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Variation in a single protoplast- and seed-derived population of *Lotus corniculatus* L.

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Summary. *Lotus corniculatus* L. is a widely cultivated, outbreeding, leguminous forage crop. Seventy-one plants, most of which were tetraploid, were regenerated from calli derived from a single protoplast. Their morphological and agronomic traits were evaluated and compared with those of the seed-produced population. The variances of most of the traits in the protoplast-derived (protoclonal) population were smaller than those of the seed-produced population. Mean values of all the traits of the protoclonal population shifted significantly towards lower values. However, new phenotypic variants with higher values than those of the plant initially used for protoplast isolation were also observed. Plants with less hydrocyanic acid (which has a toxic effect on cattle) than the initial plant were obtained in the protoclones. Generally, the pollen fertility of protoclones was significantly low compared with the seed-produced plants. This seems to be partly due to the occurrence of abnormalities in chromosome structure during protoplast and/or callus culture, as suggested by the formation of univalents, lagging, and fragment chromosomes and bridges at metaphase I and anaphase I and II of the regenerants. The changes in chromosome structure, however, did not induce any malformed morphologies.

Key words: Birdsfoot trefoil – *Lotus corniculatus* – Protoclone – Somaclonal variation – Chromosome structural changes

Introduction

Plants regenerated from tissue or cell culture are considered to be the clones of the tissue or cell donor. Generally, variation is observed among the clones in one or more traits, which was termed somaclonal variation by Larkin and Scowcroft (1981). Somaclonal variation has been found in forage legumes regenerated from cell cultures (Groose and Bingham 1984; Johnson et al. 1984; Bingham and McCoy 1986). This variation may be used in breeding programs because the variants often occur at higher frequencies than from chemical mutagenesis (Gavazzi et al. 1987). *Lotus corniculatus* L. (birdsfoot trefoil, $2n = 4\times = 24$) is an outbreeding leguminous forage crop. Its regenerants have proved to be suitable for the evaluation of somaclonal variation in morphological and agronomic traits, and for comparison with those of a seed-produced population (Damiani et al. 1985).

The purpose of this work is to evaluate morphological and agronomic traits found in the protoplast-derived regenerant population, in which preexisting variation in explants was excluded by initially using a single protoplast. The variation was also compared with that of a seed-produced population. In addition, the abnormality of chromosome structure in the regenerants was studied.

Materials and methods

Lotus corniculatus seeds from a natural population of cv 'Viking' were germinated on the medium of Nitsch and Nitsch (1969) without growth regulators. The calli were induced from hypocotyls of 10-day-old seedlings using the same medium and method of Niizeki and Saito (1986). The same seedlings, from which hypocotyls were partly cut, were placed again on the medium of Nitsch and Nitsch (1969) without growth regulators, to induce roots, and then transferred to pots. A solution containing 4% Cellulase Onozuka RS, 1% Macerozyme R-10, 0.2% Pectolyase Y-23, and 0.7 *M* mannitol (pH 5.8) was used for the protoplast isolation. The calli cultured for 2 months were used in this experiment. The enzyme-callus mixture was incubat-

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ed at 28 °C for 3 ~ 4 h in a shaker bath (60 shakes/min). The protoplasts were separated from undigested cell clumps by filtering through eight layers of cheesecloth. The enzyme was removed by four successive washings with 0.7 *M* mannitol (pH 5.8) with centrifugation at $80 \times g$ for 4 min each. The isolated protoplasts (1×10^4/ml) were cultured in a thin layer of a modified 8p medium (Kao and Michayluk 1975), containing 0.5 mg/l BA instead of zeatin, and without coconut water. The medium was solidified with 0.6% agar. The culture dishes were kept at 25 °C under light. Less than 0.1% of the protoplasts initiated division within 7 days. Divided, single protoplasts grew to form cell clusters consisting of 20 ~ 30 cells. After 1 month they developed into globular colonies. We could detect the colonies derived from single protoplasts by continuous observation using an inverted microscope. The colonies were transplanted into the medium that was used for the initial callus induction. The induced callus lines, all of which were derived from single protoplasts, were transplanted to MS medium (Murashige and Skoog 1962) with 1.5 mg/l indole-3-acetic acid (IAA) and 1.5 mg/l 6-benzyladenine (BA). One of the callus lines that produced numerous shoots was used in this experiment, and their shoots were transplanted to the medium of Nitsch and Nitsch (1969), without growth regulators, for the formation of complete plantlets. The plantlets were transferred to 15×13 cm pots. Thus, a protoclonal population was obtained from a single protoplast. To count the number of chromosomes, root tips were pretreated with 0.002 *M* hydroxyquinoline for 4 h, then fixed in an ethanol-acetic acid mixture (3:1 v/v). The determination of chromosome numbers was carried out by squashing root tips that were stained with alcoholic hydrochloric acid-carmine (Snow 1963). In each plant, at least four root tips were examined to determine its chromosome numbers.

At the time of the protoclone acclimation, a natural population from seed-derived plants was raised by planting seeds in pots. The protoclonal plants were transplanted to pots about 2 weeks later than the seedlings in order to synchronize their growth with that of the seed-derived plants.

At the first flowering in the 2nd year, the following characters were evaluated for 60 plants selected randomly from each seed- and protoplast-derived population: (1) plant height, (2) length of the longest internode, (3) diameter of the longest stem, (4) length and width of the leaflet, (5) dry matter yield, (6) pollen fertility, and (7) hydrocyanic acid (HCN) content. HCN content was analyzed according to the procedure of Grant and Sidhu (1967).

Flower buds were collected for meiotic analyses. They were fixed in an ethanol-acetic acid mixture (3:1 v/v) for 24 h and stored in 70% alcohol until use. Anthers were smeared in a drop of aceto-carmine. Meiotic analysis was carried out with pollen mother cells (PMCs) of 16 protoclonal plants and two seed-derived plants.

Results

The regenerated plants of *Lotus corniculatus* originated from a single protoplast mostly showing 24 chromosomes, indicative of their tetraploid nature. Among 71 regenerated plants, there was only one octoploid and one mixoploid plant, which had cells with 24 (tetraploid) and 48 (octoploid) chromosomes. No aneuploids were observed and chromosome structural changes were not detected under a light microscope.

Figure 1 shows the frequency and distribution of each trait. The octoploid and mixoploid plants were excluded.

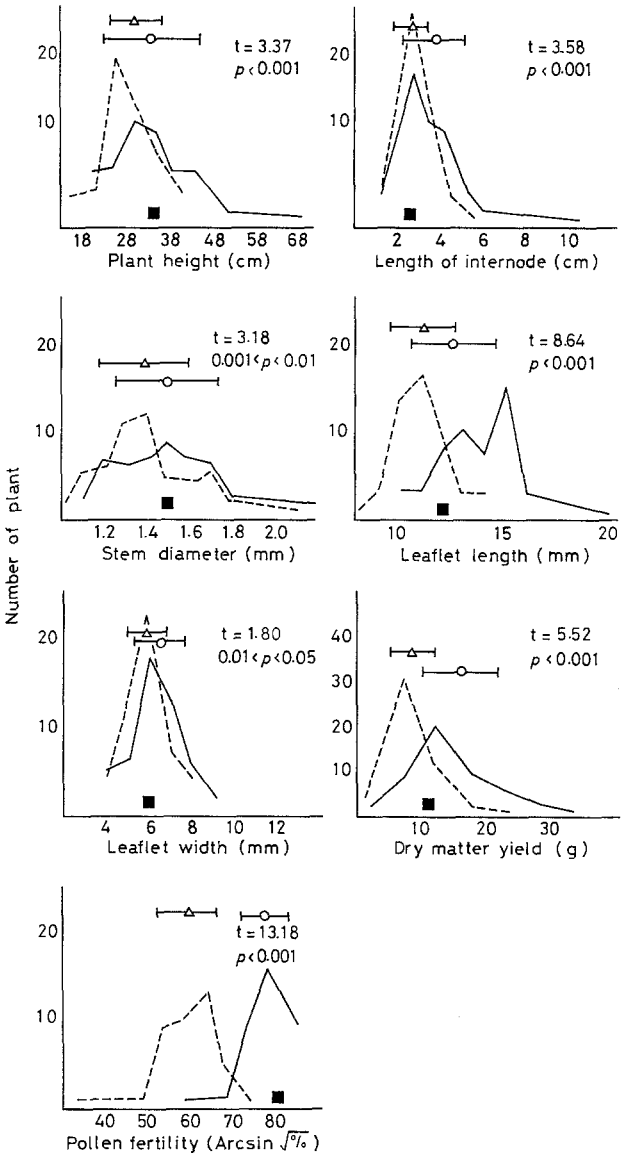

Fig. 1. Variation on seven traits of seed-derived (——) and single protoplast-derived (- - -) populations. ⊢○⊣ and ⊢△⊣, mean value and standard deviation of seed- and protoplast-derived populations, respectively. ■, the value of the plant initially used for protoplast isolation

Table 1. Number of plants having HCN, graded into five concentrations from −(minimum) to ++++(maximum)

Grade of HCN concentration	Seed-derived population	Single protoplast-derived population
−	8 (13.3%)	0 (0%)
+	11 (18.3%)	1 (1.7%)
++	13 (21.7%)	5 (8.3%)
+++	18 (30.0%)	19 (31.7%)
++++	10 (16.6%)	35 (58.3%)
Total	60	60

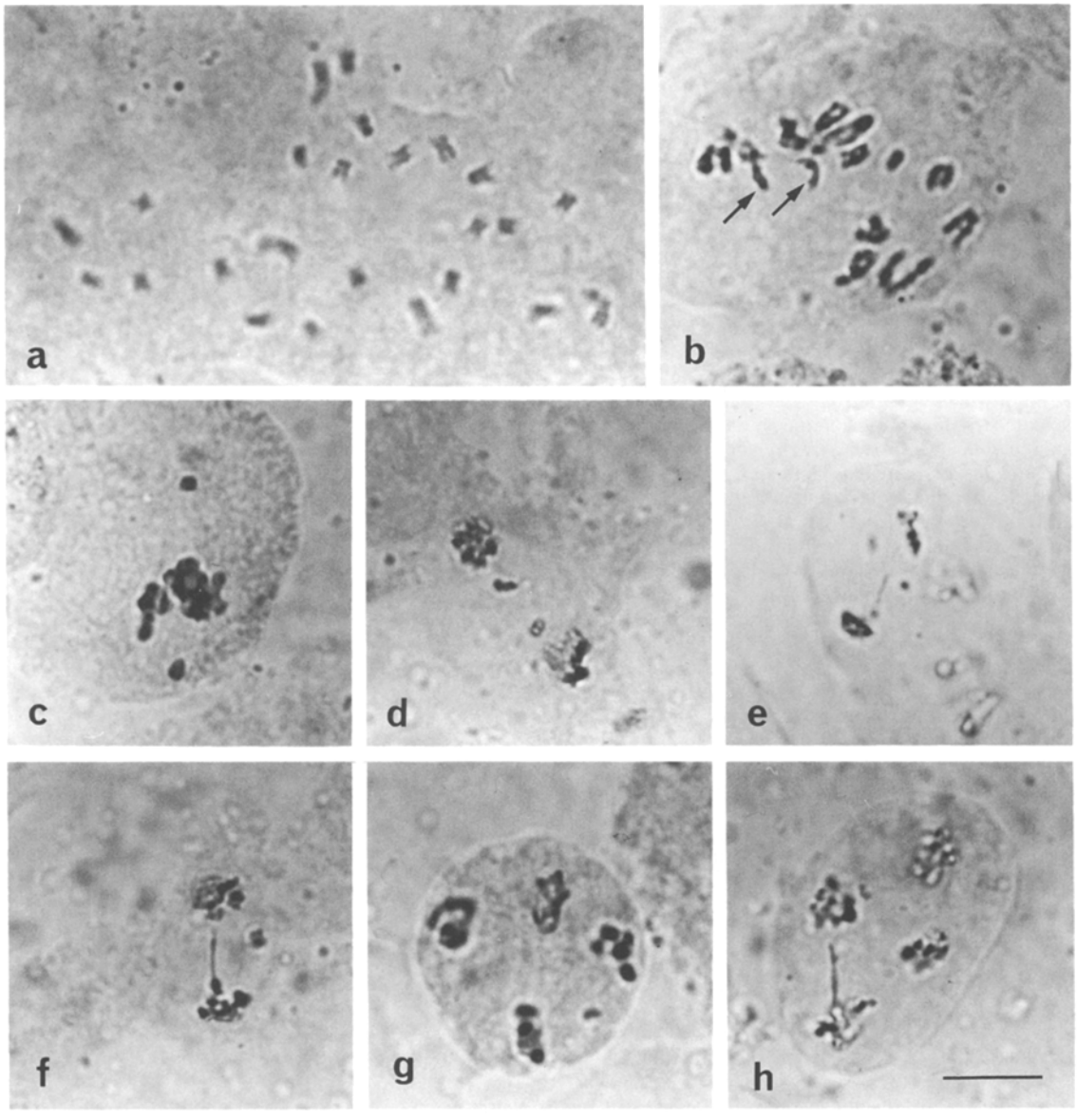

Fig. 2a–h. Mitosis and meiosis in the protoclonal plants. **a** Somatic chromosomes at metaphase of a protoclone in which no chromosome structural changes could be detected, although irregular chromosome configurations were observed in meiosis. **b** Two univalents at diakinesis (indicated by *arrows*). **c** Two univalents at metaphase I. **d** Two lagging chromosomes at anaphase I. **e** A bridge and an acentric fragment chromosome at anaphase I. **f** A bridge and an undivided, lagging bivalent at anaphase I. **g** A lagging chromosome at anaphase II. **h** A bridge at anaphase II. Bar represents 10 μm

The distribution of each character of the protoclonal population is shifted towards lower values than that of the seed-derived population. Mean values of all traits of the protoclonal population were smaller than those of the seed-derived population. The standard deviations of the protoclonal population were also generally smaller than those of the seed-derived population. Protoplast-derived variants with higher values than the highest value of the seed-derived population were not found for any of the traits. However, in all traits except pollen fertility, there were a considerable number of plants with values exceeding those of the initial plant that provided callus for the protoplast isolation. With regard to pollen fertility, the protoclonal population was extremely low and no plant had higher pollen fertility than the plant initially used for protoplast isolation.

The distribution of HCN content in the seed-derived population ranged from maximum to minimum (Table 1). On the other hand, in the protoclonal population, which was derived from a plant with the maximum HCN content, the distribution was narrow, although some variants with low HCN content were observed.

Table 2. Meiotic configurations[a] and pollen fertility of seed- and protoplast-derived plants

Plants	Metaphase I			Anaphase I					Anaphase II					Pollen fertility (%)
	No. of cells examined	A	B	No. of cells examined	A	C	D	E	No. of cells examined	A	C	D	E	
Seed-derived plant														
S10	97	98	2	50	100				28	100				98.7
S59	50	100		63	97	3			20	100				93.3
Protoplast-derived plant														
P85	60	100		71	93	4	3		41	95	5			83.6
P293	67	100		94	97	2	1		60	100				78.2
P277	32	97	3	29	100				56	100				77.8
P303	119	63	37	34	79	21			104	77	23			72.5
P285	24	67	33	31	84	16			27	74	25			77.3
P32	24	58	42	51	77	23			32	63	38			76.8
P298	34	65	35	57	82	18			49	76	25			76.0
P308	67	60	40	100	74	19	4	3	74	68	19	10	4	72.0
P316	49	57	43	38	47	42	8	3	43	54	35	7	5	64.1
P306	36	39	61	47	30	64	6		28	7	72	14	7	58.2
P296	54	91	9	39	82	10	5	3	53	85	15			70.9
P241	61	77	23	67	70	25	5		45	71	29			53.5
P312	36	56	44	23	61	35		4	36	11	89			68.1
P297	83	68	32	67	69	27	4		33	70	30			69.8
P318	60	42	58	57	58	37		5	27	44	44	4	7	70.6
P299	57	77	23	69	81	15	4		44	80	16	5		69.7

[a] Percentages of the cells of normal (A), or with univalent (B), fragment or lagging chromosome (C), bridge (D), and bridge and fragment or lagging chromosome (E)

In meiosis of the seed-derived plants, a very small number of PMCs showed abnormalities such as the univalent at metaphase I and lagging chromosomes at anaphase I (Table 2). Most of the protoclones, on the other hand, showed a high frequency of meiotic abnormalities, although we could not detect abnormal somatic chromosomes under a light microscope (Fig. 2). The abnormal chromosome set that appeared most frequently at metaphase I generally contained one or two univalents. At diakinesis, asynaptic chromosomes were also occasionally observed. These may have occurred due to deletions or translocations.

In anaphase I and II, bridges and fragments were frequently observed. These may have arisen from a crossover within the inversion. The frequencies of bridges and fragments varied among the protoclones. Besides these chromosome alterations, lagging chromosomes were frequently observed at anaphase I and II. Occurrence of these abnormal chromosome configurations at meiosis seemed to be one of the causes of the decrease in pollen fertility. Indeed, higher frequencies of chromosome abnormalities tended to relate to lower pollen fertility (Table 2).

Discussion

The cultivar 'Viking' of *Lotus corniculatus* produces calli from protoplasts with high potential for regeneration through adventitious buds (Niizeki and Saito 1986). Therefore, an investigation on somaclonal variation in a large protoplast-derived population could be carried out to determine the stability of such a population.

The ploidy level of the regenerated plants was substantially stable, which suggests that the morphogenetic ability of cells with altered chromosome numbers might be very low. The absence of aneuploids among regenerated plants supports this assumption. The presence of a mixoploid plant could result from endoreduplication during the formation of adventitious buds.

In an extensive study of *Lotus corniculatus*, Damiani et al. (1985) indicated that somaclonal variation took place in callus-derived plants. In agreement with this, our results showed somaclonal variation among regenerated plants. Most traits of the plant used for protoplast isolation were near the means of the seed-derived population. However, the presence of regenerant plants showing better performance of the traits than the initial plant suggests that tissue culture can produce useful variants, which may be useful for breeding programs of this crop.

In regenerated oat plants from cultured tissues, the most common cytogenetic alteration was chromosome

breakage, followed by loss of a chromosome segment in somatic chromosomes (McCoy et al. 1982). In cultured cells of *Haplopappus gracilis*, whole or partial chromosome loss in the form of lagging chromosomes, micronuclei, ring and fragment chromosomes in mitosis is reported (Ashmore and Shapcott 1989). In wheat × rye hybrid deletions, translocations and amplifications of heterochromatin in rye chromosomes are confirmed by C-banding analysis (Lapitan et al. 1984). Nishibayashi and Kaeriyama (1986) reported that the number and structure of somatic chromosomes in rice plants regenerated from cultured cells were stable under a light microscope, even when the plants showed morphological variation. Gill et al. (1987) observed inversions and chromosomal translocations in the analysis of pachytene, metaphase I, and anaphase I and II in mesophyll protoplast-derived, tetraploid potatoes. The meiotic analysis in the present study also gave evidence for the existence of small structural alterations in the chromosomes of many protoclones. Thus, the presence of somaclonal variants with undetected chromosome structural changes is suspected in the protoclones of *Lotus corniculatus* as well.

In the present material, the observed chromosomal alterations are not accompanied by any drastic phenotypic changes such as teratogenic morphology. A similar case is reported in potato by Secor and Shepard (1981). The quantitative alteration of various characters observed in these studies is probably caused by polygenic changes as well as minor structural alterations in somatic chromosomes. By using a single protoplast-derived population, the present investigation demonstrated that chromosome structural alterations and polygenic changes have occurred during the course of protoplast and callus culture.

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