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Phenotypic variation and ploidy level of plants regenerated from protoplasts of tetraploid potato (*Solanum tuberosum* L. cv. 'Bintje')

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Summary. A wide range of phenotypic variation occurred among protoplast – derived plants of tetraploid potato cultivar 'Bintje'. The variant plants had alterations in growth and vigour, and in leaf and stem characteristics. The results suggest that the altered morphologies are caused predominantly by changes in ploidy levels. Some alterations could be attributed typically to octoploidy and aneuploidy. The occurrence of mixoploidy indicates that at least part of the observed variation arose during culture stage. The exogeneous cytokinin or auxin level and their combination during in vitro phase influenced the frequency of the variants observed. The origin of variation is discussed.

Key words: Potato-protoplasts – Genetic stability – Growth hormones – *Solanum tuberosum* – Regenerants – Transformation of somatic cells

Introduction

Because of many advantages of the protoplast system for genetic manipulation with plant regeneration being a prerequisite, much attention is being given to reproducibility and efficiency of regeneration from protoplasts of many crop plants (Kartha et al. 1974; Thomas et al. 1976, 1982; Shepard and Totten 1977; Vasil et al. 1979; Kao and Michayluk 1980; Santos et al. 1980; Binding et al. 1981; Koblitz and Koblitz 1982 a, b). A series of recent reports on successful regeneration and on spontaneous variation among the regenerated plants from protoplasts of commercial potato cultivars (Shepard and Totten 1977; Butenko et al. 1977; Mattern et al. 1978; Shepard et al. 1980; Thomas 1981; Secor and Shepard 1981; Gunn and Shepard 1981; Shepard 1981, 1982; Thomas et al. 1982; Karp et al. 1982) stimulated interest in this variation both from the point of view of using it in plant breeding, and because it may be undesirable when stable reproduction of a specific genotype is essential.

The Dutch commercial cultivar 'Bintje' was obtained by cross breeding as early as 1904, and is still the most important and widely grown variety in The Netherlands because of its excellent quality for domestic use. However, it has one drawback, i.e. susceptibility to most potato diseases. A variety that is equal to 'Bintje', but with better disease resistance has so for not been conventionally bred using 'Bintje' as 'Bintje' is highly heterozygous and male sterile.

As an alternative approach, genetic transformation of somatic cells might be used. Presently, a procedure of plant regeneration from protoplasts of shoot cultures of 'Bintje' has been developed (Bokelmann and Roest 1983). This article reports the first results on morphological characterization and ploidy level of regenerants from protoplasts of 'Bintje'.

Materials and methods

Plant material: explant source and regenerated plants

Axenic shoot cultures of plants grown from tuber pieces (with single eyes) of the cultivar 'Bintje' of *Solanum tuberosum* L. (2 n = 4 x = 48) have been used as the source material for protoplast isolation. For details on the procedures of in vitro propagation of shoot cultures, and for protoplast isolation, culture and regeneration, reference is made to Bokelmann and Roest (1983). The method of protoplast isolation and culture described for dihaploid clones of potato by Binding et al. (1978) was used for 'Bintje' with some modifications. The culture media used were 1) V-KM medium composed of the V-47 medium of Binding (1974) without NH_4NO_3, and organ-

ic nutrients of the KM-8 p medium of Kao and Michayluk (1975) without riboflavine for protoplast culture in liquid and semi-solid media, 2) MS medium (Murashige and Skoog 1962) with benzylaminopurine (BAP) and naphthalene acetic acid (NAA) for callus growth and 3) MS medium with different combinations of BAP or zeatin riboside (ZR), and NAA and gibberellic acid (GA_3) for shoot regeneration. Under these conditions, several hundreds of plants could be obtained from adventitious shoots about 3–4 months after protoplast isolation.

Morphological characterization

All the regenerated plants and control plants of 'Bintje' (plants from tuber pieces and from in vitro cultivated shoots) were grown under controlled conditions (17 °C during the day and 15 °C during the night at 15,000 lux, a photoperiod of 12 h and a relative humidity of about 80%). To analyse the type and extent of phenotypic variation among the regenerated plants, the following morphological characters have been studied: growth and vigour of plants, leaf characters, such as colour, shape, texture and size, and axillary branching. The characters of each of the regenerated plants were scored several times during the different periods of growth and compared with those of the control plants.

Chromosome numbers

In potato the ploidy level of the plants can be reliably determined by scoring the number of chloroplasts in stomatal guard cells (L_1 layer), and this procedure has been routinely followed previously by several authors (Meinl and Rothacker 1961; Rothacker and Schäfer 1961; Frandsen 1967a, b, 1978; Jacobsen 1978, 1982; Wenzel et al. 1979; Hermsen et al. 1981). Young leaves (mostly the fourth leaf from the top) of potted plants regenerated from protoplasts as well as from control 'Bintje' plants were fixed usually between 10–12 a.m. in ethanol: acetic acid (3 : 1 v/v) and stained with potassium iodide. Chloroplast staining was more satisfactory in fixed leaves than in non fixed leaves. Chloroplasts in 50 or more stomata were counted to determine the ploidy level of a plant. This procedure enabled the ploidies of 489 regenerated plants to be tentatively characterized. However, aneuploids cannot be distinguished by this method. For a portion of the plants, the somatic chromosome numbers in root-tip cells were counted which provided additional evidence of the estimated ploidy levels. Root-tips were collected from young potted plantlets. Two different techniques of fixation and staining were followed. First, the roots were pretreated with 8-hydroxyquiniline (0.002 M) for 4 h at about 2 °C and fixed in ethanol: acetic acid (3 : 1 v/v) for 24–72 h. The root-tips were hydrolysed in 1 N HCl at 60 °C for 8 min and squashed in propionic acid haematoxylin according to Henderson and Lu (1968). Second, roots were pretreated with a saturated solution of α-bromonaphthalene for 1½ h at room temperature, fixed in ethanol: acetic acid (3 : 1 v/v) for 24 h or more and stained by the Feulgen technique. Both methods gave satisfactory condensation and staining of chromosomes.

Results

Morphological variation and ploidy level of regenerated plants

The morphological description of plants showing variation in a few (1, 2 and 3) characters or more (> 3) is given in Table 1. The protoplast-derived plants showed a wide range of morphological variation. In all, 620 plants have been analysed. Among these plants, 394 (64%) were normal-looking, i.e. they resembled 'Bintje', and 226 (36%) differed in morphological characters. Some of the variant plants showed alteration in a few (1, 2 or 3) characters and others in several (> 3) characters (gross aberrants) (Figs. 1–6). The grossly aberrant plants can be easily distinguished from the other types.

The tetraploid (4 x) and octoploid (8 x) stomata (i.e. a pair of guard cells) contain the average numbers of 24 and 36 chloroplasts respectively, and they can be clearly distinguished from each other (Figs. 7 and 8). The plants which showed markedly deviating chloro-

Table 1. Alterations in morphological characters of some representative examples of plants derived from protoplasts of cv. 'Bintje'

Alterations in a few characters		Alterations in several characters (gross aberrations)	
Plant no.	Type of alterations	Plant no.	Type of alterations
1	Sparse hairs on leaves	6	Plant less vigorous and thin, leaf chimeric with light yellow sectors of different size on entire leaf, shrivelled and thick, curly and dense hairs on leaves
2	Short plant, secondary leaflets absent		
3	Narrow leaves, sparse hairs on leaves, secondary leaflets absent	7	Plant taller but weak, leaves with small yellow sectors of different size occurring on a few leaves, sparse hairs on leaves, more axillary shoots present
4	Shrivelled and irregular leaves		
5	Thin plant, small leaves		

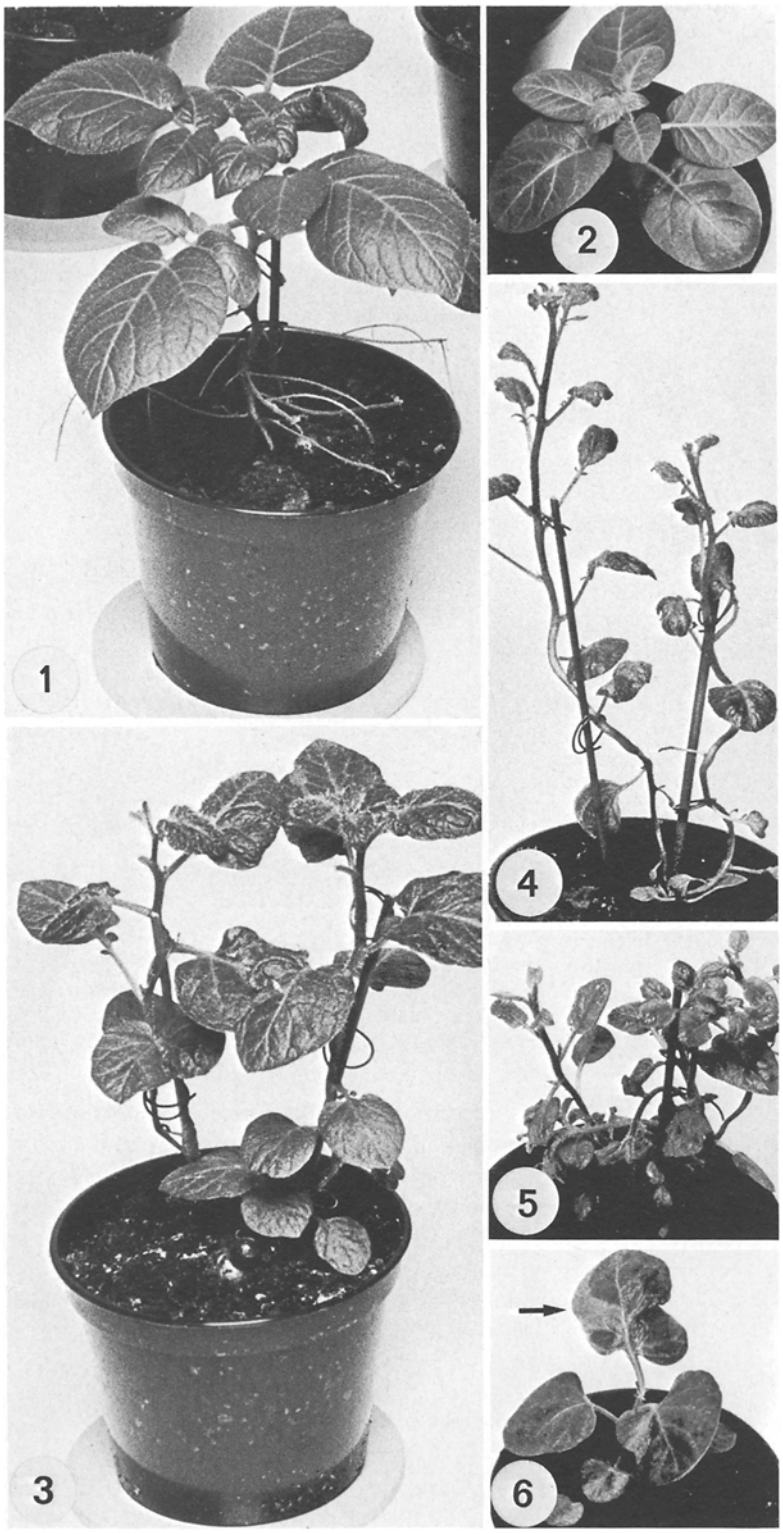
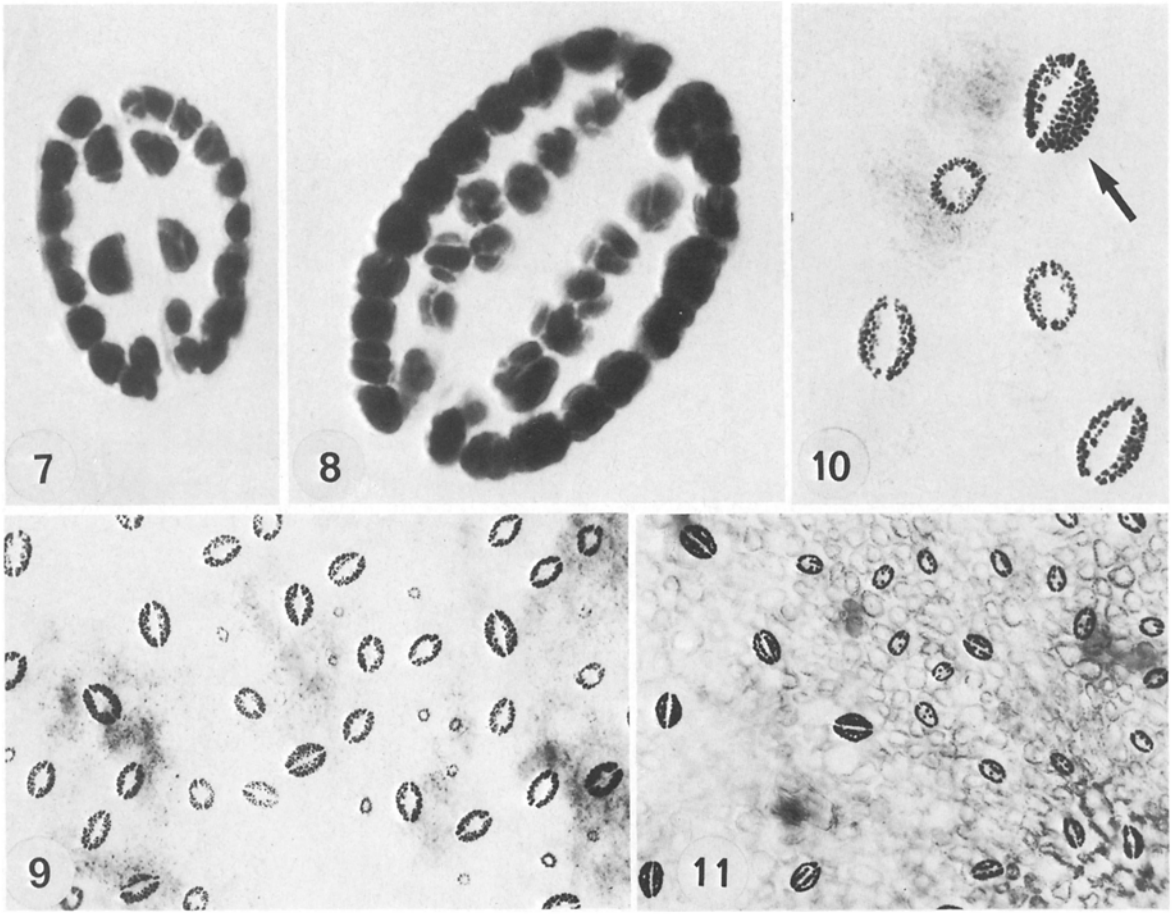


Fig. 1. Control plant of cv. 'Bintje' of *Solanum tuberosum* grown from in vitro cultured shoot

Figs. 2–6. Plants regenerated from protoplasts of cv. 'Bintje'. **2** A plant with round leaves and sparse hairs on leaves; **3** Grossly aberrant plant having chimeric leaves with chlorophyll deficient (yellow) sectors, deformed, single (secondary leaflets very poorly developed or absent), dense hairs on leaves; **4** Thin plant, small leaves with chlorophyll deficient sectors, single, and irregular in shape; **5** Plant having small and single leaves which were irregular in shape, chlorotic and rough in texture with dense hairs; **6** A plant with distinct chlorophyll deficient (yellow) sectors (arrow marked), single and deformed leaves, and sparse hairs on leaves



Figs. 7–11. Chloroplasts in stomatal guard cells of leaf epidermis in protoplast-derived plants of cv. 'Bintje' of *Solanum tuberosum*. **7** Stomata with 25 chloroplasts in both guard cells of tetraploid plant; **8** stomata with 35 chloroplasts in both guard cells of an octoploid regenerant; **9** Stomata showing mixoploidy in a grossly aberrant plant; **10** Stomata of a mixoploid plant with two guard cells, one containing 20 chloroplasts and the other more than 30 (arrow marked); **11** Stomata of a mixoploid plant: note one part (left side of the figure) containing octoploid stomata and the other tetraploid and "other ploid" stomata

plast numbers from those of the tetraploid or octoploid level, were tentatively classified as "other ploids".

About 80% of the 316 normal-looking plants showed tetraploid chloroplast numbers. A high frequency of the morphologically variable plants (82% of 173 plants) had altered ploidy levels (Table 2). Among the grossly aberrant plants octoploids occurred at a high frequency.

In the course of screening for chloroplast numbers, it was found that most of the grossly aberrant plants contained not only the stomata of octoploid level, but also the stomata of "other ploid" levels (Fig. 9). Therefore, for 27 plants randomly chosen from the group of gross aberrants, chloroplast counts were made in a large number of stomatal guard cells (Table 3). The results showed that 22 were mixoploid. Among them,

Table 2. Morphological variation and ploidy level of plants regenerated from protoplasts of tetraploid cv. 'Bintje' of *Solanum tuberosum* (2n = 4x = 48)

	Normal-looking plants			Plants showing variation in					
				a few characters			several characters		
	4x	8x	other	4x	8x	other	4x	8x	other
No.	250	0	66	27	8	42	4	73	19
%	51.1	0	13.5	5.5	1.6	8.6	0.8	14.9	3.9

Table 3. Chloroplast counts in stomatal guard cells of grossly aberrant plants. Four groups of plants can be distinguished, namely plants with 1) octoploid cells only, 2) octoploid and tetraploid cells, 3) octoploid, tetraploid and diploid cells and 4) octoploid, tetraploid, diploid and haploid cells

Group no.	No. of plants	Numbers of guard cells containing the mean number of chloroplasts corresponding to each ploidy level, i.e. (x), etc.			
		4.2 (x)	7.4 (2x)	12.7 (4x)	18–32 (8x–> 8x)
1	5	0	0	0	2508
2	12	0	0	85	3202
3	3	0	8	18	595
4	7	22	26	134	331

15 plants had a high frequency of octoploid cells and a low proportion of tetraploid and/or diploid cells. However, the 7 other plants contained, in addition to octoploid cells, a considerable proportion of "other ploid" cells.

In 4 of the 22 mixoploids a few stomata had different chloroplast numbers in the two guard cells, with two combinations: 13 [4 x] and 19 [8 x]; 20 [8 x] and 30 [probably 16 x] (Fig. 10). In two other mixoploid plants, one contained a very small sector of 13 tetraploid stomata among the several octoploid stomata, and the other had two distinct ploidy-sectors in a leaf, one showing octoploid stomata and the other tetraploid as well as "other ploid" stomata (Fig. 11).

In mixoploid plants gross morphological aberrations similar to those of plants 6 and 7 (Table 1) were observed. In particular, the mixoploids were characterised by having leaves with chlorophyll deficient sectors.

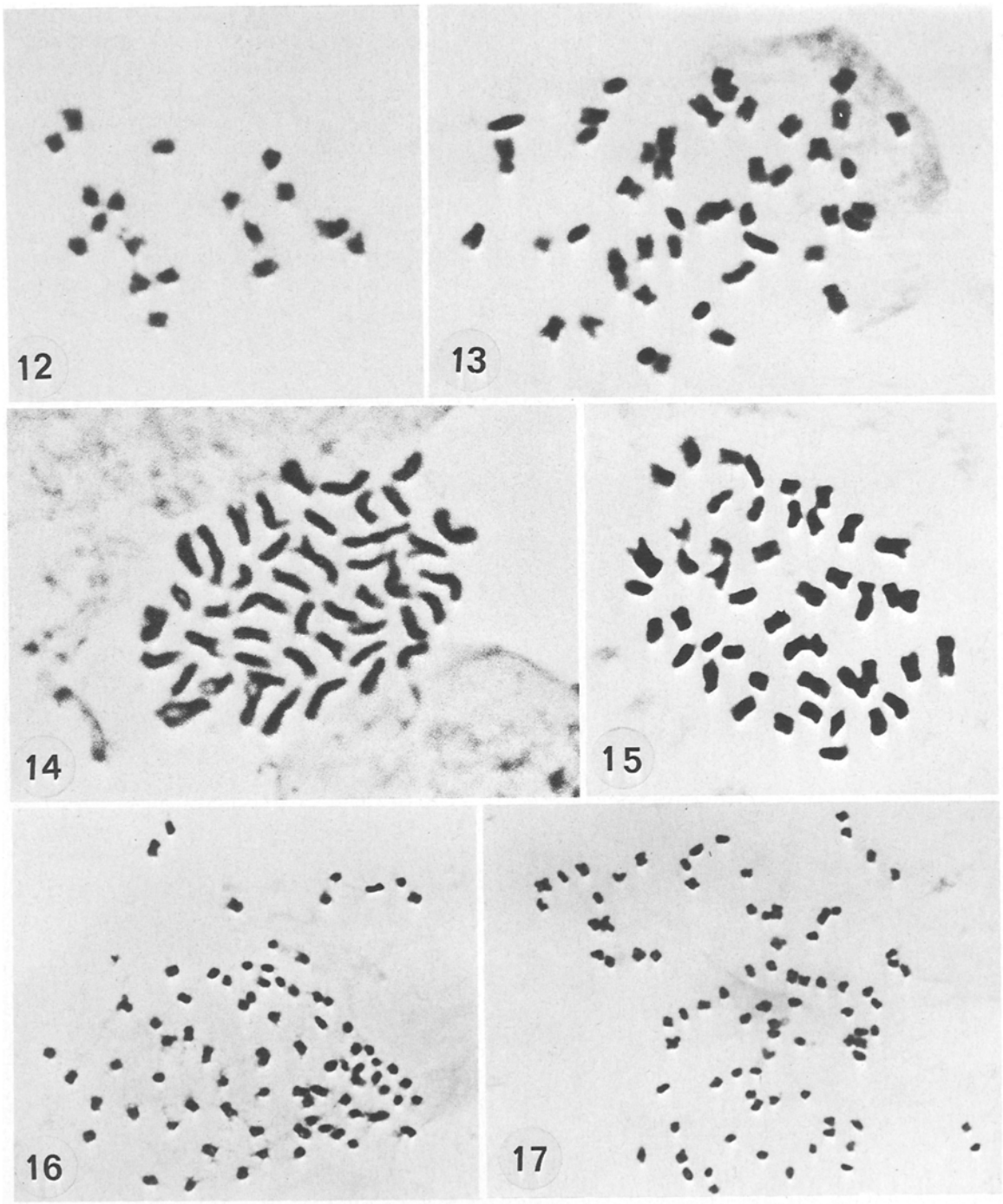
Chromosome counts in root-tip cells of regenerated plants

The data on chromosome counts of root-tip cells in each of the three groups of regenerated plants, namely 1) normal-looking plants, 2) plants showing variation in a few characters and 3) plants showing variation in several characters (gross aberrants), are given in Table 4. The ploidy levels estimated from chloroplast numbers for the plants of these groups were tetraploid, "other ploid" and mixoploid respectively. The data on chromosome counts made for plants of groups 1 and 3

Table 4. Chromosome counts in root-apices of plants regenerated from protoplasts of tetraploid cv. 'Bintje' of *Solanum tuberosum* (2n = 4x = 48). Chromosome numbers (mostly aneuploid) are reported in parentheses

Type of regenerated plants	No. of plants[a]	Distribution of mitotic cells with chromosome numbers as grouped below						
		12 (x)	13–23	24 (2x)	25–47	48 (4x)	49–95	96 (8x)
Normal-looking plants (group 1)	12					35		
Plants showing variation in a few characters (group 2)	1[b]				7 (39)			
	1[b]				4 (43)			
	3				7 (44)			
	1				4 (45)			
	7[c]				20 (46)			
	2				8 (47)			
	2					5		
	1	3	2 (18)	2	22 (28, 30, 32, 36, 40, 41, 43, 44)	7		
Plants showing variation in several characters (group 3)	1				2 (40)			
	2				6 (46)			
	1	1	1 (23)	4	2 (36)	3	4 (60, 66, 89)	5
	1						6 (56, 60, 83, 88, 93)	
	1					1	3 (72, 77, 86)	
	1			2	2 (36, 45)	3	5 (60, 72, 87)	
	1						2 (60, 72)	1
	1			1	3 (36)	2		
	1	1	1 (18)		7 (44, 47)	2	1 (72)	
	1			1	5 (30, 40, 44)			
	1				3 (38, 46)			
	1				2 (44)	2		

[a] Plants with specific chromosome number
[b] These plants were classified as tetraploids from chloroplast number
[c] One classified as tetraploid from chloroplast number



Figs. 12–17. Root-tip metaphase cells showing different chromosome numbers in mixoploid plants regenerated from protoplasts of cv. 'Bintje' of *Solanum tuberosum.* **12** A cell with 18 chromosomes; **13, 14** and **15** Hypotetraploid cells with 45, 46 and 47 chromosomes respectively; **16** and **17** Aneuploid cells showing 78 and 83 chromosomes respectively

correlate rather well with the estimates of ploidy levels based on chloroplast numbers. In the case of plants of group 2, three plants estimated as tetraploid each contained an aneuploid chromosome number, thus indicating that aneuploids cannot be distinguished by the procedure of chloroplast counts.

Fourteen of the eighteen plants of group 2 were aneuploids with chromosome numbers ranging from 39–47. The plants of group 3 contained mitotic cells with different chromosome numbers in addition to octoploid chromosome numbers (chromosome mosaicism) (Figs. 12–17).

Table 5. Frequencies of protoplast-calli that regenerated only normal-looking plants (group 1), only variant plants (group 2) and mixtures of normal and variant plants (group 3), and the ploidy of regenerated plants. Percentages are reported in parentheses

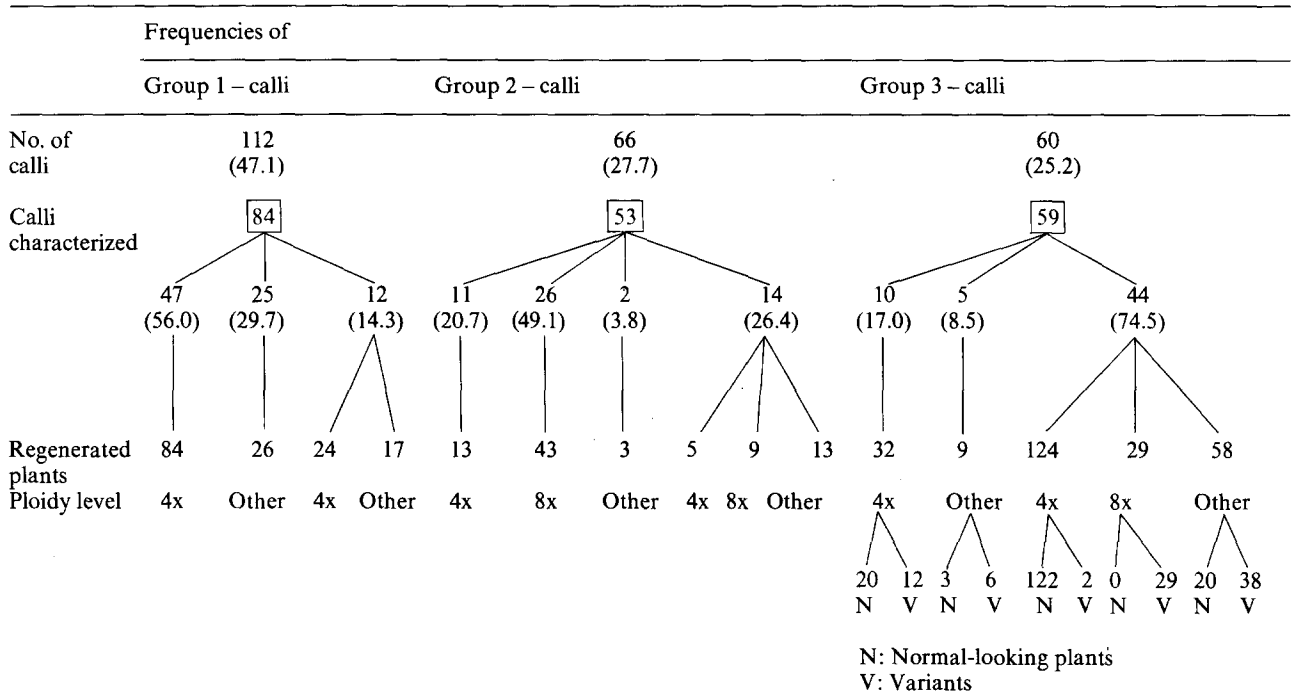


Table 6. Growth hormones in regeneration medium and the percentages of P-calli that regenerated only normal-looking plants (group 1), only variant plants (group 2) and mixtures of normal and variant plants (group 3). Observations were made on regenerated plants. The numbers of regenerated plants are given in parentheses

Growth hormones (mg/l)	No. of calli analysed	% group 1 calli	% group 2 calli	% group 3 calli	Average no. of plants regenerated per callus
ZR 1.0	41	53.7	17.1	29.2	2.5 (102)
ZR 1.0 + NAA 0.01	27	44.4	33.3	22.3	3.0 (81)
ZR 1.0 + NAA 0.01 + GA_3 0.01	39	38.5	33.3	28.2	2.7 (104)
BAP 1.0	17	82.3	5.9	11.8	1.3 (23)
BAP 1.0 + NAA 0.01	12	83.3	16.7	0	1.3 (16)
BAP 1.0 + NAA 0.01 + GA_3 0.01	25	64.0	20.0	16.0	1.6 (41)

Characterization of protoplast-calli

As such the calli were not analysed, but the morphological characters and the ploidy level (based on chloroplast number) of each plant regenerated from a given callus of a protoplast have been individually registered so that each protoplast-callus (p-callus) could be characterized separately. From the type of plants regenerated, the calli could be classified into three groups, namely those yielding: 1) only normal-looking plants, 2) only variant plants and 3) mixtures of normal and variant plants (Table 5).

In all, 238 p-calli which regenerated 589 plants were characterized. The calli of group 1 occurred at a higher frequency (47.1%) than those of group 2 or group 3. Group 1 also contained a high percentage (56.0) of tetraploids. By contrast, octoploid plants were regenerated only from the calli of groups 2 and 3.

In addition, it can be seen from the data that in each group some calli gave plants of the same ploidy while others produced mixtures of plants that showed different ploidy levels. The latter category occurred at higher frequencies in groups 2 (26.4%) and 3 (74.5%).

The phenotypic variability within the group of plants derived from a given p-callus was also higher in these two groups.

Growth hormones in regeneration medium and frequency of the variants

As compared to ZR or ZR + NAA, BAP or BAP + NAA gave higher percentage of group 1 calli that regenerated only normal-looking plants, and lower frequencies of calli of groups 2 and 3 which gave rise to abnormal plants and mixtures of normal and variant plants respectively (Table 6). The addition of GA_3 resulted in the decrease of the frequency of group 1 calli.

However, the media containing ZR alone, or together with NAA and/or GA_3, gave relatively more plants per callus. These treatments increased the frequency of plants with abnormal ploidy level (data not given).

In addition, ZR (1.0 mg/l) with a higher concentration of NAA (0.03 mg/l) or GA_3 (0.03 and 3.0 mg/l) gave rise to calli of groups 2 and 3 that regenerated a greater proportion of morphologically aberrant plants characterized by having predominantly abnormal ploidy levels.

Discussion

The results show a wide range of phenotypic variation among regenerants from protoplasts of potato cv. 'Bintje'. This phenomenon has previously been reported in plants regenerated from protoplast or tissue cultures of other potato cultivars and also of other crop species.

The probable causative factors, such as polyploidy, aneuploidy, chromosome aberrations and gene mutations, have been extensively discussed (Matern et al. 1978; Shepard et al. 1980; Secor and Shepard 1981; Shepard 1981, 1982; Thomas et al. 1979, 1982; Van Harten et al. 1981; Jacobsen 1981; McCoy et al. 1982; Sree Ramulu 1982; reviews in Skirvin 1978; D'Amato 1978; Larkin and Scowcroft 1981; Sybenga 1981).

The morphologically abnormal regenerants showed abnormal ploidy levels at a high frequency. They contained aneuploids and mixoploids. Mixoploidy (chromosome mosaicism) occurred predominantly in grossly aberrant plants (Tables 3 and 4). The phenomenon of chromosome mosaicism has previously been observed in plants regenerated from in vitro cultures of a wide range of plant species, including potato (Sacristan and Melchers 1969; Heinz et al. 1969; Ogura 1976; Mix et al. 1978; Bennici and D'Amato 1978; Binding et al. 1978; Bennici et al. 1979; D'Amato et al. 1980; Novak 1980; Nuti Ronchi et al. 1981; Browers and Orton 1982; Jacobsen 1981; Lupi et al. 1981; Caffaro et al. 1982).

Karp et al. (1982) obtained a high degree of aneuploidy among the protoplast-derived plants of two potato cultivars ('Maris Bard', 'Fortyfold') and concluded that some of the observed morphological variation can be accounted for by variation in chromosome number. The grossly aberrant plants regenerated from protoplasts of another tetraploid cultivar 'Russet Burbank' may be expected to contain altered chromosome numbers (Shepard 1982).

In addition, the results by Gill (pers. commun.) on the occurrence of chromosomal translocations, bridges, fragments and laggards in meiosis of some protoclones of 'Russet Burbank' suggest that chromosome aberrations also contribute to the total variation. However, with regard to gene mutations, the detection and analysis are not easy because of high heterozygosity, tetraploidy and male sterility of the commercial potato cultivars, including 'Bintje'.

More recently, Karp et al. (1982) have attempted to explain the origin of chromosome variation in shoot cultures of plants regenerated from protoplasts of tetraploid potato cultivars. Three possibilities have been mentioned, i.e. variation occurring 1) in explant source (fully expanded leaves, or shoot cultures of control plants), 2) in callus phase and 3) during the propagation through shoot cultures of regenerated plants.

In potato occasionally plants with doubled chromosome numbers were obtained in control material after propagation through tuber pieces (Howard 1961; Frandsen 1967a). The occurrence of polyploidy as well as mixoploidy has also been found in control shoot cultures of dihaploid potato clones (Binding et al. 1978). Chromosome loss found during the passages of shoot cultures of plants regenerated from protoplasts of tetraploid cultivars (Karp et al. 1982) is a further evidence of generation of chromosome instability in shoot cultures. In addition, preliminary results on the ploidy level of control shoot cultures of 'Bintje' indicated the occurrence of octoploidy (unpublished results). Mixoploidy (chromosome mosaicism) has also been found in control shoot cultures of monohaploid potato (E. Jacobsen, pers. commun.).

Unless an efficient technique of detecting ploidy changes in shoot cultures is routinely followed, the possibility of explant source variation cannot be completely excluded. In this case, it is desirable to use a technique of multiplication of explants which assures genetic stability, e.g. culture of shoot apices. Furthermore, mitotic reactivation of endoreduplicated nuclei, if present in explants, can give rise upon culture to cells of various ploidies. Cytophotometric measurements of nuclear DNA content in explants, protoplasts and calli might resolve this problem.

The observation that in a high frequency of calli (43%) the plants from the same callus were different in phenotype and/or ploidy (Table 5) implies that variation arose during culture phase. However, the possibility that the calli are not derived from single protoplasts cannot be excluded. If this is true, it may probably account only for a low fraction of calli. Furthermore, it should be mentioned that the phenomenon of nuclear fragmentation followed by mitosis which occurs at an earlier stage of culture, can give rise to a wide variation in ploidy level both in calli and in regenerated plants (D'Amato et al. 1980; Lupi et al. 1981).

The chromosome mosaicism of the regenerated plants is probably originated from one or more initial cells in a callus, which are cytologically unstable giving rise during ontogeny, to variable chromosome numbers (Nuti Ronchi et al. 1981; Caffaro et al. 1982).

The demonstration that exogenous auxin and cytokinin levels can influence chromosome variation (Torrey 1961, 1967; Matthijsse and Torrey 1967; Bennici et al. 1968, 1971; Singh and Harvey 1975; D'Amato et al. 1980; Bayliss 1980; Evans and Gamborg 1982) suggest that conditions favouring stability might be established experimentally. The present data support this suggestion: in the presence of BAP or BAP+NAA, the frequencies of calli that regenerated normal-looking plants were higher than those in the presence of ZR or ZR+NAA. ZR or ZR+NAA increased the frequency of plants with abnormal ploidy level.

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