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Phenotypic and karyotypic status of *Beta vulgaris* plants regenerated from direct organogenesis in petiole culture

C. Detrez*, R.S. Sangwan and B.S. Sangwan-Norreel

Laboratoire Androgenèse et Biotechnologie, U.F.R. de Sciences, 33 rue St. Leu, F-80039 Amiens Cedex, France

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Summary. A method for high frequency in vitro regeneration from petiole explants was tested on nine breeding lines of *Beta vulgaris* L. from the haploid, diploid and tetraploid levels. Regenerants could be obtained without a callus step, from excised petioles derived either from axillary buds sprouted in vitro or from field grown plants, by plating the explants on MS medium supplemented with TIBA (2,3,5-triiodobenzoïc acid) and BAP (6-Benzylaminopurine). The multiple shoots obtained were then rooted in vitro and transferred to soil. In some cases, these adventitious shoots were also used as a petiole explant source for further petiole culture cycles, and the phenotypic characteristics and ploidy status of the regenerants were investigated after one or three petiole culture cycles. Conventional shoot apex culture was used as an in vitro control. Phenotypic variations such as differences in morphology and changes in in vitro growth behaviour, were noticed. Chloroplast and chromosome counts indicated that the alterations in morphogenetic pathway could not be explained by the occurrence of gross cytogenetic abnormalities such as aneuploidy or myxoploidy. Our results suggest that the altered morphology is caused by the presence of the exogenous antiauxin (TIBA) during the in vitro phase. Following transfer to the greenhouse, none of these variations persisted and cytogenetic analyses revealed karyotypic stability in all the plants studied, even after three petiole culture cycles. An assessment of the in vitro petiole culture method as a true-to-type multiplication method for *Beta vulgaris* is made.

Key words: Direct organogenesis – Somaclonal variation – *Beta vulgaris* – Beet breeding

* To whom correspondence should be addressed

Introduction

The techniques commonly used in plant tissue culture for large scale multiplication involve regeneration either of axillary buds from shoot apex or of adventitious shoots from non-meristematic material. The shoot meristem provides a starting material in which cell division and DNA replication is strictly controlled, so genetic stability in the tissue culture cycle can be maintained. For *Beta vulgaris*, germplasm preservation and clonal plant micropropagation from shoot apex culture of young seedlings (Margara 1977; Hussey and Hepher 1978; Atanassov 1980) or of inflorescence pieces (Margara 1977; Coumans-Gilles et al. 1981; Miedema 1982) are well established.

In contrast, adventitious regeneration, and also techniques such as callus and protoplast culture, are well known to generate somaclonal variation (D'Amato 1977; Larkin and Scowcroft 1981; Meins 1983). This can be advantageous in agricultural applications, as the production of genetic variability from tissue culture may contribute to the overall breeding strategy for the development of improved crops. In sugar beet, callus can be initiated from almost any tissue (Atanassov 1986) and its subsequent growth is easily achieved. On the contrary, regeneration of viable plants has only been possible at a low frequency (Margara 1970; Butenko et al. 1973; Hooker and Nabors 1977), except in some callus lines which have a high regeneration capacity (De Greef and Jacobs 1979; Saunders and Daub 1984; Van Geyt and Jacobs 1985; Saunders and Doley 1986). In our laboratory, somatic embryogenesis from callus of *Beta vulgaris* has recently been achieved (Tetu et al. 1987). However, improvements of all these techniques are necessary before it will be possible to assess the potential application of using a callus step to generate variation for plant

breeding purposes. Moreover, determination of the underlying genetic basis and analysis of the sexual transmittance to the progeny must be undertaken.

In a previous report (Detrez et al. 1988), we described a routine protocol for high frequency direct organogenesis from petioles and thin cell layer explants containing no pre-existing meristem. The morphogenetic capacity of petiole explants of *Beta vulgaris* has been observed by other workers (Saunders and Mahoney 1982; Hussey and Hepher 1978; Rogozinska and Goska 1978; Saunders 1982; Freytag et al. 1988) and this protocol is well known as a suitable method for propagation of other species (Van Harten et al. 1981; Cassells and Carney 1987; Pais et al. 1987; Cheema and Sood 1987). However, in some cases, for instance in *Pelargonium* (Cassels and Carney 1987), some of the regenerants and their progeny have shown variation in foliar or flower characteristics (up to 16% of the adventitious shoots). This was achieved without a callus step. In fact, all techniques of in vitro culture which use non-meristematic explants are considered by numerous authors to produce genetic variability (Meins 1983; Reisch 1983), even without concomittant biochemical cellular selection.

Somaclonal variation in sugar beet is very little documented, except for a cytological investigation in callus tissues cultivated in vitro (Atanassov et al. 1976). Reports concerning the generation of in vitro variability by organogenesis from petioles of *Beta vulgaris* are restricted to some observations concerning variations in leaf structure (Saunders 1982; Saunders and Mahoney 1982; Freytag et al. 1988) or to some inconclusive remarks (Rogozinska and Goska 1978). We thus sought to examine the technique of high frequency adventitious regeneration from petiole explants of *Beta vulgaris*, with the objective of investigating somatic cell variation and of determining genome stability in vitro. To analyse whether our protocol could be considered as a true-to-type in vitro method, we report phenotypic diversity and changes in chromosome numbers among plants derived from petiole culture of *Beta vulgaris*. One and three cycles of adventitious regeneration have been studied.

Materials and methods

Plant material

The sugar beet lines used in the present study were: two diploid clones initiated from a genetic monogerm population (C64A, C64D), two triploid clones from the Rizor cultivar (RI5, RI7), two haploid clones (G-93, G-84, obtained by Doctrinal 1988) and two haplodiploid clones (G-VEC29, kindly given by Cérés-France, G-A48). For initiation of axenic shoot cultures, the sterilization of the seeds was carried out as previously described (Tetu et al. 1987).

In vitro plants used as source material for petiole isolation were multiplied by subculture of axillary buds in culture tubes (25 × 100 mm) on inorganic salts, aminoacids and vitamins of MS (Murashige and Skoog 1962) supplemented with H_3BO_3 (3.8 mg/l) and BAP (0.8 mg/l) (referred to here as M medium). For detail on this procedure, reference is made to Detrez et al. (1988).

Field-grown adult sugarbeet plants of strain E321 (2N, polygerm) were kept on vermiculite:soil mixture (1:3) in a glasshouse natural daylight cycle, with supplementary artificial lighting (about 32 $\mu Em^{-2} s^{-1}$). They were treated weekly with half-strengh MS nutrient formulation. Petiole explants were taken from several expanded leaves. The detached leaves were surface-sterilized for 20 min by soaking in a 10% (W/V) calcium hypochlorite solution, followed by three rinses with sterile distilled water. Petioles were obtained from three sources: (i) non-vernalized plants, (ii) vernalized plants before elongation of inflorescence pieces, and (iii) flowering sugarbeet.

In vitro culture

Petiole explants (0.5–1 cm), excised from the blade petiole transition zone of in vitro cultured axillary shoots or of field-grown plants, were plated in Petri dishes (90 mm in diameter, 5 explants/dish) containing 30 ml of medium and sealed with polyvinyl film (Scelofrais).

All shoots, rooted plants and petiole cultures were maintained in 16 h daily light (125 $\mu Em^{-2} s^{-1}$), at 24 + 1 °C, with no humidity control.

Establishment of p1 and p3 populations of petiole-derived plants

Plant regeneration from excised petioles derived from shoot apex cultures was efficiently achieved on MS medium supplemented with TIBA (0.5 mg/l) and BAP (1 mg/l) (OD4-medium) (Fig. 1). This first regenerant population, referred to as p1, was used for a new in vitro bud induction cycle on the same medium and according to the same protocole. This led to a p2 population which, in turn, was submitted to a third petiole culture cycle to give the p3 generation.

From various combinations of factors, the best recovery of organogenesis from excised petiole explants from field grown plants was obtained with a sequence of three media: MS + BAP 5 mg/l + TIBA 0.1 mg/l; MS + BAP 2.5 mg/l + TIBA 0.5 mg/l; MS + BAP 1 mg/l + TIBA 1 mg/l. Subculturing was done every 15 days and macroscopic structures (Fig. 2) appeared approximately 40 days after the first inoculation. Rods and small calli were frequently observed on the blade petiole transition zone and, in addition, buds appeared on non-vernalized or on vernalized explants isolated before elongation of the inflorescence. In this study, only variations in the p1 population were analysed.

Plant rooting

De novo buds obtained from petiole culture were subcultured on M as necessary to provide sufficient clonal material of each line for experimental studies. Rooting was improved as compared to our previous paper (Detrez et al. 1988) by using R medium: major salts of MS at ⅓ strength for KNO_3 and NH_4NO_3, minor salts and vitamins of MS with IBA (1.5 mg/l). Development of viable plants proceeds via adventitious shoot formation to rooted plantlets in 5–7 weeks. Plantlets were then potted in vermiculite:soil mixture (1:1) in a greenhouse. They were covered with a plastic cup for the first ten days in order to maintain a high degree of humidity.

Studies of ploidy level

Determination of ploidy level is conventionally performed by means of chloroplast scoring in stomatal guard cells of the lower epidermis, using young leaves (Deuter 1970). These were fixed for 1 h in ethanol:acetic acid (3:1 v/v) and stained with potas-

sium iodide. Counts were established from a minimum of 15 stomata in each adventitious bud studied.

For a portion of plants, the chromosome numbers were determined in leaf meristematic tissue of individual plants. Young leaves collected from potted plantlets were pretreated with 8-hydroxyquiniline (0.002 *M*) for 4 h at about 4°C. They were fixed in ethanol: acetic acid (3:1 v/v) for 24–72 h. DNA-stained squash preparations were carried out in acetocarmin at room temperature and observations were made in 45% acetic acid solution. Screening analysis was performed from a minimum of five well spread cells, and on at least two different leaves, each at metaphase stage.

Control populations

In vitro axillary shoot populations acted as control material to study in vitro behaviour and for data concerning chloroplast and metaphasic chromosome counts. The control plants were propagated routinely according to the method of Detrez et al. (1988) and grown under the same conditions as the regenerated plants.

Results

Culture and regeneration

Since our studies have begun, 54 genotypes (i.e. seeds/in vitro clones), including the N, 2N, 3N and 4N ploidy levels, have been screened and assessed for their ability to regenerate plants in petiole culture. Genotypic differences have been observed as shown by the percentage of organogenic explants and the numbers of buds obtained per explant. Table 1 illustrates the differences in morphogenic capacity between the nine tested sugarbeet lines. However, no relation between "culturability" in petiole culture and ploidy of the donor was noticed. The considerable effect on "culturability" appeared to be rather a consequence of the pregrowth conditions of the donor plants. For instance, the best results for de novo bud formation have been obtained on OD4 medium from progenitor shoots cultured on media containing BAP, as compared to shoots grown in the presence of auxin (IBA, NAA) (data not shown).

Morphological variations and in vitro behaviour of the regenerated plants

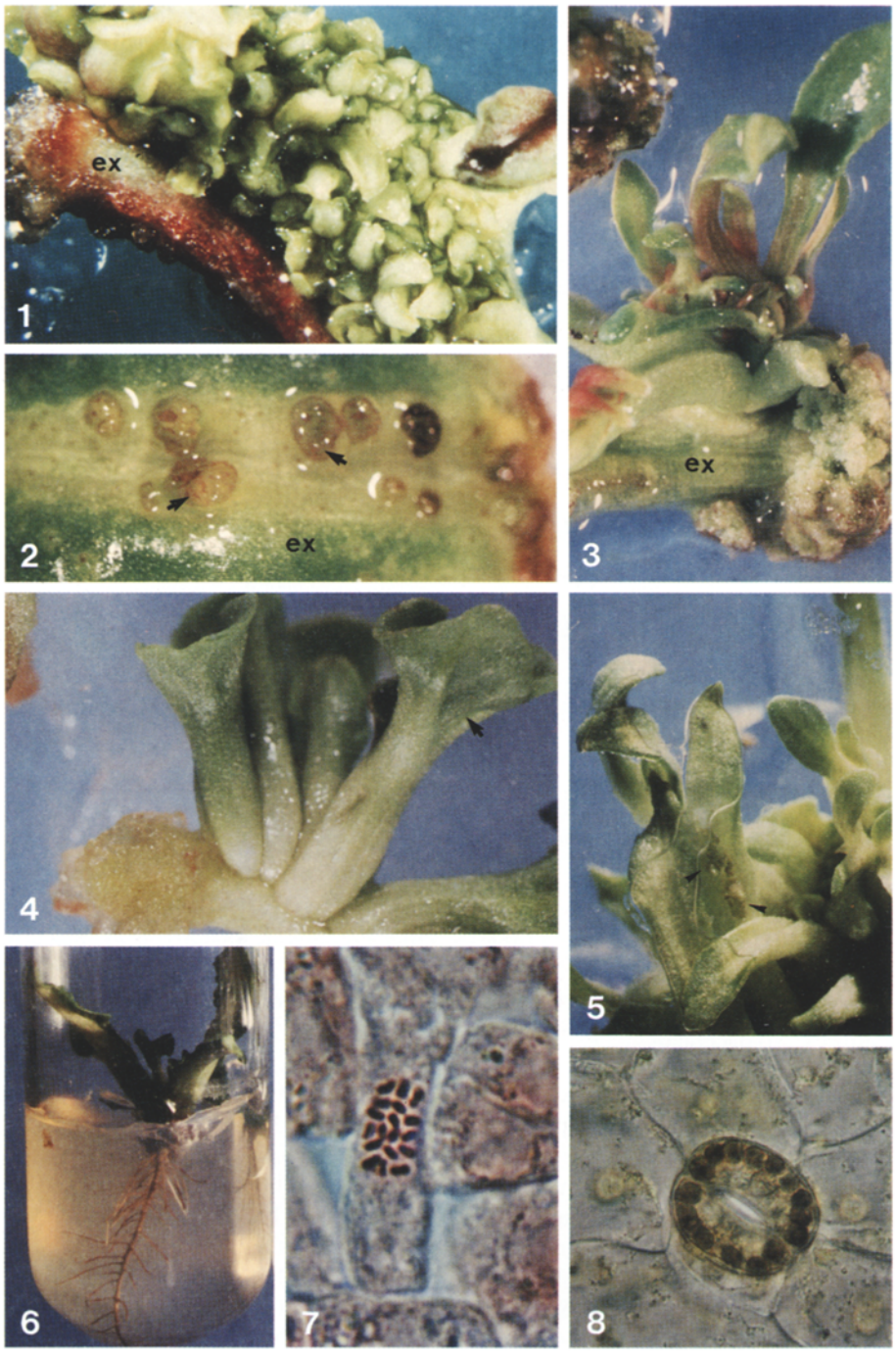
The regenerants from petiole culture could be grouped into two main classes:

– healthy and normal looking shoots as compared with typical seedlings, i.e. characteristics such as leaf color, shape and arrangement, rosette character, petiole/blade length ratio, etc. ..., appeared normal (Fig. 3);

Table 1. Morphogenetic response of nine sugarbeet germplasm in petiole culture. Total number of morphological-variated plants studied are given in parenthesis

Genotype	No. p1 explants cultured	% p1 explants forming buds	No. buds per p1 explants	No. of buds obtained in p1	Percentage of shoots with deviating morphology after	
					1 culture cycle (p1 population)	3 culture cycles (p3 population)
N						
G-93	140	15	4–9 (7.4±0.8)	196	12.7 (25)	15.2 (29)
G-84	128	28.9	6–13 (9.3±1.7)	296	5 (15)	5.3 (7)
2N						
G-A48	320	68.2	6–20 (13.9±2.5)	1,612	0.7 (12)	3.3 (9)
G-VEC29	206	39.3	4–7 (6.5±1.1)	518	5.7 (30)	8.1 (7)
C64A	159	40.1	4–10 (6.9±1.1)	204	20 (10)	16.4 (6)
C64D	151	27.1	4–10 (6.8±1.1)	314	17.5 (55)	7.8 (11)
E321*	379	1.8	1–2 (1.5±0)	12	–	–
3N						
RI5	45	21.2	2–6 (4.1±1.2)	31	6.4 (2)	3.9 (5)
RI7	106	22.6	1–6 (4.6±1.3)	124	7.2 (9)	4.9 (12)
Total	1,255	34.1	3.9–10.1	3,295	4.8 (158)	6.9 (86)

* Field-grown plants subjugated to only one petiole culture cycle. They were not counted in "Total" line because of kind of protocol used



Figs. 1–8. 1 High frequency regeneration from petiole explant (ex) obtained from in vitro cultured plant. **2** Initiation of buds (*arrows*) on petiole explant (ex) obtained from field-grown plant. **3 and 4.** Regenerants from one petiole culture cycle; **3** – normal-looking plant (Type I); **4** – variegated shoots (Type II): leafy plants (*arrows*) with tubular leaves. **5.** Subsequent shoot proliferation (*arrows*) on petiole without isolation and subculture (OD4 medium). **6.** Rooting of a leafy plant (3-week culture). Large petiole is still evident. **7.** Diploid cell metaphase with $2N = 18$ from Type II plants. **8.** Stomata guard cell with 16 chloroplasts in a regenerant from three petiole culture cycles

– shoots with obvious morphological leaf deviations (Fig. 4). Among this class, grossly aberrant buds could be easily distinguished but others showed alterations in fewer characters, and a gradation between leafy and normal shoot was observed. Variations, such as leaves appearing petiole-like, were common.

These two classes will be referred to throughout this report as Type I and Type II plants, respectively. Table 1 shows the frequency of shoots, with aberrant morphology which arose for each sugarbeet line studied.

Whereas Type I plants keep the characteristics of the control population, the in vitro behaviour of Type II plants is quite different. Subsequent shoot proliferation frequently takes place on the petiole of the leafy plant, on OD4 medium, without a new subculture cycle (Fig. 5). The majority of these grossly abnormal shoots also failed

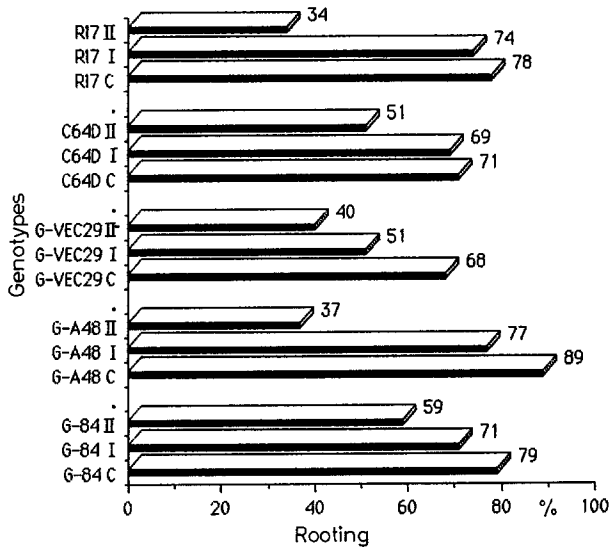


Fig. 9. Rooting percentage of Type I and II shoots grown from one petiole culture cycle, as compared with the control shoot apex culture population. *C* – shoot apex population; *I* –normal looking plants; *II* – variated population

to root or at best established themselves poorly. Thus, a lower frequency of rooting as compared to the shoot apex culture control population was observed (Fig. 9). However, in some cases, when isolation of Type II buds and subculture on an elongation medium (MS + GA3 0.5 mg/l + BAP 0.05 mg/l) is made early (aberrant plants are about 0.5 mm in length), normal-looking shoots emerge from the leafy buds, which are then easily rooted (Fig. 6).

Regeneration of p2 and p3 populations followed the same pattern as described above for the original p1 samples isolated from the shoot apex cultures. No decline or increase in regeneration potential was observed and frequency in Type 1 or Type II plants also remained the same. On the contrary, the capacity for bud induction and the frequency of leafy buds formation appeared to increase when the regenerated variants (Type II plants) were submitted to a second and third in vitro culture cycle, before reverting to the typical morphological pattern (unpublished data).

In all cases, 2–3 weeks after subculture to soil and transfer to the greenhouse, no morphological abnormalities could be detected.

Ploidy level and chromosome number

Cytological examinations were carried out on petiole-derived plants (Type I and Type II) together with their respective control plants. All Type II buds, together with 50% of the normal buds, chosen at random, were studied. The analysis of chloroplast and chromosome numbers (Figs. 7 and 8) revealed that none of the shoots (neither Type I nor Type II) contained changes in ploidy

Table 2. Range in chloroplast numbers, and chromosome numbers of control plants and petiole-derived populations (Type I and Type II)

Genotype	Control material (shoot apex culture)[b]	Petiole-derived populations		
		normal-looking plants (Type I)	variated plants (Type II)	
			one cycle	three cycles
G-93	7.8±1.2 (N=9)	7.7±0.9	8.9±1.1	8.0±1.2
G-84	8.6±1.3 (N=9)	7.9±1.4	7.5±0.9	8.3±1.2
G-A48	16.8±1.2 (2N=18)	17.0±1.1	16.8±1.3	17.0±0.8
G-VEC29	15.2±1.2 (2N=18)	14.8±1.0	15.2±1.1	15.3±1.3
C64A	14.8±2.0 (2N=18)	14.2±1.2	15.4±1.4	14.9±0.9
C64D	13.6±1.9 (2N=18)	15.0±1.0	14.5±1.6	16.1±1.6
E321[a]	14.2±1.8 (2N=18)	14.6±1.1		
RI5	19±0.4 (3N=27)	21.6±0.9	18.2±1.2	19.4±1.0
RI7	20±1.5 (3N=27)	20.8±1.7	21.8±1.7	20.9±1.1

[a] Petiole of field grown plants subjugated to only one culture cycle
[b] Chromosome numbers are given in parenthesis

level (Table 2). Thus, no obvious phenotypic change was the result of a change in ploidy and, moreover, no change in the ploidy level was observed after successive culture petiole cycles.

In a second investigation, cytogenetic analysis was systematically carried out for 147 p1 shoots derived from 15 petiole explants of the G-93 clone. This is a breeding line with one of the highest frequencies for inducing leafy plants. Precise comparisons in morphology and chromosome number between the p1 buds from one petiole explant and between buds of the 14 other organogenic explants were made. Generally, all buds of the same petiole explant were either leafy or normal; mixed populations were rare. Growth of buds was not synchronous on one petiole. However, neither intra-petiole nor inter-petiole variation concerning the range of morphological deviations was noticed among the regenerants. All shoots subsequently followed identical morphological development and contained the euploid chromosome number.

Discussion

Somaclonal variation present in adventitious regenerants may reflect either pre-existing cellular genetic differences

or physiological, epigenetic and/or genetic variation induced by tissue culture, as has been extensively discussed by Larkin and Scowcroft (1981) or Meins (1983).

In the work described herein, a rapid and efficient protocol, applicable to a wide range of germplasm of *Beta vulgaris,* has been used for the recovery of many plants from a selected donor genotype. Among the 7,000 adventitious buds obtained since our studies began, only phenotypic variants in leaf characters (Type II plants) have been observed and, contrary to Freytag et al. (1988), no albino plant was obtained. Moreover, we have reported a loss of the changed character following transfer to the greenhouse. According to this, phenotypic alterations would be a consequence of physiological state rather than any heritable change in morphology, and may be regarded as a response to hormonal stress during in vitro culture. This is confirmed by other investigations, not detailed in this report, in which we have observed that an anti-auxin dilution step (TIBA < 0.5 mg/l) or a reduction in culture time on OD4 medium (12 days culture and subculture on M medium for instance), caused a lower frequency of leafy plant formation. Variants having leaves with no blade or appearing petiole-like and features such as bumps rods were previously reported to arise from petiole culture (Saunders 1982; Saunders and Mahoney 1982; Freytag et al. 1988). Such disturbances in growth have been found to be a consequence of hormone concentrations, especially of the BAP level (Miedema 1982; Saunders 1982). The auxin/cytokinin ratio is, of course, determinant in the morphogenic response.

Because mitotic reactivation of endoreplicated nuclei, if present in the explant, can give rise to cells of various ploidy upon in vitro culture, the explant used can greatly affect the quantity and type of variation produced (Reisch 1983). In particular, leaf explants are considered to be a specific plant tissue responsible for variation in the regenerant plants. In *Beta vulgaris,* the occurrence of polyploid cells in leaf tissue is frequent and is well known by beet breeders (Lenee, personal communication). Abnormal ploidy levels might, therefore, be expected to arise from petiole culture-derived plants. However, no karyological change has been observed among the first generation of adventitious buds. Similarly, chimerism, mixoploidy and mosaicism were not noticed in the regenerants. It is the best evidence of no or low participation of endoreplicated nuclei of the mature petiole in the in vitro organization of adventitious buds. Moreover, no polyploidization and no increase in the frequence of variations in the morphogenic pathways occur through three petiole culture cycles; this tends to show a good genome stability.

Contrary to our results, Cassells and Carney (1987), and Van Harten et al. (1981) have observed variations in petiole-derived populations. Two points underlie this difference. On the one hand, regeneration and rooting act somewhat as a barrier for several altered variants due to their genetic instability. Therefore, although aneuploid cells may be present in culture, viable plants are less likely to be regenerated from these cells, especially for species/genotypes less buffered against chromosomal changes and less tolerant of aneuploidy. On the other hand, because of the small size of the chromosomes, cytological procedures for the determination of chromosome numbers have been restricted. As a consequence, the possibility of genetic modifications such as chromosomal rearrangements, recombination, sequence copy number changes, etc. (Larkin et al. 1985), cannot be completely excluded. Moreover, the detection and analysis of these phenomena are not easy, due to the high heterozygosity of sugar beet.

In conclusion, no cytological change has been recorded and no morphological variations have occurred as a result of a change in ploidy. Based on these results and on the morphogenic competence of the petiole, direct regeneration from this explant might be adopted in *Beta vulgaris* for true-to-type high frequency micropropagation and production aims. However, we feel that field testing of plant populations cultured from petioles by traditional breeding approaches is necessary, to assess their conformity and their relative performance.

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