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Flow cytometric and karyological analysis of polysomaty and polyploidization during callus formation from leaf segments of various potato genotypes

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Summary. Flow cytometry and karyological analysis were used to study polysomaty and polyploidization during the first 15 days of callus formation in leaf segments from shoot cultures and greenhouse-grown plants of various lines and genotypes of *Solanum tuberosum* and *S. phureja*. The greenhouse-grown plants showed a higher degree of polysomaty (77% and 49% of polyploidized nuclei) than the shoot cultures (< 3%). During the in vitro culture period, polyploidization occurred through endoreduplication. Segments of the five shoot cultures showed up to 87%, 53%, 59%, 45% and 56% polyploidization, respectively; the DNA content of corresponding interphase nuclei amounted to 8C, 16C, 16C, 16C and 8C, and the chromosome numbers to 96. Segments from the two greenhouse-grown genotypes showed up to 87% and 84% polyploidization; the DNA content amounted to 32C and 16C, and the chromosome numbers to 192 and 96. The number of reduplication cycles was species-dependent; the degree of polyploidization was dependent on the initial ploidy level of the genotypes. Cell proliferation did not take place at a constant rate. The maximum frequencies of metaphases (52–171 per segment) occurred after 1 week of culture and were correlated with the ploidy level of the genotypes. Cells were triggered to mitosis rather than to endoreduplication. Cell cycles with normal monochromosomes could be shorter than 1 day, and those with diplochromosomes lasted at least 1 day. Polysomaty, degree of polyploidization and abnormal nuclear processes are discussed in relation to the origin of genetic instability early in culture.

Key words: Potatoes – Callus – Polysomaty – Polyploidization – Cell proliferation

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Introduction

Plants of several plant species often exhibit phenotypic variability or somaclonal variation when regenerated from tissue cultures, cells or protoplasts. The exact mechanisms underlying the genetic changes causing this phenotypic diversity are still poorly understood. While numerical and structural chromosome variation may arise during the in vitro cell or tissue culture phase, the occurrence of chromosome and genome mutations, including polysomaty (the existence of somatic cells with chromosome numbers corresponding to different levels of ploidy), in the plant tissue from which the cultures are derived may also contribute to the variation observed. The relative contribution of each of these may be different due to variations in genotype, type of culture, culture medium and culture age (reviews in D'Amato 1978, 1985; Skirvin 1978; Bayliss 1980; Larkin and Scowcroft 1981; Evans et al. 1984; Karp and Bright 1985).

In the cultivated potato, *Solanum tuberosum*, a wide range of phenotypic variability and a high degree of chromosomal variation have been reported among plants regenerated from protoplasts and tissue cultures (reviews in Sree Ramulu 1986; Jacobsen 1987). Both polysomaty of the parental plants and karyotypic instability during the in vitro phase may underlie the genetic changes (Wenzel et al. 1979; Carlberg et al. 1984; Sree Ramulu et al. 1984, 1985; Sree Ramulu and Dijkhuis 1986; Tempelaar et al. 1985; Pijnacker et al. 1986a, b; Pijnacker and Ferwerda 1987; Hänisch ten Cate and Sree Ramulu 1987; Uijtewaal 1987).

This study investigates in vivo and in vitro genetic alterations that contribute to DNA and chromosomal variation in initial callus cultures. The results of a DNA flow cytometric and karyological analysis of leaf

segments from shoot cultures and greenhouse-grown plants of five *S. tuberosum* genotypes and two *S. phureja* genotypes cultured in vitro for 15 days are compared. These genotypes are currently being used in various cell genetic studies, including somatic hybridization and genetic transformation.

Materials and methods

Plant material

One monohaploid line (7322, $2n = x = 12$, Jacobsen 1981), two dihaploid lines (HH260, $2n = 2x = 24$, supplied by Prof. O. Schieder, Berlin, FRG; and SVP3, SH77-78-904, $2n = 2x = 24$, provided by the Foundation for Plant Breeding SVP, Wageningen, NL), one tetraploid cultivar ('Bintje', $2n = 4x = 48$) of *S. tuberosum* and one diploid clone of *S. phureja* (SVP5, PH77-1445-2242, $2n = 2x = 24$) were propagated as shoot cultures (Bokelmann and Roest 1983) and used as the source material for callus induction. In addition, plants of cv. 'Bintje' and of a diploid clone of *S. phureja* (SVP7, PH77-1426-1872) were grown in the greenhouse (designated as 'Bintje'-gr and SVP7-gr) and used for comparison with the shoot cultures.

Culture

For callus initiation from shoot cultures, 80–175 leaf segments of about 10 mm^2 from 10–20 shoots per genotype were cultured on solidified basal medium (Murashige and Skoog 1962) supplemented with 5 mg/l α-naphtalene acetic acid and 0.1 mg/l benzyl aminopurine. Similar numbers of segments were cultured on this medium supplemented with 10 mg/l 5-bromodeoxycytidine (BrdC) for differential staining (Pijnacker et al. 1986b). Leaves of 'Bintje'-gr and SVP7-gr were selected randomly from ten plants and surface-sterilized in ethanol (70%, 10 s) followed by immersion in calcium hypochlorite (5%, 12 min). After 3–4 rinses in sterile distilled water, 80–100 leaf segments per genotype were processed in the same manner as the shoot cultures.

Flow cytometry

For flow cytometric analysis of the nuclear DNA content, leaf segments cultured on medium without BrdC were collected on days 0, 3, 8 and 15. The nuclei were obtained from non-fixed samples (about ten leaf segments including callus) by chopping the leaf segments with a sharp razor blade in a glass petri dish containing chopping buffer (Hänisch ten Cate et al. 1986; Sree Ramulu and Dijkhuis 1986). Nuclei, 3,000–27,000 per sample, were stained with ethidium bromide, and the relative nuclear DNA content was measured with a Fluorescence Activated Cell Sorter (FACS)-IV (Becton Dickinson, Sunnyvale, USA) equipped with a Spectra Physics argon Laser (model 164-05) operated at 0.3 W/488 nm with a LP 620 filter in the emission beam. Fluorescent microspheres (Duke Scientific, Palo Alto, CA, USA) were used to check the instrument alignment and served as a reference standard. Based on nuclear DNA content, flow cytometry can distinguish cells occurring with a frequency as low as 2%–3% as a separate peak. The nuclear DNA content was expressed in C values (Sree Ramulu and Dijkhuis 1986). The contribution of cellular and nuclear debris in the flow histograms was removed by an interactive computer analysis (Van der Linden 1980).

Staining procedures and karyotyping

Feulgen-stained squashes were made of 4–5 leaf segments per plant of each genotype at day 0 according to standard procedures. Provided that mitoses were present, the chromosomes of ten metaphases per plant were counted. About 5–20 BrdC-treated and untreated segments were collected on days 1–8 and on day 15. A slide was prepared from each segment and either normally or differentially stained with Giemsa (Pijnacker et al. 1986b). The total numbers of metaphases karyotyped in the untreated segments varied from 355–3128 dependent on the genotype; the total numbers in BrdC-treated segments varied from 4–461.

Results

Nuclear DNA content in interphase nuclei

Flow cytometric analysis revealed that shoot cultures of the five genotypes (day 0) contained nuclei of two C levels (Table 1). The lower C level corresponds to the amount of DNA present in the normal somatic chromosome complement of that genotype in G1 phase, and the other C level corresponds to double that amount of DNA and may belong to G2-phase nuclei. The percentages of nuclei in G1 phase (and G2 phase) differed between the various genotypes, irrespective of the ploidy level. In contrast, leaves of the two greenhouse-grown genotypes contained nuclei with four times the G1-phase amount of DNA (7% in SVP7-gr, 13% in 'Bintje'-gr) indicating the occurrence of polysomaty.

When leaf segments were grown on callus induction medium, polyploidization of cells occurred in all genotypes (Table 1). One extra C level appeared in SVP5, SVP7-gr, 'Bintje' and 'Bintje'-gr, and two in 7322, HH260 and SVP3. In general, within a given genotype the percentages of nuclei with higher C values increased with increasing age of the cultures. Differences in the percentages of nuclei with various C values existed between diploid as well as tetraploid genotypes after the same culture period.

Assuming that at day 0 the nuclei with double the amount of DNA were G2-phase nuclei and not polyploidized (i.e. G1-phase nuclei with double the number of chromosomes) and that the percentage of nuclei in the G2 phase remained constant during the 15 days of culture, the minimum percentage of all polyploid nuclei on days 3, 8 and 15 can be calculated for each genotype. For example: at day 3, 7322 has $1/3 \times 55\% = 18\%$ G2-phase nuclei and $(33\text{-}18) + 12\% = 27\%$ polyploid nuclei. The same calculation can also be carried

Table 1. Relative nuclear DNA contents in leaf segments of shoot cultures and greenhouse-grown plants of *S. tuberosum* and *S. phureja* genotypes cultured in vitro on nutrient medium for various periods

Genotype		Percentages of nuclei with various C values																			
Line or Cultivar	Ploidy level	Day 0					Day 3					Day 8					Day 15				
		1C	2C	4C	8C	16C	1C	2C	4C	8C	16C	1C	2C	4C	8C	16C	32C	1C	2C	4C	8C	16C	32C
Shoot cultures																							
S.t. 7322	1×	75	25				55	33	12			26	47	24	3			10	38	36	16		
S.t. HH260	2×		75	25				52	38	10			31	47	20	2			35	45	18	2	
S.t. SVP3	2×		88	12				53	47				46	43	11				36	44	18	2	
S.p. SVP5	2×		68	32				53	43	4			34	50	16				30	47	23		
S.t. "Bintje"	4×			86	14				67	33				56	41	3				55	42	3	
Greenhouse-grown plants																							
S.p. SVP7-gr	2×		35	58	7			20	36	44			18	50	30	2			11	45	37	7	
S.t. "Bintje"-gr	4×			20	67	13			17	53	30			11	47	39	3			11	44	41	4

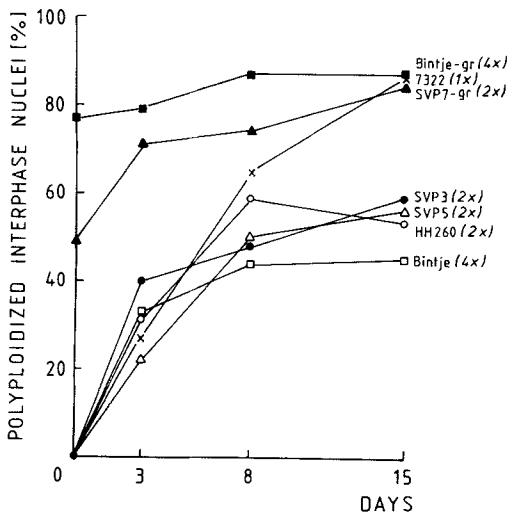

Fig. 1. Effect of the duration of in vitro culture on the frequency of polyploidized interphase nuclei in leaf segments from monohaploid (×), dihaploid (○, ●) and tetraploid (□, ■) *Solanum tuberosum* genotypes and diploid (△, ▲) *S. phureja* genotypes

out for SVP7-gr and 'Bintje'-gr if it is supposed that at day 0 the percentages of nuclei with double the amount of DNA per genotype are 32% (as in SVP5) and 14% (as in 'Bintje') of the percentages of nuclei with the amount of DNA in the G1 phase, respectively. The data thus obtained (Fig. 1) reveal that in the leaf segments of the shoot cultures, the increase in polyploidization was highest in the monohaploid 7322, intermediate in the diploids HH 260, SVP3 and SVP5, and lowest in the tetraploid 'Bintje'. Apparently, the degree of polyploidization is dependent on the initial ploidy level of the genotypes. Since the diploid genotypes of *S. tuberosum* and *S. phureja* had the same degree of polyploidization, these species do not differ in their response to polyploidization. In the cultured leaf explants from the greenhouse-grown plants, the increase in polyploidization was also higher in the diploid SVP7-gr than in the tetraploid 'Bintje'-gr, but the degree was basically determined by the presence of polysomaty. In all of the cultures, the increase in polyploidization was highest during the first week of culture.

In the same way, the minimum number of polyploidized nuclei can be calculated from the total number of interphase nuclei measured by the flow cytometer. Using these (high) numbers for Chi-square tests at the 5% level, it appeared that in the segments of each genotype the number of polyploidized nuclei had increased significantly from days 0–3, 3–8 and 8–15; only 'Bintje' and 'Bintje'-gr did not show further polyploidization between days 8–15, while HH260 showed

Table 2. Percentages of metaphases with various numbers of chromosomes (12–192) or diplochromosomes (12d–96d) in leaf segments of shoot cultures and greenhouse-grown plants of *S. tuberosum* and *S. phureja* genotypes cultured in vitro on nutrient medium for various periods

Genotype		Percentages of metaphases in each chromosome number class[a]												
Line or Cultivar	Ploidy level	Day 0			Day 1			Day 2			Days 3+4[b]			
		12	24	48	12	24	48	12	24^{24d}	48^{48d}	12^{12d}	24^{24d}	48^{48d}	96
Shoot cultures														
S.t. 7322	1×	100			–			50	50		75^{9}	14	2	
S.t. HH260	2×		100			–			76^{7}	17		90^{4}	7	
S.t. SVP3	2×		100			–			97^{3}			66^{7}	28	
S.p. SVP5	2×		–			–			93	7		38	63	
S.t. 'Bintje'	4×			100			100			95^{5}			92^{2}	6
Greenhouse-grown plants														
S.p. SVP7-gr	2×		–			–			100			33^{13}	49^{4}	1
S.t. 'Bintje'-gr	4×			–			–			–			70^{3}	27

Genotype		Percentages of metaphases in each chromosome number class[a]												
Line or Cultivar	Ploidy level	Days 5+6[b]				Days 7+8[b]					Day 15			
		12^{12d}	24^{24d}	48^{48d}	96^{96d}	12^{12d}	24^{24d}	48^{48d}	96	192	12^{12d}	25^{24d}	48^{48d}	96
Shoot cultures														
S.t. 7322	1×	53^{7}	36^{1}	$3^{\cdot 2}$		39^{4}	46^{2}	$8^{\cdot 2}$	.2		24^{1}	66	9	
S.t. HH260	2×		57^{3}	39^{1}	.3		83^{4}	$13^{\cdot 3}$				29^{8}	58^{1}	4
S.t. SVP3	2×		84^{1}	15	1		68^{5}	$27^{\cdot 3}$	.3			80	18^{2}	
S.p. SVP5	2×		75^{1}	23	1		$59^{\cdot 2}$	41				88	12	
S.t. 'Bintje'	4×			96^{2}	3			96^{1}	4				98^{1}	2
Greenhouse-grown plants														
S.p. SVP7-gr	2×		38^{5}	57^{1}			18^{1}	70^{2}	9			6	42^{2}	51
S.t. 'Bintje'-gr	4×			83	16^{1}			38^{3}	56	3			71	29

[a] Percentage > 0.5 rounded off
[b] Pooled data

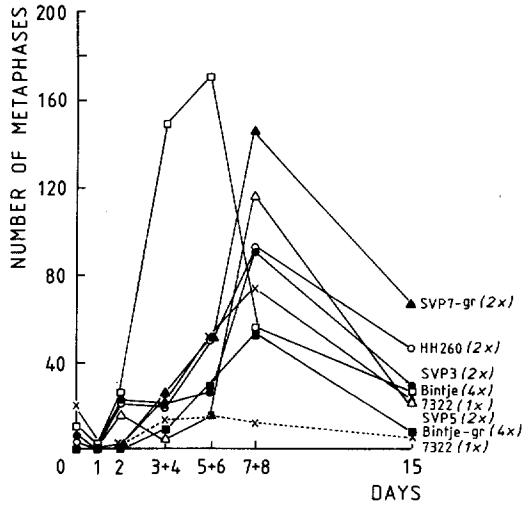

Fig. 2. Changes in the number of metaphases per leaf segment in monohaploid (×), dihaploid (○, ●) and tetraploid (□, ■) *Solanum tuberosum* genotypes and diploid (△, ▲) *S. phureja* genotypes cultured in vitro on medium without and with (7322 only, *dotted line*) bromodeoxycytidine for 15 days

a significant decrease at day 15 (reason unknown). At day 15 there was a significant difference in the numbers of (non-)polyploidized nuclei between the genotypes, except for 7322 and 'Bintje'-gr.

Metaphase frequency in leaf segments cultured on medium without BrdC

The number of metaphases and the number of chromosomes or diplochromosomes per metaphase were scored for each leaf segment (Table 2, Fig. 2). In the leaves of 7322, HH260, SVP3 and 'Bintje' prior to explant culture (day 0), only metaphases having a normal chromosome complement occurred, while in the leaves of SVP5, SVP7-gr and 'Bintje'-gr, no mitotic divisions could be observed. During the first day of culture, metaphases were observed in 'Bintje' only. However, from day 2 ('Bintje'-gr, day 3) onwards, more metaphases, also with diplochromosomes, occurred in all genotypes, thus indicating the onset of cell prolif-

eration for callogenesis at almost the same time after a dedifferentiation period of 2 or 3 days.

Because the number of cells per cultured leaf segment was not determined, the exact frequency of metaphases per leaf segment could not be established. (A leaf segment consists of at least 20,000 cells.) If it is assumed that the mean number of metaphases of the (5–20) segments of 1 day is a reflection of the real frequency (and so the mitotic index), the data in Fig. 2 show that the metaphase frequency increased up to days 5 + 6 in 'Bintje' and up to days 7 + 8 (or later?) in the other genotypes, and that it decreased considerably after 15 days of culture. The frequencies varied among the genotypes. The maximum frequencies of metaphases in the segments of the shoot cultures, and so the speed of earlier callus growth, are positively correlated with the ploidy level of the genotypes.

The percentages of metaphases with diplochromosomes were rather low (in general < 5%), in particular on day 15, when compared to those with monochromosomes (Table 2). During meta-anaphase, the diplochromosomes separated into two-chromatid chromosomes. Until telophase, the two chromatids either remained together or separated.

Chromosome numbers in leaf segments cultured on medium without BrdC

In the cultured leaf segments, metaphases with polyploidized chromosome numbers (mono- or diplochromosomes) were observed from day 2 (SVP7-gr and 'Bintje'-gr, day 3) onwards (Table 2). It should be noted that a set of diplochromosomes is numerically identical with a duplicated set of monochromosomes. In 7322, HH260, SVP5 and 'Bintje'-gr, the first metaphases with a doubled number of chromosomes appeared on the same day as metaphases with the normal chromosome complement. The chromosome numbers could be reduplicated as geometrical series, 1–3 times in 7322, 1–2 times in HH260, SVP3, SVP5, SVP7-gr and 'Bintje'-gr, and only once in 'Bintje'. The percentages of metaphases with normal or polyploidized numbers of mono- or diplochromosomes varied considerably within a given genotype and differed between diploid and tetraploid genotypes after the same period of culture. Only 7322 and SVP7-gr showed a gradual shift towards metaphases having higher chromosome numbers. In addition, the total percentages of polyploidized metaphases varied in such a way that differences between the genotypes could not be related to the difference in ploidy level of the genotypes or the presence of polysomaty (Fig. 3). The percentages increased in 7322, HH260 and SVP7-gr until the end of the culture period, whereas they decreased in the other genotypes during the second week. Over the total culture period, the lowest percentage of polyploidized metaphases were found in 'Bintje' (maximum 8%) and the highest in SVP7-gr (maximum 94%).

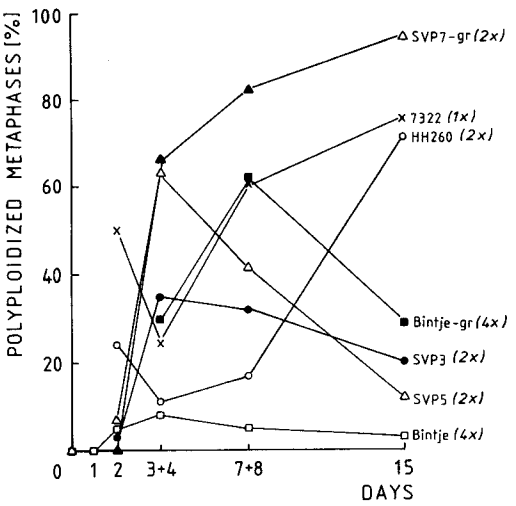

Fig. 3. Effect of the duration of in vitro culture on the frequency of polyploidized metaphases in leaf segments from monohaploid (×), dihaploid (○, ●) and tetraploid (□, ■) *Solanum tuberosum* genotypes and diploid (△, ▲) *S. phureja* genotypes

When chromosome numbers are expressed in C DNA values (12 metaphase chromosomes contain 2C DNA), the data on chromosome numbers presented in Table 2 show that the metaphases of all genotypes attained a maximum amount of 16C, except for 'Bintje'-gr which attained 32C. These maximum C values correspond to those of the interphase nuclei (Table 1) except that the nuclei of 7322 and SVP5 contained maximally 8C. The rare occurrence of metaphases with a 16C DNA value in 7322 (0.2% on days 5 + 6 and 0.2% + 0.2% on days 7 + 8) and in SVP5 (1% on days 5 + 6) may explain this discrepancy.

Metaphases in leaf segments cultured on medium with BrdC

By culturing leaf segments on BrdC-containing medium, BrdC is incorporated into the DNA of the chromosomes during the S phase and, as a consequence, the chromosomes are stained differentially. The following types of metaphases were detected (Table 3): (1) metaphases with normally stained monochromosomes or diplochromosomes (i.e. those which underwent one S phase in the presence of BrdC, or none at all), (2) metaphases with differentially stained monochromosomes or with differentially stained diplochromosomes consisting of two darkly stained inner chromatids and two lightly stained outer ones (having passed through two S phases), and (3) metaphases with a mixture of

differentially and lightly stained monochromosomes or with diplochromosomes having one darkly stained inner chromatid and three lightly stained chromatids (having passed three S phases) (cf. Pijnacker et al. 1986b).

The metaphase frequency per cultured leaf segment was lower than that found in the leaf segments cultured on medium without BrdC. The highest mean frequency was found in 7322:15 on days 5 + 6 (Fig. 2). In HH260 and 'Bintje'-gr, only ten and four metaphases were found, respectively. The distribution of the metaphases with various numbers of chromosomes and diplochromosomes (the normally and differentially stained ones with the same number pooled) was similar to that of the metaphases of the cultured leaf segments not treated with BrdC. Metaphases with mono- or diplochromosomes which had passed through two or three S phases according to their type of staining appeared in increasing frequencies from day 2 ('Bintje'), 3 (7322, SVP3, SVP5, SVP7-gr) or 5 ('Bintje'-gr) onwards until the end of the culture period (Table 3). Of the total number of metaphases of a given genotype, less than 40% had undergone two S phases and less than 3%, three S phases.

In cultured segments of 7322 and SVP3, metaphases were observed with four times the somatic number of monochromosomes while in 7322, SVP3 and SVP5, metaphases with double the somatic number of diplochromosomes were present – all of which were not differentially stained (Table 3). These metaphases had thus undergone a maximum of one S phase during the culture period and therefore must have arisen from cells having a minimum nuclear DNA content of 4C, 8C and 4C, 8C, 8C, respectively, before the start of the culture period. Differentially stained metaphases with 96 chromosomes were found in 7322. Since they had undergone two S phases, they must have originated from original leaf cells with 4C nuclei. The occurrence of these cells with four times the amount of DNA present in the somatic number of chromosomes in G1 phase indicates that the shoot cultures of 7322, SVP3 and SVP5 were polysomatic.

Whether or not polyploidized, metaphases containing differentially stained monochromosomes could be found on the same day or up to 2 days later than normally stained ones having the same chromosome number (Table 3). This indicates that in these segments, the second cell cycle, i.e. from metaphase to metaphase including the second S phase, had a minimum duration of less than 1 day irrespective of the ploidy level of the genotype. Metaphases that had passed through three S phases were observed in 7322, SVP3 and 'SVP7'-gr only. In SVP7-gr, these metaphases had occurred as soon as 3 days after culture initiation and on the same day as the metaphases which had passed through two

Table 3. Day of culture on which a metaphase with a certain number of normally or differentially stained chromosomes (12–96) or diplochromosomes (12 d–48 d) appeared for the first time in leaf segments of shoot cultures and greenhouse-grown plants of *S. tuberosum* and *S. phureja* genotypes cultured in vitro on nutrient medium with bromodeoxycytidine for various periods

Genotype		Day of first appearance of a type of metaphase																					
Line or cultivar	Ploidy level	12			12 d			24			24 d			48			48 d			96			
		[a]																					
Shoot cultures																							
S. t. 7322	1×	3	3	4	2	4	6	3	3	6	3	5	6	8	3	5					6	6	
S. t. HH260	2×							1						2									
S. t. SVP3	2×							1	3	5	2	4	7	2	3	6	3	5		6	4		
S. p. SVP5	2×							1	3		2	5		3	3		3						
S. t. 'Bintje'	4×													2	2		2			2	3		
Greenhouse – grown plants																							
S. p. SVP7-gr	2×							2	3		2	4		2	3	3	3	8		3	4		
S. t. 'Bintje'-gr	4×													5	5								

[a] = darkly stained chromatid; = lightly stained chromatid

S phases: this indicates that the third cell cycle could also last less than 1 day.

Metaphases with differentially stained diplochromosomes appeared 1–5 days later than the normally stained ones having the same chromosome number and those having passed through three S phases appeared 1–3 days later than the differentially stained ones (Table 3). Cell cycles in which diplochromosomes were involved thus lasted at least 1–2 days and were longer than the cell cycles with monochromosomes.

Discussion

Polysomaty

Flow cytometry on interphase nuclei of *S. tuberosum* and *S. phureja* genotypes maintained as shoot cultures did not reveal any polysomaty. However, in leaf segments from shoot cultures that had been cultured on medium with ('Bintje') or without BrdC (7322, HH260, SVP5), metaphases with double the somatic number of chromosomes were observed at day 2. These occurred simultaneously with the first metaphases having normal chromosome numbers, and may have originated from two types of nuclei: (1) G2-phase nuclei which had undergone an extra S phase, and the resulting diplochromosomes (see below) subsequently separated into monochromosomes and (2) G1-phase cells with the doubled chromosome number which underwent a normal mitosis (i.e. without an extra S phase). Since the metaphase chromosomes were not observed to arrange into pairs, which would occur in the separation of diplochromosomes into monochromosomes, the first explanation is not likely. If they originated from G1-phase cells, a number of the cells determined by flow cytometry to be in the G2-phase (Table 1) might have actually been in the G1 phase with a doubled somatic number of chromosomes (i.e. with a similar C value). This would mean the occurrence of a polysomatic condition in the shoot cultures of these genotypes, which normally cannot be detected by flow cytometry. Consequently, the percentages of polyploidized interphase nuclei as shown for these genotypes in Fig. 1 would then be underestimates.

Differential staining of metaphases from cultured leaf segments showed that shoot cultures of genotypes 7322, SVP5 and SVP3 were polysomatic for cells having as much as four times the DNA amount of the somatic number of chromosomes in G1 phase. With flow cytometry these nuclei with 4C, 8C and 8C DNA content, respectively, were not detected probably because they were present at a frequency below the detection level (< 3%; Sree Ramulu and Dijkhuis 1986). Polysomaty in shoot cultures of the monohaploid, dihaploid and diploid genotypes used in this study has been observed before (Tempelaar et al. 1985; Sree Ramulu and Dijkhuis 1986; Uijtewaal 1987).

Flow cytometry revealed that greenhouse-grown plants of SVP7 and 'Bintje' were polysomatic and had one additional higher ploidy level in the interphase nuclei. Similar results have been reported previously in other potato genotypes (Sree Ramulu and Dijkhuis 1986; Uijtewaal 1987). The leaves of plants grown in the greenhouse contained hardly any dividing cells when compared to those of shoot cultures. The presence of polyploidized cells in the greenhouse-grown plants may be due to the fact that differentiation is often involved with polyploidization (Nagl 1978; D'Amato 1985).

Degree of polyploidization

Polyploidization occurred in the leaf segments of all potato genotypes when cultured on callus-inducing medium; this has been reported for several plant species, including potato (reviews in D'Amato 1985; Karp and Bright 1985; Sree Ramulu 1986; Pijnacker et al. 1986b; Hänisch ten Cate and Sree Ramulu 1987). In the cultured leaf segments of the tetraploid 'Bintje', irrespective of whether polysomatic or not, one additional doubling of the nuclear DNA content and chromosome number was induced during 15 days of in vitro culture. In the same period, two successive duplications occurred in the cells of the dihaploids HH260 and SVP3 and three in the cells of the monohaploid 7322 resulting in ploidy levels identical to those observed in cultures of 'Bintje'. Thus, genotypes with a lower ploidy level have an initial tendency to undergo polyploidization up to a tetraploid level and then, as in 'Bintje', continue to higher levels thereafter. The genotypes 7322, HH260 and SVP3 originated from tetraploid *S. tuberosum* genotypes. The degree of polyploidization was dependent on the ploidy level. A similar situation was found by Sacristàn (1971) when comparing haploid and diploid callus cultures of *Crepis capillaris*.

With the exception of one leaf segment of SVP5 which showed one octoploid metaphase, i.e. two duplication rounds, the cells of the cultured leaf segments of diploid *S. phureja* genotypes SVP5 and SVP7-gr showed a doubling of the nuclear DNA content and chromosome number similar to those of the tetraploid 'Bintje' and 'Bintje'-gr, respectively. This indicates that the number of reduplication cycles taking place during a certain culture period is dependent on the genotype of the species, irrespective of the parental chromosome number.

Nuclear processes

Cells from the leaves of the shoot cultures and the greenhouse-grown plants that were already polyploidized were able to undergo mitosis during in vitro culture of the leaf segments. Dedifferentiation and subsequent cell division are thus not inhibited by polyploidization. These cells are the source of variation in the euploid chromosome numbers in callus cultures of potato genotypes; this variation has been observed in other polysomatic plant species also (reviews in Skirvin 1978; Bayliss 1980; D'Amato 1985).

The occurrence of diplochromosomes in the cultured leaf segments demonstrates that endoreduplication of the chromosomes had taken place (D'Amato 1952; Nagl 1978; Pijnacker et al. 1986b). The first metaphases containing diplochromosomes originate from leaf cells in G2 phase that were activated to undergo an additional S phase during in vitro culture before entering mitosis. They were not from G1-phase cells; otherwise they would have been differentially stained when the leaf segments were cultured on medium with BrdC due to having passed through two S phases. The frequency of metaphases with diplochromosomes remained, in general, below 5% during the culture period. In the plant leaves, a minimum of 12% of the cells were found in the G2 phase (SVP3, Table 1). Consequently, cells were triggered to normal mitosis rather than to endoreduplication.

In leaf segments cultured on medium with BrdC (except for HH260 and 'Bintje'-gr) normally stained metaphases with diplochromosomes were found at day 15. Consequently, even after this period of culture, endoreduplication could take place in G2-phase cells which already existed before the start of the culture period. Because the frequency of these metaphases was considerably lower at day 15, the highest frequency of endoreduplication in such G2-phase cells was maintained only during a short initial culture period. Whether endoreduplication also occurred in G2-phase cells that had arisen during the culture period, i.e. in cells which began in the G1-phase, is not known. When cultured on BrdC medium, the resulting diplochromosomes show a differential staining pattern. However, this pattern can also be found if cells with diplochromosomes continue cycling as such up to the next metaphase. In the present study, this most likely occurred as diplochromosomes were observed throughout the mitotic cycle. For similar reasons, the presence of metaphases with diplochromosomes consisting of three lightly stained and one darkly stained chromatid does not provide conclusive evidence. This staining pattern occurs both when the above-mentioned G1 phase is preceded by a cell cycle with an S phase and when the chromatids of the diplochromosomes remain together until the third cell cycle. Through the induction of endoreduplication in G2-phase cells of pre-culture origin and their subsequent divisions, the population of polyploidized cells increased during culture. Increases by endomitosis, restitution, spindle fusion during bimitosis, or nuclear fusion was not observed (Nagl 1978).

The cell cycles with normal monochromosomes took place within 1 day, and those with diplochromosomes within 1–2 days. These times are within the limits established for cell cycle durations in cell suspensions and explant cultures of other species (Gould 1983). As was shown by differential staining, within the in vitro culture period of 15 days, cells containing the normal somatic number of chromosomes were able to divide twice in SVP5, SVP7-gr, 'Bintje' and 'Bintje'-gr and at least three times in 7322 and SVP3, and cells with a polyploidized number of chromosomes, two times in SVP5 and 'Bintje' and at least three times in 7322, SVP3 and SVP7-gr. Within a genotype, the cells with various numbers of chromosomes apparently proliferated rather similarly. It should be noted that after three cell cycles with BrdC incorporation, cells are no longer viable.

Cell multiplication did not take place at a constant mitotic index in the cultured leaf segments. As shown by the mean number of metaphases, the maximum frequencies in all genotypes occurred after about 1 week of culture, and they were correlated with the level of ploidy of the genotypes. The subsequent decrease during the second week of culture may be due to exhaustion of the medium. In primary explant cultures of other plant species also, eg. *Nicotiana tabacum* (Shimada and Tabata 1967), *Allium sativum* (Doležel and Novák 1985) and *Bellevalia romana* (Cavallini et al. 1986), a maximum index was found after about 1 week. Therefore, 1-week-old explant cultures can be used for karyological investigations.

In conclusion, the results demonstrate that polysomaty of the explant source and nuclear instability of cells and of differentiated tissue segments cultured in vitro results in the early occurrence of polyploidization and karyotypic changes. Investigations aimed at establishing appropriate culture conditions in vivo and in vitro to reduce or avoid genetic instability are in progress. This is of practical importance for the application of somatic cell genetic methods in fundamental and breeding research on potato.

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