 to 11.26 seeds per pollinated flower, but at least one flower within each fertile cross combination was characterized by seed set much higher than the average value of the plant. Two plants failed to produce any seed (J19 × *L. tenuis* no. 7 and J1 × *L. tenuis* no. 4); nevertheless, it is worth noting that these plants presented good values with regard to the other two fertility estimates.

Plant F20 × *L. tenuis* no. 4 ($2n=2x=12$) always showed good fertility indices and, in particular, appeared to be fertile when backcrossed to *L. corniculatus,* though the number of seeds obtained was low (6). Fertility indices of this plant in the cross with *L. corniculatus,* ploidy level of its progenies and its pollen characteristics will be further verified.

On the whole, both cytological and morphological observations, showing that all but one of the plants from *L. corniculatus* × *L. tenuis* were normal tetraploids, suggest the existence of diplandrous gametes in *L. tenuis.* Without cytological confirmation, we cannot exclude that apomixis has played a role. Nevertheless, the existence of differences both in phenotypic appearance and fertility indices among plants within a cross, though not statistically verified, does not give evidence to apomixis. Besides, it seems improbable that apomixis could have simultaneously occurred in three different genotypes, while the existence of 2n gametes as a remnant of old evolutive processes or as a means of actual evolutive processes is a much more feasible alternative hypothesis.

Haploid parthenogenesis, on the other hand, appears to have given origin to the di-haploid plant F20 × *L. tenuis* no. 4. The occurrence of this phenomenon in interploid crosses has already been described and used as a method of obtaining haploids in alfalfa and potato (Hougas et al. 1958; Bingham 1971).

Conclusion

L. tenuis appears to be involved in the evolution of *L. corniculatus* and, due to the presence of 2n gametes, the transfer of positive characters from one species to the other appears to be easy. The finding of 2n gametes, if cytological analysis would show the existence of meiotic mutants producing 2n gametes at high frequencies, would also suggest the possibility of maximizing heterozygosity in birdsfoot trefoil through breeding at the diploid level, restoring the tetraploid condition later.

In any case, the feasibility of such a breeding procedure is a long way off. First, it is necessary to assess the effective weight that heterozygosity has on vigor in this species since few data are available at the moment (Negri 1987), then to find genotypes producing diploandrous and possibly diploginous gametes through an unreductive process and, finally, to increase their production through selection.

It is also necessary to assess whether analytic breeding can be used in manipulating genes conditioning important quantitative traits in birdsfoot trefoil. Some data on another important forage crop, alfalfa, suggest that it may not be possible to improve quantitative traits via analytic breeding scheme (Groose et al. 1988).

Presently plants of *L. tenuis* from the isolation cages have been cloned and will be evaluated by hand pollination crosses in order to assess if some of the randomly chosen genotypes produce 2n gametes at high frequency. Then, analysis of meiosis will be carried out on these plants in order to assess the mechanism of production of diploandrous gametes.

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