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Behaviour of chromosomes in potato leaf tissue cultured in vitro as studied by BrdC-Giemsa labelling

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Summary. Cells of leaf explants of a monohaploid potato (*Solanum tuberosum*) were stimulated to mitosis on a medium with 5-bromodeoxycytidine during a period of 7 days. The cells cycled with mono- or diplochromosomes which showed differential staining of the sister chromatids and sister chromatid exchanges by the fluorescent plus Giemsa technique after two rounds of BrdC incorporation. Through the staining pattern the course of the first three cell cycles could be traced and the duration of the cycles estimated. Polyploidisation was enhanced by selective stimulation of polyploid cells and by endoreduplication of G2-phase cells. The percentage of polyploid mitoses increased from 10 to 70.

Key words: Potato – Tissue culture – Differential staining – Diplochromosomes – Polyploidisation

Introduction

Variability in chromosome number and morphology is a common phenomenon in plant cell cultures and is generally induced or enhanced by the in vitro conditions.

The changes have generally been established by karyotyping the metaphases and by estimating the amounts of nuclear DNA (D'Amato 1977; Bayliss 1980; Krikorian et al. 1983). The behaviour of chromosomes has been studied by autoradiography, especially in connection with polyploidisation during callus initiation (Patau and Das 1961; Torrey 1965; Phillips and Torrey 1973). Chromosomal behaviour can also be investigated by differential staining of the sister chromatids (Schubert 1979). The pattern of chromatid staining gives information about what happened to the chromosomes, and consequently to that particular cell, during two or three rounds of replication. This method has some advantages with respect to autoradiography: it is quicker (no exposure time of film), handier (no radioactivity), and suited for small chromosomes (the staining has a higher effective resolution than silver grains over the chromosomes). To our knowledge the method has not yet been used to study the chromosomes in plant tissue cultures. In cultured explants and calli of the potato *Solanum tuberosum,* a high degree of chromosomal variation has been found (Karp and Bright 1985; Sree Ramulu et al. 1985; Tempelaar et al. 1985), but the origin of this varation has not yet been clarified.

By using the differential staining technique we tried to trace the first mitotic divisions during callus initiation in leaf explants of a monohaploid potato to elucidate the mechanisms responsible for chromosomal variation.

Materials and methods

For callus initiation, cut leaf pieces of about $5 \times 2\ mm^2$ randomly sampled from in vitro shoot cultures of a monohaploid potato (*Solanum tuberosum*) genotype 7322 (Jacobsen 1981) were placed on solidified (8 g/l agar) basal medium of Murashige and Skoog (1962) supplemented with 5 mg/l α-naphtaleneacetic acid (NAA), 0.1 mg/l benzylaminopurine (BAP) and 10 mg/l 5-bromodeoxycytidine (BrdC), and incubated at 25 °C in the dark. As a control, callus was also initiated on medium without BrdC. As BrdC is heat- and light-sensitive, it was supplemented as follows: 270 µg of BrdC in 2 ml distilled water was filter sterilized and added to a Petri dish with 25 ml solid medium. After 24 h the BrdC had permeated sufficiently into the medium and the leaf explants were positioned. BrdC was used instead of BrdU because it was less detrimental to the explants.

The leaf explants (with developing callus) were collected on days 0, 1, 2, 3, 4, 5, 6 and 7 and either fixed directly in Carnoy (ethanol 100% : glacial acetic acid = 3 : 1) at 4 °C or pretreated in saturated α-bromonaphtalene for 3 h at room temperature before fixation in Carnoy. Slides were prepared following a modified procedure of Pijnacker and Ferwerda (1984): after about 24 h in the fixative, the leaf pieces were rinsed in distilled water, incubated in 15% (v/v) pectinase (Sigma P5146)/1.5% (w/v) cellulase R10 (Yakult) in citrate

buffer pH 4.8 for 45 min at 37 °C, rinsed, and then kept in distilled water for a minimum of 2 h. One leaf piece was transferred to a clean slide and a drop of acetic acid 60% was added. With fine needles, the leaf piece was made into a suspension. This suspension was surrounded with cold Carnoy, then about 3 drops of Carnoy put on top of the suspension, and the slide left to air-dry.

Differential staining was carried out according to the fluorescent plus Giemsa technique (Perry and Wolff 1974; Goto et al. 1975). Slides were stained in Hoechst 33258 (1 mg Hoechst in 1 ml ethanol and 0.3 ml of this solution in 100 ml 0.5 × SSC) for 15 min at room temperature. After washing in 0.5 × SSC, coverslips were added and the slides exposed to 285–385 nm UV-light at a distance of 10 cm for 15 min. Then the coverslips were removed in 0.5 × SSC and the slides incubated in 2 × SSC at 60 °C for 30 min, rinsed in distilled water and stained in 2% Giemsa in Sörensen buffer pH 6.9 for 30 min, rinsed in buffer and distilled water, air-dried and, via xylene, mounted in DePeX.

The principle of differential staining is as follows (see Fig. 1): cells are grown in the presence of the base analogue BrdC. After one round of replication, both sister chromatids are unifilarly substituted with BrdC (i.e. after the semiconservative replication of the DNA only one strand has incorporated BrdC in place of thymidine) and they both stain darkly. After two rounds of replication, one of the sister chromatids is unifilarly substituted, whereas the other is bifilarly substituted. Now the sister chromatids stain differentially because the unifilarly substituted chromatid stains more darkly than the bifilarly substituted one (Conner et al. 1978; Bayramian and Zakharov 1979). After three rounds of replication, the one chromosome has a unifilarly and a bifilarly substituted chromatid whereas the other chromosome consists of two bifilarly substituted chromatids which both stain lightly. Differentially stained chromosomes are also found after one round of incorporation with BrdC followed by one round of incorporation without BrdC (i.e. with thymidine), but in the present experiments BrdC was constantly present.

To locate the places with mitotic divisions, Feulgen squashes were made of whole explants.

Results and discussion

General

Three experiments were carried out to study the mitoses in leaf explants cultured over a period of 7 days. The results have been pooled and are presented in Table 1. The frequency of explants with mitoses increased considerably at day 3 of culture and from then onwards, and indicates the induction of cell divisions through the in vitro conditions. However, even after 7 days, explants were found without mitoses. The number of metaphases per explant varied over the whole culture period, i.e. 1–78 metaphases per explant of 20,000–40,000 cells. So, concerning mitotic stimulation, there is a considerable variation in the reaction pattern of the explants to in vitro culture. This variation is not due to BrdC for it was also found in explants not cultured on BrdC. The first induced divisions took place alongside the veins as seen in Feulgen squashes. Localized swellings, indicating centres of callus formation, were visible after 5 days of culture. The explants did not survive a period of 10 days on BrdC.

Number of S-phases

The karyotype consists of the haploid number (x) of 12 chromosomes which are similar to the Giemsa stained chromosomes of the potato cv. 'Gineke' described by Pijnacker and Ferwerda (1984). (The common potato is tetraploid (4x = 48).) The metaphases showed the euploid numbers 1x, 2x and 4x of mono- or diplochromosomes which were stained either normally or differentially (Figs. 2–8; Table 1). Monochromosomes are the normal two-chromatid chromosomes and diplochromosomes consist of four co-aligned chromatids (see "Origin of diplochromosomes"). Whenever differential staining was present, it had taken place in all the chromosomes of a metaphase, indicating that BrdC was available as precursor in sufficient quantity. While it is not known whether all the cells took up BrdC, it will be assumed they did. Cells with normally stained metaphases, whether with mono- or diplochromosomes, had passed one S-phase (= DNA synthesis phase) or none (Fig. 1; see "Methods"). In this aspect BrdC-labelling is at a disadvantage with respect to radioactive labelling of DNA, because the latter can be traced after only the first round of incorporation (Patau and Das 1961). Cells having metaphases with differentially stained monochromosomes (Fig. 2) had passed through two S-phases and cells with metaphases consisting of a mixture of differentially stained chromosomes and chromosomes with both chromatids stained lightly (Fig. 3), three S-phases. In addition, the staining pattern of diplochromosomes (Figs. 4–7) gives the number of S-phases. This pattern is fixed because the newly formed polynucleotide chains are found towards the outside of the chromatids (Fig. 1; Weber and Hoegerman 1980; Takanari and Izutsu 1981; Goyanes and Schvartzman 1981). Cells having diplochromosomes with the two inner chromatids stained darkly (Figs. 5–6) had passed through two S-phases and those with one inner chromatid stained darkly (Fig. 7), three S-phases. Mitotic restitution cycles do not interfere with the number of S-phases but they reduce the number of cell cycles. They were not observed and consequently the number of cell divisions which a particular cell had passed through until it was fixed in metaphase, was the same as the number of S-phases. A course of three cell cycles could thus be followed.

Cell cycle duration

Table 1 shows that metaphases (1x, 2x, 4x) with differentially stained monochromosomes which had passed through two replication cycles appeared for the first

Table 1. Numbers of metaphases and of normally or differentially stained monochromosomes and diplochromosomes in leaf pieces of a monohaploid *Solanum tuberosum* (2n = x = 12) cultivated on nutrient medium for 7 days

Duration of culture in days	No. of explants					No. of metaphases	No. of metaphases with yx[c] normally stained						No. of metaphases with yx differentially stained											
							monochromosomes			diplochromosomes			monochromosomes						diplochromosomes					
	Total	Without mitoses	With n.s.m.[a]	With n.s.m. + d.s.m.	With d.s.m.[b]		1x[d]	2x	4x	1x	2x	4x	1x[e]	1x	2x	2x	4x	4x	1x	1x	2x	2x	4x	
0	27	18	9			23	21	2																
1	27	21	6			35	35																	
2	24	16	8			58	31	10		14	3													
3	26	6	9			33	20	2	1	10														
				11		163	61	16	3	46	15		15		3		1				3			
4	22	5	8			19	13	5		1														
				9		111	35	12		8	8	2	7		8	2	5	1	6		16		1	
5	26	2	2			2	1	1																
				21		280	31	32	3	46	19		14	1	18		3	3	53		54	2	1	
					1	1														1				
6	23	0	2			6	4			1	1													
				18		177	29	19	2	15	17		13		17	1	6	5	35	2	16			
					3	22									3	3	2		7		7			
7	25	3		21		247	46	20	7	15	9		28	6	9	7	2	2	56		32	7	1	
					1	4													4					

[a] n.s.m. = normally stained metaphases;
[b] d.s.m. = metaphases with differentially stained mono- or diplochromosomes;
[c] yx = y haploid sets of (x = 12) mono- or diplochromosomes;
[d] ┼ = normally (darker) stained chromatid;
[e] ┼ = differentially (lighter) stained chromatid

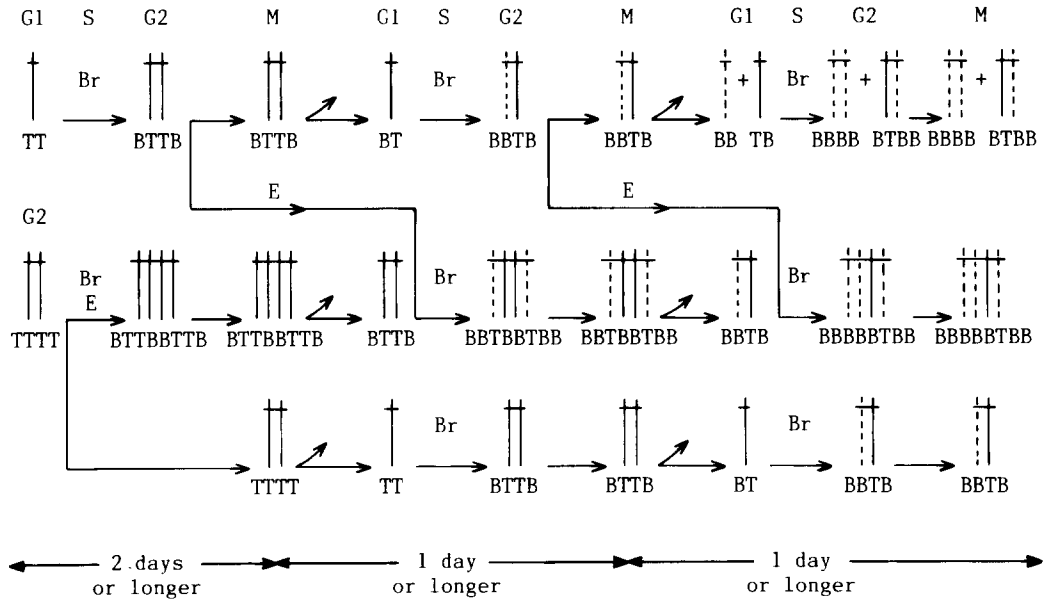
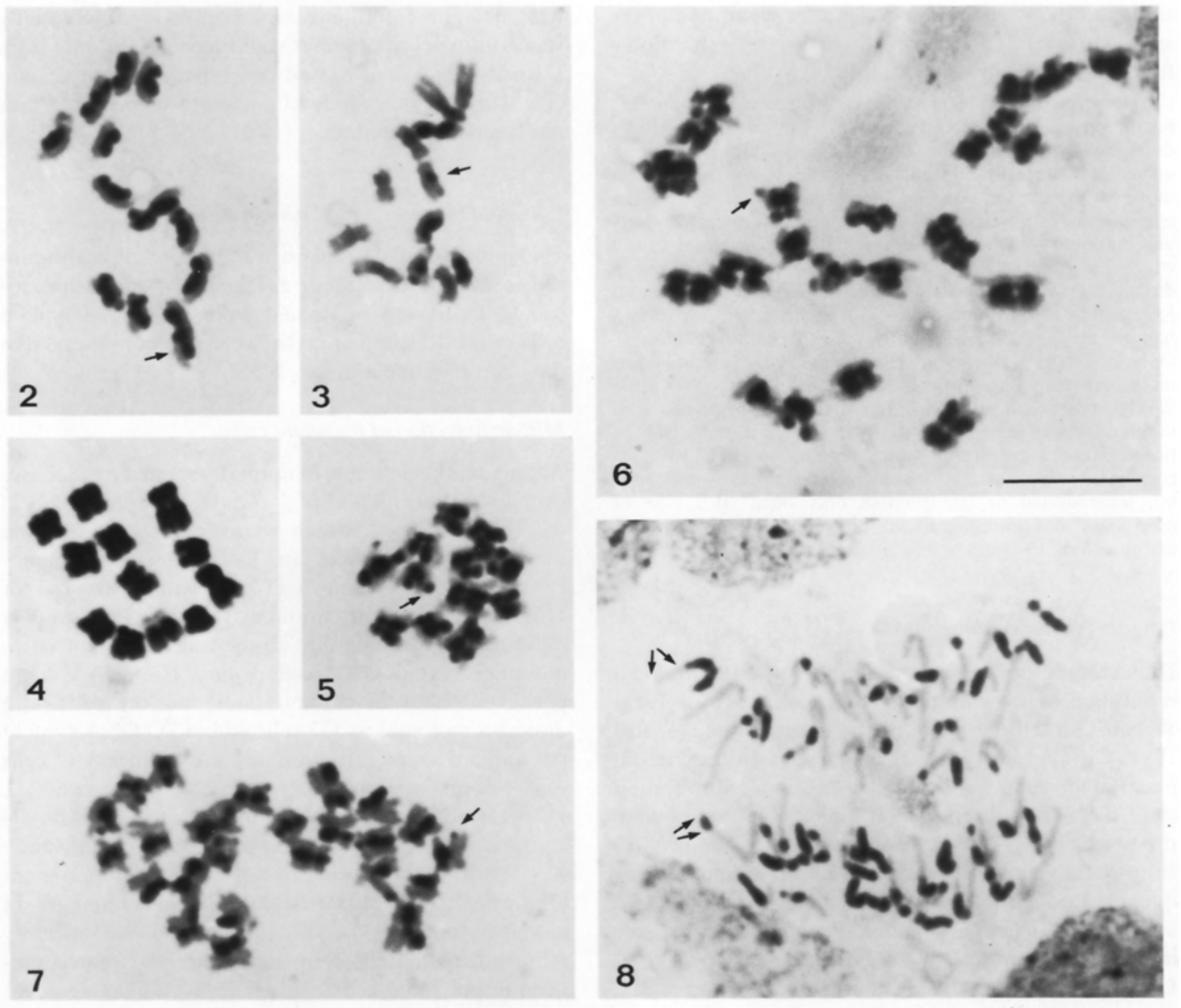


Fig. 1. Schematic representation of the incorporation of 5-bromodeoxycytidine into the chromosomal DNA and of the differentially stained chromosomes of leaf explants of monohaploid *S. tuberosum* during 4 days of cultivation. BrdC (Br or B) is incorporated into the DNA in place of thymidine (T) during each S-phase (S) of the cell cycle. Three cycles are presented. A TT helix becomes two BT helices, a BT helix becomes one BT and one BB helix, and so on. TT and BT chromatids (*solid lines*) stain darkly and BB chromatids (*broken lines*) lightly by the FPG-technique. An endoreduplication (E) starts either from a G1-phase (*connection between first and second row*) or from a G2-phase (*second row*) and results in diplochromosomes. The pattern of differentially stained chromatids of a diplochromosome is the result of the location of the new-formed DNA chain on the outside of the chromatids. After metaphase (M) the (diplo)chromosomes divide (⇜), but only one of the daughter(diplo)chromosomes is presented. See text

time at day 3 of culture, and metaphases which had undergone three cycles, at day 4 (2x, 4x) or at day 5 (1x). From such data the minimum duration of the third cell cycle in the presence of BrdC can be estimated, even in relation to the degree of polyploidy. The third division of diploid and tetraploid cells lasted one day and of haploid cells one day longer, perhaps because of deleterious effects of three rounds of BrdC incorporation (see below). If the cells cycled with a minimum duration of one day, which is sustained by the duration of cell cycles with diplochromosomes (see below), then a (time) scheme can be made as presented in Fig. 1. The only cell cycle which may be different is the first one because the cells have to return to the undifferentiated state. According to this scheme the first induced cell cycle from the onset of the experiment until metaphase was indeed one day longer than the following ones: this day apparently is the minimum time needed for dedifferentiation. Whether the G1 (=pre-DNA synthesis phase) cells and G2 (=post-DNA synthesis phase) cells reached the "first" metaphase after different times cannot be concluded from the data; for both an equal time has been assumed. The metaphases found at days 0 and 1 may then be considered to originate from cells which started mitosis before the experiment. Most explants showed the first induced mitoses on day 3 (see General) and normally stained metaphases remained to appear in explants of three days and older. This means that in cells of an explant mitosis could be triggered at any moment of the culture period. Consequently, the present data do not allow a calculation of the variation in the cell cycle durations, which also contributed to the asynchrony of the divisions.

Metaphases with diplochromosomes were not found in the explants at days 0 and 1 (Table 1) and the diplochromosomes were thus induced by the culture conditions as has been reported before (D'Amato 1977). The appearance of metaphases with normally or differentially stained diplochromosomes (1x, 2x, 4x), at day 2 and later, can be explained in the same way as has been done for monochromosomes (Fig. 1). Also, the minimum duration of a cell cycle with diplochromosomes was one day, since the first normally stained 2x diplochromosomes appeared at day 2 and the differentially stained ones at day 3 (second cycle), and 1x differentially stained diplochromosomes with two dark chromatids appeared at day 4 and with one dark chromatid at day 5 (third cycle).

The frequency of metaphases with monochromosomes which had incorporated BrdC three times, was rather low and positively correlated with the degree of polyploidy (Table 1). This correlation may be caused by the production of chromosomes with bifilarly substituted



Figs. 2–8. Mitotic figures of leaf explant cells of a monohaploid potato (2n = x = 12); **2** Differentially stained monochromosomes after two rounds of BrdC incorporation; **3** Idem after three rounds; **4** Normally stained diplochromosomes; **5** Differentially stained diplochromosomes with two darkly stained inner chromatids after two rounds of BrdC incorporation; **6** Idem of a polyploidised cell with 2x = 24; **7** Differentially stained diplochromosomes (2x = 24) with one darkly stained inner chromatid after three rounds of BrdC incorporation; **8** Anaphase of differentially stained diplochromosomes (2x = 24). *Arrows* point to some of the sister chromatid exchanges; Bar represents 10 μm

DNA only as such chromosomes can no longer function in transcription. This effect will be particularly deleterious in the haploid condition where it can cause a lengthening of the cell cycle or may be lethal to the cell before metaphase has been reached. In a similar way the frequency of cells with diplochromosomes which had passed three replications may have been influenced.

Polyploid numbers of monochromosomes

The metaphases were haploid at day 0, except for two (= 10%) which were diploid and occurred in two explants (Table 1). The plants were thus slightly mixoploid which has been verified by cytophotometric measurements on nuclear DNA of leaves of shoot cultures of the same genotype by Tempelaar et al. (1985). They found 58% of the cells with 1C DNA content indicating 12 chromosomes in G1-phase, 37% with 2C indicating 12 chromosomes in G2-phase and/or 24 chromosomes in G1-phase and < 5% with 4C indicating 24 chromosomes in G2 and/or 48 chromosomes in G1. Since the metaphase indices are < 0.4% (see "General"), the diploid and tetraploid metaphases with monochromosomes may have originated from polyploid cells already existing in the plants. During the first divisions polyploid cells may also have arisen

through mitotic restitution cycles after separation of the sister chromatids has taken place or through spindle fusion in binucleate cells (Nagl 1978).

However, these processes were not observed. Supposing restitution and fusion would occur, they cannot be detected by differential staining of the chromosomes. Chromosomes involved in such restitution and fusion cycles stain similarly to those which pass the same number of normal cell cycles. The chromosomes of a tetraploid cell, for instance, stain similarly and differentially whether they originate from a tetraploid cell which has passed two cell cycles (with two S-phases), from a diploid cell which has passed one cell cycle (with one S-phase) followed by a restitution cycle (with one S-phase), or from a haploid cell with two restitution cycles (with two S-phases). Only the origin of polyploid cells of which complete basic sets of chromosomes stain differently, that is to say the one set(s) darkly and the other set(s) differentially, can be traced with differential staining. These cells can originate only from a fusion between a cell(s) of which the chromosomes have incorporated twice, and a cell(s) of which the chromosomes have not incorporated or incorporated only once. This type of metaphase, and consequently this type of polyploidisation, was not observed.

Origin of diplochromosomes

Diplochromosomes are the result of an additional replication of two-chromatid chromosomes (Nagl 1978; Weber and Hoegerman 1980; Takanari and Izutsu 1981; Goyanes and Schvartzman 1981). In the present material this replication was likely an endoreduplication since it was not observed that diplochromosomes were the product of a mitotic restitution cycle. The in vitro culture thus induced endoreduplication and, consequently, through that polyploidisation. It remains to be investigated whether medium components as, for instance, the hormones NAA and BAP (Partanen 1963; Libbenga and Torrey 1973), are involved in the induction process.

Diplochromosomes arise, in general, from G2-phase chromosomes which have been stimulated to an additional S-phase. The presence of mitotic cells in some explants, already at day 0 (Table 1), and the nuclear DNA contents (Tempelaar et al. 1985; see "Polyploid number of monochromosomes") indicate the presence of G2 cells. So, in this material the diplochromosomes may also have been the result of an additional S-phase triggered in a G2 cell. Differentially stained diplochromosomes are obtained if the diplochromosomes cycle further as diplochromosomes (Fig. 1) and do not fall apart into monochromosomes as generally occurs (D'Amato 1952, 1977). In the present material the diplochromosomes indeed cycled normally because the two chromatids of the daughter chromosomes did not separate during anaphase (Fig. 8). However, it cannot be excluded that the chromatids did separate (invisibly) during the next interphase and prophase and appeared as differentially stained monochromosomes during the next metaphase. In that case the cells with differentially stained diplochromosomes could have arisen only from G1 cells which had passed two S-phases in succession (Fig. 1). This latter mode of polyploidisation could thus not be demonstrated.

Polyploid numbers of diplochromosomes

The diploid and tetraploid numbers of diplochromosomes can be explained, as above for monochromosomes, as having originated from existing polyploid cells in G2. Restitution cycles were also not observed in diplochromosome mitoses.

Mitotic induction of polyploid cells

Adding up the numbers of normally stained metaphases over the culture period of 2–7 days, the frequencies of the cells are found which were dividing for the first time. These frequencies are for cells with 1x monochromosomes 271, with 2x 117 and with 4x 16, and for cells with 1x diplochromosomes 156, with 2x 72 and 4x 2 (Table 1). Assuming that all the cells with monochromosomes started as G1 cells with a 1C DNA content per haploid set of chromosomes and the cells with diplochromosomes as G2 cells with a 2C DNA content per haploid set of chromosomes, the frequency of cells can be presented as follows: 271 cells with 1C, $117+156=273$ cells with 2C, $16+72=88$ cells with 4C and 2 cells with 8C. These data differ significantly ($P=1\%$) from the frequencies of nuclear DNA content (Tempelaar et al. 1985; see "Polyploid numbers of monochromosomes"). This means that significantly more polyploid cells were in mitosis and that consequently polyploid cells had a selective advantage over haploid cells in being triggered to mitosis.

Increase in polyploid cells

The total percentage of polyploid metaphases increased to about 68 at day 7 of culture (Table 1). About 19% of these metaphases showed monochromosomes (2x = diploid and 4x = tetraploid) and about 49% diplochromosomes (1x = diploid, 2x = tetraploid and 4x = octoploid). These percentages indicate that the frequency of polyploid cells can increase considerably during callus initiation, which is in accordance with earlier investigations on polyploidisation in tissue cultures of various plants (D'Amato 1977; Bayliss 1980).

Sister chromatid exchanges

Sister chromatid exchanges were found in differentially stained mono- and diplochromosomes of almost all the explants (Figs. 2, 3, 5–8). They were visible even in the smallest chromosomes of the complement. In the same

explant the numbers could range from 0 to 8 per basic set of chromosomes. It remains to be resolved which factors are involved in the induction of these variable numbers of exchanges. Sister chromatid exchange analysis is used for detecting mutagenic agents (Kato 1977; Wolff 1977; Latt 1981). So the present method can also be used to test the effect of the (chemical) environment on the chromosomes during in vitro culture.

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