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Nuclear Cytology of Callus and Plantlets Regenerated from Pea (*Pisum sativum* L.) Meristems

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Summary

The chromosomal status of calli and plantlets regenerated from *Pisum sativum* shoot apical meristems was studied. Chromosome mosaicism (aneusomaty) occurs during callus induction and proliferation, mostly owing to nuclear fragmentation prior to mitosis in the first days of culture. Plantlets regenerated from calli are diploid or aneusomatic, but a selective advantage of diploid cells (diplontic selection) takes place with plantlet growth. The results are discussed in relation to the possibility of inducing chromosomal and/or genetic variability by using meristematic tissues as explants.

Keywords: Callus; Chromosome mosaicism, Meristem culture, *Pisum sativum*, Plant regeneration.

1. Introduction

Although a considerable amount of literature has been published on plant regeneration through cell and tissue culture, difficulties are still being encountered for *in vitro* regeneration of many plant species. Differences in response may be observed from genotype to genotype within the same species (KEYES *et al.* 1980, DIETERT *et al.* 1982, NATALI and CAVALLINI 1987). Screening of nutrient media with the aim to achieve "totipotent" cultures has met only with limited success. Therefore, many workers resorted to selecting immature tissue explants, which often proved to show a great morphogenetic ability.

As to the important legume *Pisum sativum* L., regeneration was obtained only from calli originated from immature tissue explants, *i.e.* vegetative shoots (GAMBORG *et al.* 1974, KARTHA *et al.* 1974), young epycotyls (MALMBERG 1979), immature leaflets (MROGINSKI and KARTHA 1981), immature embryos (NATALI and CAVALLINI 1987).

Callus formation prior to plant regeneration is a potential source of genetic instability, especially when differentiated tissues are used as explants (cf. D'AMATO 1985). Little information is known on genetic and/or cytological conditions of calli and regenerated plants from immature tissues. In the pea, RUBLUO *et al.* (1984) indicated genetic stability in regenerants from callus culture of immature leaflets.

In this paper we report the results of a cytological study on callus formation and plantlet regeneration from pea shoot apices, that consist of meristematic cells.

2. Materials and Methods

In vitro culture

In vitro culture of vegetative pea shoots was performed according to GAMBORG *et al.* (1974). Seeds of *Pisum sativum* L., cv. "Dolce Provenza" and line "5075" (kindly supplied by Prof. L. M. MONTI, Centro di Miglioramento Genetico delle Piante Orticole, C.N.R., Portici, Italy) were surface sterilized with 2% sodium hypochlorite for 20 min and then rinsed twice with sterile distilled water. Seeds were germinated on hormone-free MURASHIGE and SKOOG (1962) (MS) medium solidified with 0.8% agar.

When shoots were 3–4 days (d) old, vegetative apices were excised and mechanically macerated under sterile conditions with a scalpel in a drop of hormone-free MS medium under a dissection microscope. The explant was composed of the apical dome plus 3 to 4 leaf primordia. The cell masses were placed on solid MS medium supplemented with 3% sucrose, 0.5 mg/l 6-benzylaminopurine (BAP) and 0.2 mg/l napthaleneacetic acid (NAA) to promote callus for-

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Table 1. *Chromosome counts in calli from P. sativum shoot meristems (cv. "Dolce Provenza") after different times of culture on MS medium containing 0.5 mg/l BAP plus 0.2 mg/l NAA*

Days of culture	No. of mitoses	Percentage of cells in each chromosome number class																			
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	...	27	28
0	35	—	—	—	—	—	—	—	100.0	—	—	—	—	—	—	—		—	—
2	67	1.5	—	—	—	3.0	6.0	3.0	83.6	3.0	—	—	—	—	—	—		—	—
4	44	—	—	—	—	—	4.5	4.5	88.6	2.3	—	—	—	—	—	—		—	—
6	50	—	—	—	—	—	4.0	6.0	82.0	6.0	2.0	—	—	—	—	—		—	—
8	50	4.0	—	—	—	—	2.0	8.0	78.0	4.0	—	—	—	—	—	—		—	4.0
12	50	2.0	—	—	—	2.0	—	4.0	86.0	4.0	—	—	—	—	—	—		—	2.0
16	50	—	—	—	—	—	—	2.0	76.0	—	4.0	—	—	—	2.0	—		—	16.0
20	49	—	—	—	—	—	—	2.0	63.3	2.0	2.0	—	—	—	—	—		—	30.6

Table 2. *Chromosome counts in calli from P. sativum shoot meristems ("5075" line) after different times of culture on MS medium containing 0.5 mg/l BAP plus 0.2 mg/l NAA*

Days of culture	No. of mitoses	Percentage of cells in each chromosome number class																			
		7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	...	27	28
0	32	—	—	—	—	—	—	—	100.0	—	—	—	—	—	—	—		—	—
2	46	—	—	2.2	—	—	2.2	4.3	80.4	2.2	—	—	2.2	—	—	—		—	6.5
4	36	—	—	—	—	2.8	—	5.6	80.5	2.8	2.8	—	—	—	—	—		—	5.6
6	40	—	—	—	—	—	5.0	2.5	77.5	7.5	—	—	—	—	—	—		—	7.5
8	34	—	—	—	—	—	5.9	5.9	76.5	2.9	—	—	—	—	—	—		—	8.8
12	50	—	—	—	2.0	—	2.0	2.0	72.0	4.0	4.0	—	—	—	—	—		—	14.0
16	50	4.0	—	—	—	—	—	6.0	72.0	6.0	2.0	2.0	—	—	—	—		—	8.0
20	50	4.0	2.0	—	2.0	—	—	6.0	70.0	2.0	2.0	—	—	—	—	—		—	12.0

mation. The pH of the medium was 5.7. The explants were kept in a temperature controlled room, maintained at 25 ± 1°C under cool white fluorescent light of 2,000 lux, using a light and dark cycle of 16/8 hours (h).

After 20 d of culture, multiple adventitious shoots developed on the callus surface. These shoots were then excised from callus and cultured on the same medium in order to achieve plantlet development. Regeneration was tested using at least 40 explants in each experiment.

Cytological Analyses

For caryological and cytophotometric analyses, vegetative shoots, portions of callus at different growth stages (up to 20 d of culture) and adventitious shoot apices in two different developmental stages (1–2 cm and 4–5 cm long) were collected.

For chromosome counts the material was pretreated with 0.2% colchicine (Sigma) at 25°C for 4 h and then fixed in ethanol-acetic acid 3:1 (v/v). Small pieces of callus and vegetative apices were hydrolyzed in N HCl at 60 °C for 7 min, stained in 0.5% fuchsin-Schiff at room temperature for 1 h and squashed under a cover-slip in a drop of 45% acetic acid. The cover-slips were removed by the dry-ice method and slides were dehydrated and mounted with DPX (BDH) mountant.

For DNA cytophotometry, vegetative apices and small samples of callus were fixed in ethanol-acetic acid 3:1 (v/v), treated with a 4% solution of pectinase (Sigma) for 30 min at 38 °C, squashed under a cover-slip in a drop of 45% acetic acid. After removal of the cover-slips, the slides were simultaneously hydrolyzed in N HCl at 60 °C for 7 min and then stained in 0.5% fuchsin-Schiff for 1 h at room temperature. After three 10 min washes in SO_2 water, the slides were dehydrated and mounted as above. The amount of DNA *per* nucleus was measured by a Barr & Stroud integrating microdensitometer, type GN5, at the wavelength of 550 nm.

3. Results

In vitro culture of macerated apices allowed a very high percentage of regenerants from callus: 60.0% and 45.9% for the cv. "Dolce Provenza" and the "5075" line, respectively.

In Tables 1 and 2 are reported the chromosome counts in calli of cv. "Dolce Provenza" and "5075", respectively, during callus induction and proliferation. All metaphases in the explant (0 d) were diploid (2 n = 14). In the first phases of callus induction already, in addition to a majority of diploid cells, aneuploid (hypo- and hyperdiploid) cells and a few cells with 7 chromosomes ("haploids") were present. After 20 d of cul-

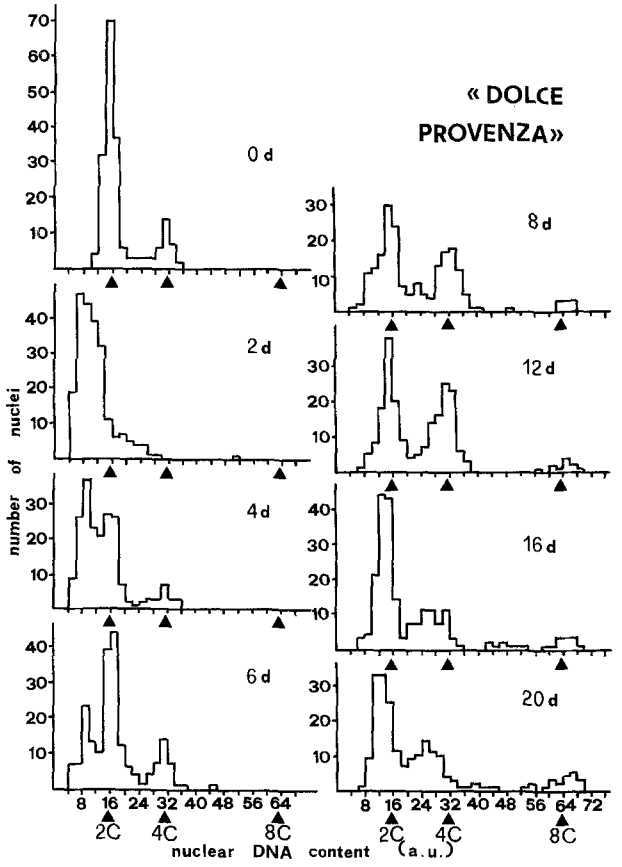

Fig. 1. Nuclear DNA content (arbitrary units) in cells of calli from *P. sativum* shoot apical meristems (cv. "Dolce Provenza") after different times (days-d) of culture on MS containing 0.5 mg/l BAP plus 0.2 mg/l NAA. Nuclear DNA content of cells of the initial explants are given (0 d). The 4C value is calculated on Feulgen mean absorption of early prophases in shoot apices *in vivo*; 2C and 8C values are calculated on 4C value (1/2x and 2xn respectively)

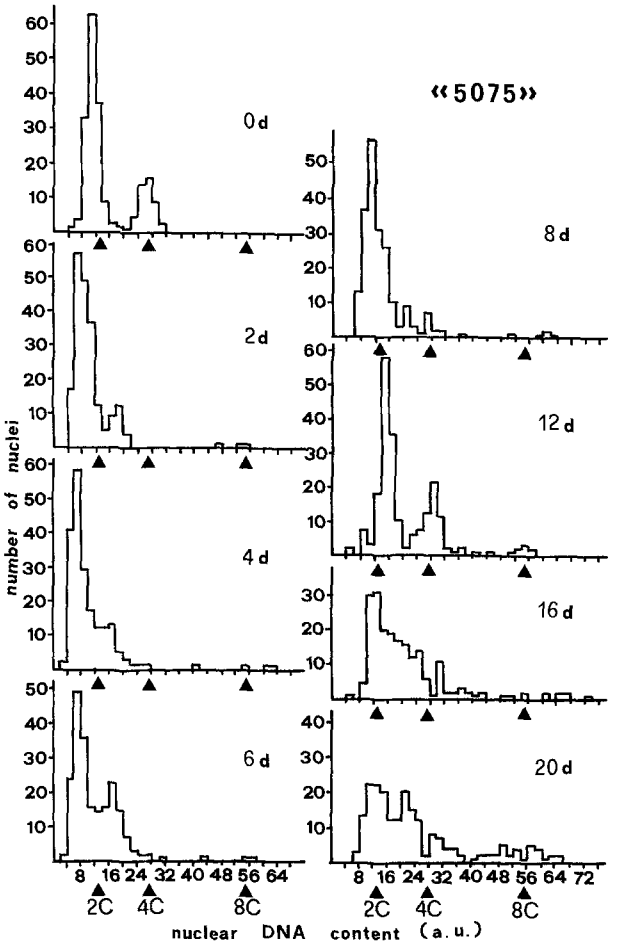

Fig. 2. Nuclear DNA content (arbitrary units) in cells of calli from *P. sativum* shoot apical meristems ("5075" line) after different times (days-d) of culture on MS containing 0.5 mg/l BAP plus 0.2 mg/l NAA. Nuclear DNA content of cells of the initial explants are given (0 d). C values are calculated as in Fig. 1

ture, diploid cells were 63.3% and 70.0% in the cv. "Dolce Provenza" and the "5075" line, respectively (Tables 1, 2). Polyploid cells occurred even in the first phases of callus induction in the "5075" line (Table 2), while in the cv. "Dolce Provenza", these started to appear on the 8th day of culture (Table 1).

In the first days of culture (up to the 8th day), in both genotypes, we observed both lobation of nuclei commonly ending with nuclear fragmentation and aberrant mitoses (chiefly anaphase chromosome bridges).

The above described nuclear status of the callus was confirmed by the cytophometric analysis (Figs. 1, 2). In particular, the distribution of Feulgen-DNA values clearly shows that: i) in the explant, the majority of nuclei belong to the G_1 (2C) and G_2 (4C) peaks; ii) after 2 days of culture, there occurs a shift of the G_1 peak towards a mean absorbance value lower than 2C; iii) in later days, both 2C and 4C peaks progressively reappear in addition to nuclei with less than 2C DNA contents and nuclei with DNA values between 2C and 4C, between 4C and 8C, and polyploid are present.

Chromosome counts were made on the shoot apex of the regenerated plantlets at two growth stages. In the cv. "Dolce Provenza", 6 out of 20 1–2 cm long plantlets were diploid and 14 were chromosomal mosaics (aneusomatics). Chromosome counts in these aneusomatic plantlets are reported in Table 3. When 4–5 cm long, 10 out of 20 plantlets were diploid and the rest (10) were aneusomatic (Table 4).

In the "5075" line, 9 out of 20 1–2 cm long plantlets were diploid and 11 aneusomatic (Table 5), while, when 4–5 cm long plantlets were analyzed, 13 out of 20 were diploid and only 7 plantlets were aneusomatic (Table 6).

Table 3. *Chromosome counts in 14 aneusomatic plantlets (1–2 cm in length) of P. sativum (cv. "Dolce Provenza") regenerated from shoot apical meristem derived calli*

Plantlet of no.	No. of mitoses	Percentage of cells in each chromosome number class												
		7	8	9	10	11	12	13	14	15	16	17	18	... 28
1)	20	—	5.0	—	—	—	—	10.0	85.0	—	—	—	—	—
2)	28	—	—	—	—	—	3.6	3.6	85.7	3.6	—	—	—	3.6
3)	30	—	—	—	—	—	—	10.0	80.0	—	—	—	10.0	—
4)	25	—	—	—	—	—	4.0	8.0	88.0	—	—	—	—	—
5)	20	—	—	—	—	—	—	5.0	75.0	15.0	—	—	5.0	—
6)	21	4.8	—	—	—	—	—	9.5	57.1	28.6	—	—	—	—
7)	31	—	—	—	—	3.2	—	29.0	58.1	9.7	—	—	—	—
8)	20	—	—	—	—	—	—	10.0	85.0	5.0	—	—	—	—
9)	30	—	—	—	3.3	—	—	30.0	56.7	6.7	3.3	—	—	—
10)	29	—	—	—	—	—	3.4	3.4	82.8	10.3	—	—	—	—
11)	14	—	—	—	—	—	7.1	—	64.3	21.4	7.1	—	—	—
12)	30	—	—	—	—	—	—	5.0	80.0	5.0	10.0	—	—	—
13)	22	—	—	—	—	—	—	4.5	77.3	13.6	—	—	4.5	—
14)	25	—	—	—	—	—	4.0	8.0	88.0	—	—	—	—	—

Table 4. *Chromosome counts in 10 aneusomatic plantlets (4–5 cm in length) of P. sativum (cv. "Dolce Provenza") regenerated from shoot apical meristem derived calli*

Plantlet no.	No. of mitoses	Percentage of cells chromosome number class					
		12	13	14	15	... 19	... 28
1)	30	10.0	6.7	80.0	3.3	—	—
2)	32	—	6.3	93.7	—	—	—
3)	21	—	4.8	81.0	9.5	4.8	—
4)	20	10.0	10.0	80.0	—	—	—
5)	30	3.3	3.3	90.0	3.3	—	—
6)	30	—	3.3	90.0	6.7	—	—
7)	30	6.7	—	93.3	—	—	—
8)	30	—	3.3	80.0	10.0	—	6.7
9)	23	8.7	—	91.3	—	—	—
10)	30	—	3.3	86.7	10.0	—	—

Table 5. *Chromosome counts in 11 aneusomatic plantlets (1–2 cm in length) of P. sativum ("5075" line) regenerated from shoot apical meristem derived calli*

Plantlet no.	No. of mitoses	Percentage of cells in each chromosome number class				
		12	13	14	15	16
1)	27	—	—	85.2	14.8	—
2)	23	—	8.7	91.3	—	—
3)	17	—	—	88.2	5.9	5.9
4)	22	—	—	90.9	9.1	—
5)	30	—	—	76.7	6.7	16.7
6)	30	3.3	3.3	93.3	—	—
7)	28	—	—	82.1	14.3	3.6
8)	24	—	8.3	87.5	4.2	—
9)	20	—	—	80.0	10.0	10.0
10)	30	—	—	73.3	10.0	16.7
11)	15	—	—	86.7	13.3	—

4. Discussion

While shoot tip culture under conditions that avoid callus formation is usually utilized for vegetative propagation (micropropagation), callus induction and culture may allow to achieve genetic and chromosomal variation in regenerated plants (cf. D'AMATO 1985).

Our results clearly show that:

i) callus from shoot tip meristems contains cells with different chromosome numbers ("haploid", hypodiploid, hyperdiploid and tetraploid) in high frequency;

ii) aneuploid cells and cells with 7 chromosomes in the callus originate mainly from processes of nuclear fragmentation (amitosis) and aberrant mitoses in the first days of culture;

iii) chromosome counts are corroborated by DNA microdensitometric analyses of calli at different days of culture;

iv) regenerated plantlets are diploid or chromosomal mosaics, *i.e.* they reflect the nuclear condition of the callus.

The observation reported at point iv) is in contrast with other cytological studies on regenerated plants of *Pisum sativum*: RUBLUO *et al.* (1984) and GRIGA *et al.* (1986)

Table 6. *Chromosome counts in 7 aneusomatic plantlets (4–5 cm in length) of P. sativum ("5075" line) regenerated from shoot apical meristem derived calli*

Plantlet no.	No. of mitoses	Percentage of cells in each chromosome number class				
		12	13	14	15	16
1)	30	—	—	93.3	3.3	3.3
2)	20	10.0	—	80.0	10.0	—
3)	30	6.7	3.3	90.0	—	—
4)	30	6.7	—	93.3	—	—
5)	20	10.0	10.0	80.0	—	—
6)	30	3.3	3.3	90.0	3.3	—
7)	30	—	3.3	90.0	6.7	—

found karyotypic stability in root tips of the regenerated and propagated plants, respectively. By contrast, plantlets regenerated from immature embryo derived calli were either diploid or aneusomatic (NATALI and CAVALLINI 1987). In this study, we sometimes observed a very broad aneusomaty in shoot apices of regenerants. These discrepancies might depend on the different organ analyzed (adventitious root tips *vs.* shoot apices) and/or on genetic differences among cultivars.

Differences in cytological conditions of calli (SINGH 1986) and regenerated plants from different genotypes within the same species may be found in the literature (cf. D'AMATO 1985). It is worth noting that the two genotypes tested gave different cytological responses during callus proliferation, *i.e.* nuclear polyploidization occurs earlier (and in different final frequencies) in the "5075" line than in the cv. "Dolce Provenza" (see Tables 1, 2). Moreover, regenerated plantlets (at both developments stages) show a lower aneusomaty in the "5075" line than in the cv. "Dolce Provenza".

The origin of aneuploid cells found in regenerated plants is chiefly due to processes of nuclear fragmentation during callus induction; similar processes were described in calli originating from differentiated tissues (cf. D'AMATO 1985).

As aneusomaty was reduced during plantlet development (see Tables 3–4 and 5–6) one may suppose the operation of a selective advantage of diploid over aneuploid cells (diplontic selection) as already ascertained by LUPI *et al.* (1981) for regenerated plantlets of *Triticum durum*. It will be, however, necessary to analyze the progeny of the regenerated plants to assess whether chromosomal variation is maintained.

In conclusion, plantlets regenerated from immature tissues derived calli can carry chromosomal variation in *Pisum sativum*. More extensive studies are in progress in order to analyze genetic variability in regenerated plants from shoot derived calli of the "5075" line, carrying genetic markers.

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