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# Measurement of the extent of somaclonal variation in begonia plants regenerated under various conditions. Comparison of three assays

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**Abstract** *Begonia* plants were regenerated from leaf explants treated with increasing concentrations of the chemical mutagen nitrosomethylurea (NMU). In these plants, we evaluated three methods to assess the extent of variation: a qualitative, phenotypic assay (the percentage of aberrant plants), a molecular assay (changes in RAPD patterns) and a quantitative, phenotypic assay (variation in a quantitative trait). The qualitative, phenotypic assay required a large number of plants per treatment (approx. 100) and careful, skilled judgement. It was sensitive to physiological variation. The RAPD assay was not sufficiently sensitive: even at the highest NMU concentration there were no changes in RAPD patterns. The quantitative, phenotypic assay gave the best results: it was simple, objective and sensitive, and required few plants per treatment (approx. 30). Plants were also regenerated from different types of intermediate callus, and their variation was assessed. The performance of the three assays was essentially the same as with plants obtained after mutagenesis with NMU. An intermediate nodular- or non-nodular-callus phase resulted in slightly or strongly increased variation, respectively. In contrast to NMU-induced variation, callus-related variation, as determined in the quantitative, phenotypic assay, appeared to be to a large extent transient since it decreased strongly after a second direct-regeneration step. An intermediate callus phase resulted in 2.5% juvenile plants. This aberration, which might be related with changes in the methylation status of DNA, was not observed in NMU-treated plants.

**Keywords** Auxins · *Begonia* · RAPD · Regeneration · Somaclonal variation

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## Introduction

Plants produced vegetatively in tissue culture may be different from the plants from which they originate because of physiological changes (e.g. bushiness), genetic changes, loss of pathogens and/or loss of chimerical structure. The term ‘somaclonal variation’ has been introduced to describe genetic changes due to tissue culture (Larkin and Scowcroft 1981). It should be noted that the difference between somaclonal and physiological variation is not an all-or-nothing phenomenon but a matter of degree (De Klerk 1990). DNA methylation, for example, may be very long lasting and even transmittable through meiosis (Jorgensen 1994).

The potential of somaclonal variation to generate new, desirable traits for breeders has been advocated (among others by Larkin and Scowcroft 1981), and although valuable cultivars have been obtained (Bouman and De Klerk 1996), the results have not lived up to expectations. Since the highest level of somaclonal variation occurs in plants generated via adventitious shoot or somatic embryo formation, in particular after an intermediate callus phase (Yamagishi et al. 1996; Piccioni et al. 1997; Plader et al. 1998), somaclonal variation is a problem in breeding and propagation techniques that involve adventitious regeneration. In micropropagation, somatic embryogenesis via suspension cultures may suffer from somaclonal variation. In breeding, undesirable variation may be introduced during genetic engineering or haploid culture.

An assay to measure the extent of somaclonal variation will assist research into the backgrounds of somaclonal variation and, from a practical point of view, enable the selection of cultivars and tissue culture procedures with little or no somaclonal variation. In this paper, we evaluate three putative assays using plants regenerated from explants treated with the chemical mutagen nitrosomethylurea (NMU). We have also studied the effect of tissue culture conditions on somaclonal variation.

## Materials and methods

### Plant material and tissue culture procedures

Plants from *Begonia×hiemalis* (Fotsch.) cv. Schwabenland Red were obtained from a local grower. The standard procedure for direct regeneration has been described previously (De Klerk et al. 1990). Briefly, leaf explants were cultured on half-strength Murashige and Skoog (1962) salts, 100 mg $l^{-1}$ m-inositol, 0.4 mg $l^{-1}$ thiamine-HCl, 20 g $l^{-1}$ sucrose, 0.5 µ*M* 1-naphthaleneacetic acid (NAA), 0.9 µ*M* benzyladenine and 6 g $l^{-1}$ agar (BBL granulated). After 10 days at 25°C in the dark, the explants were transferred to basal medium without hormones, and grown at 25°C under 16-h light per day provided by cool-white fluorescent lamps (35 $mmol^{-2}$ $s^{-1}$). After 6–8 weeks, shoots were excised from the explants and rooted in glass jars on the same medium at 20°C. Three weeks later the plants were transferred to soil.

For chemical mutagenesis, leaf explants were treated for 1 h with an aqueous solution of 0.2, 2 or 10 m*M* nitrosomethylurea (NMU) directly after cutting. After three rinses in water, plants were regenerated as described above.

Callus was induced on the same medium as used for regeneration but instead of NAA, 2,4-dichlorophenoxyacetic acid (2,4-D), 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) or picloram were added at different concentrations. Cultures were kept in the dark at 25°C and subcultured at 4-week intervals. Plant regeneration from callus was achieved by culturing callus fragments of approximately 2×2×2 mm on the medium for direct regeneration. This callus was subcultured every 4 weeks until shoots appeared. After another 4 weeks, callus fragments with shoots were placed on medium without plant growth regulators. After 4 weeks, the shoots were rooted.

In one experiment, leaf explants excised from juvenile plants were cultured on medium for direct shoot induction. To this medium, 0.1 m*M* 5-azacytidine had been added after autoclaving. After 2 weeks in the dark, the explants were transferred to fresh medium without plant growth regulators and without azacytidine.

Plants obtained from different regeneration procedures were grown to flowering in 3 months in glasshouses at the Research Station for Floriculture and Glasshouse Crops, Aalsmeer, The Netherlands. In commercial practice, this begonia cultivar is sprayed with trimethylammoniumchloride to improve the plant form. Because this may mask variation, especially in plant size and leaf form, spraying was omitted. During the winter, additional light was given to prevent early flowering.

### Phenotypic assays for somaclonal variation

In populations of regenerated plants, we determined the percentages of adult plants with somaclonal and physiological changes. Qualitative phenotypic changes (dwarf plants, abnormal venation, etc.) were considered as somaclonal when they were stable in a second direct regeneration step from leaf explants taken from the adult plant. Otherwise, they were considered to be physiological. An exception to this were plantsdeficient in chlorophyl; these were always considered to be somaclonal (see Results section), For some plants this procedure was repeated in a third regeneration step. We also determined the percentages of juvenile plants. These plants were characterised by round leaves and did not flower.

In each population of regenerated plants, we also determined variation by measuring spreading of the leaf shape. This method has been described in detail by De Klerk et al. (1990). In short, the lengths of two ribs were measured from two leaves (the third and fourth from the apex). These were the longest rib and the rib perpendicular to it. The shape of the leaf was expressed as the natural logarithm of the ratio of both lengths ('ln r') to obtain a normal distribution. The coefficient of variation of 'ln r' was determined in each population of regenerated plants. Only normal-looking plants and intact leaves were selected for this assay. It should be noted that because of this selection, the figures presented here are underestimations.

### Molecular assay for somaclonal variation

For DNA isolation we initially used a modified version of the protocol of Hu and Quiros (1991). Leaf tissue (150 mg) was frozen in liquid nitrogen and stored at –80°C until use. It was homogenised in an all-glass homogeniser with 0.5 ml extraction buffer [50 m*M* EDTA, 0.5 *M* NaCl, 1.25% (w/v) SDS, 100 m*M* TRIS-HCl pH 8.0]. The homogenate was transferred to a 1.5-ml microtube and heated for 15 min at 65°C. After the addition of 0.2 ml 5 *M* K-acetate, the mixture was refrigerated for 10 min at 0°C and centrifuged for 5 min in a microfuge. Isopropanol (2/3 volume) was added to the supernatant. After 30 min at –20°C, DNA was precipitated and then washed with 70% ethanol. The pellet was dissolved in 0.25 ml buffer (1 m*M* EDTA, 10 m*M* TRIS-HCl pH 8.0) and treated with 5 µl RNase (10 mg $ml^{-1}$) for 30 min at 37°C. The DNA was again precipitated with 2 vol. ethanol, washed and dissolved in 0.1 ml TE-buffer (0.1 m*M* EDTA; 1 m*M* TRIS-HCl, pH 8).

During our investigations we changed to a shorter procedure according to Yu and Pauls (1993). After the same homogenisation step as above, the homogenate was centrifuged. The DNA was precipitated from the supernatant in a new tube by adding an equal volume of isopropanol. After centrifugation, the pellet was dried and dissolved in 100 µl TE-buffer. The non-dissolved debris was discarded after a subsequent centrifugation step.

Arbitrary oligonucleotide 10-mer primers of Operon Technologies were used for amplification by the polymerase chain reaction (PCR). The protocol of Williams et al. (1990) was slightly modified. In a 50-µl volume, the reaction mixture was composed of reaction buffer [5 µl of a 10×Super*Taq* buffer (HT Biotechnologies, UK)], 0.2 m*M* dNTP, 0.2 m*M* primer, 20–100 ng DNA and 0.125 U *Taq* polymerase (HT Biotechnologies, UK). In a thermocycler 42 cycles were applied (1 min at 92°C, 1 min at 35°C and 2 min 72°C), whereas the first 92°C period was extended for 4 min and the last 72°C period for 6 min. After completion of the amplification 15 µl of the amplified DNA mixture was loaded on a 2% agarose gel (100 ml with 90 µg ethidium bromide) in TBE buffer (90 m*M* TRIS-HCl, 90 m*M* boric acid, 2.5 m*M* EDTA, pH 8.3) and run for approximately 3 h at 70 V, or equivalent times at higher or lower voltages. The gel was photographed under UV light with a Polaroid camera. The random amplified polymorphic DNA (RAPD) patterns were compared with the patterns of the mother plant, and only reproducible differences were taken in account.

## Results

### Assessment of NMU-induced variation

Plants were regenerated from leaf explants that had been exposed to increasing concentrations of NMU. In the absence of NMU plants did regenerate from almost all leaf fragments. After treatment with 0.2 m*M* NMU, 4% of the leaf fragments did not show regeneration; with 2 or 10 m*M* NMU, this percentage increased to 11 or 36, respectively. The time required for the regeneration of shoots also increased with the NMU concentration. Shoots regenerated after NMU treatment were rooted, grown to flowering and evaluated. Variations that were persistent in a second regeneration step using explants taken from leaves of the regenerated plants were considered to be genetic. Occasionally, a third direct regeneration step was applied, but in all cases any variation present after the second step remained after the third step. The percentage of physiological variation was 11.2 for the normal population, but it should be noted that we included in this group plants with only minor variations in,

among others, height, start of flowering or shape. These slightly aberrant plants were within the limits of variation occurring in populations of commercial growers. After the NMU treatment, the percentage of plants with physiological aberrations was also approximately 10.

Regeneration without NMU treatment resulted in a low incidence of genetic variation (Table 1) that included very retarded growth and 1 chimerical plant. After treatment with NMU, several severe mutations were observed: dwarfs, venation deviations, chlorophyll mutants (mosaics, sectorial chimeras), change in flower colour from red to rose, liverwort-like growth, among others. Apart from the chlorophyll mutants, all mutations were still present after direct regeneration from leaves of the mutant plant. Regeneration from leaf fragments of chlorophyll mutants yielded mostly normal plants, presumably due to the better regeneration capacity of the cells containing chloroplasts. Table 1 shows that the percentages of aberrant plants increased with increasing NMU concentration. Even at the lowest concentration (0.2 m*M*), the percentage of genetically aberrant plants was significantly different from the control (P<0.001); at 2 m*M* and 10 m*M*, the percentage of aberrant plants increased significantly. It should be noted that large numbers of plants have been evaluated: more than 1,000 plants for direct regeneration without NMU and for each NMU concentration approximately 100 plants.

We also determined a parameter for the leaf shape in the plants: the natural logarithm of the ratio of two ribs ('ln r'; see Materials and methods section). The mean 'ln r' was the same within each experiment (thus independent of the regeneration conditions), but its value was dependent on glasshouse conditions (temperature, light, etc). For instance, at the various NMU concentrations, the 'ln r' of the third leaf was 0.41, 0.43, 0.41 or 0.42, and the 'ln r' of the fourth leaf 0.42, 0.39, 0.42 or 0.39 at 0, 0.2, 2 or 10 m*M* NMU, respectively. In another experiment, the 'ln r' of the third leaf was 0.49, 0.47 or 0.48 and of the fourth leaf 0.49, 0.48 or 0.50 after regeneration at 0, 0.5 or 5 µ*M* 2,4-D, respectively. The coefficient of variation of 'ln r' in a population of regenerated plants increased with increasing NMU concentration and reached a maximum value of 20% at 2 m*M* (Table 2). Note that only normal-looking plants had been selected for this assay. The number of plants included in the assay was much less than in the previous assay (in the range of

**Table 1** Variation in begonia plants regenerated in various ways (accumulated numbers of several experiments). Nodular callus was cultured for a short time (less than 3 months) on 0.5 µ*M* 2,4-D and non-nodular callus for longer times (more than 6 months) on 0.5 µ*M* 2,4-D. The significance of the difference from the control (direct generation) was evaluated in a $\chi^2$ test

| Way of regeneration | Number of plants | Somaclonal variation (%) | Juvenile plants (%) |
|---|---|---|---|
| Direct regeneration | 1,265 | 1.5 | 0 |
| Direct regeneration after 0.2 m*M* NMU | 102 | 7.8*** | 0 |
| Direct regeneration after 2 m*M* NMU | 101 | 27.7*** | 0 |
| Direct regeneration after 10 m*M* NMU | 119 | 46.2*** | 0 |
| Regenerated from nodular callus | 483 | 4.6*** | 2.5 |
| Regenerated from non-nodular callus | 508 | 10.6*** | 2.4 |

*** *P*<0.001

**Table 2** Coefficient of variation of 'ln r' in a somaclonal population of normal-looking begonia plants regenerated under various conditions. Experiments were carried out twice for the NMU plants and 5–12 times for the 2,4-D plants. The presented data are from a typical experiment. The experiments with picloram and 2,4,5,-T were carried out only once. The significance of the difference from the control (direct regeneration) was evaluated in an *F*-test

| | Number of plants | Occurrence of nodular callus[a] | Coefficient of variation of third leaf | Coefficient of variation of fourth leaf |
|---|---|---|---|---|
| Direct regeneration | | | | |
| +0 m*M* NMU (control) | 25 | No callus | 9.9 | 10.1 |
| +0.2 m*M* NMU | 45 | No callus | 13.1* | 15.3** |
| +2 m*M* NMU | 29 | No callus | 20.8*** | 21.1*** |
| +10 m*M* NMU | 21 | No callus | 19.1*** | 22.5*** |
| Regeneration after callus phase on: | | | | |
| 0.5 µ*M* 2,4-D (after 2 months) | 30 | + | 13.7* | 13.2 |
| 0.5 µ*M* 2,4-D (after approx. 6 months) | 28 | – | 18.2*** | 17.5*** |
| 5.0 µ*M* 2,4-D | 27 | – | 18.9*** | 20.0*** |
| 0.5 µ*M* 2,4,5-T | 38 | ++ | 8.8 | 8.3 |
| 0.5 µ*M* picloram | 47 | ++ | 11.4 | 11.5 |
| 2.5 µ*M* 2,4,5-T | 16 | ++ | 10.7 | 9.9 |
| 2.5 µ*M* picloram | 22 | + | 12.5 | 15.5* |
| 5.0 µ*M* 2,4,5-T | 18 | + | 12.5 | 13.4* |
| 5.0 µ*M* picloram | 26 | + | 11.6 | 17.5*** |

* *P*<0.05, ** *P*<0.01, *** *P*<0.001

[a] Presence of nodular structures in the callus: ++, many; +, occasionally; –, absent

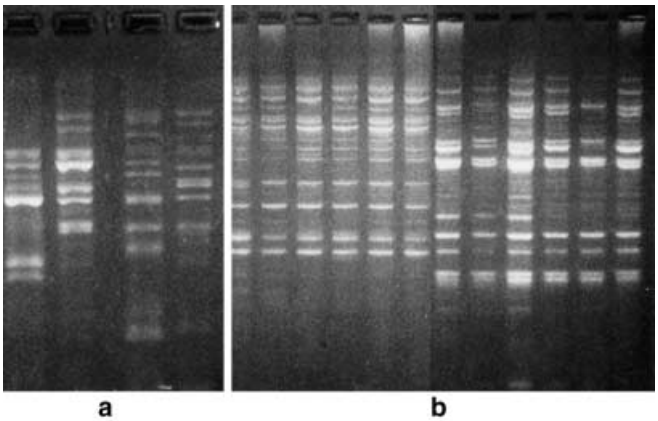


**Fig. 1a, b** RAPD patterns of four *Begonia elatior* cultivars (**a**) and of 6 *B. elatior* cv. Schwabenland Red plants regenerated from leaf explants after treatment with 10 m*M* NMU (**b**). The latter 6 plants all showed a strongly aberrant phenotype. Primers used were A19 for the four cultivars (**a**) and A19 (*lanes 1–6*) and E1 (*lanes 7–12*) for the 6 NMU-treated plants (**b**)

**Table 3** Change in the coefficient of variation of the 'ln r' after an additional step of direct regeneration. The coefficient of variation of 'ln r' was determined after direct regeneration with various concentrations of NMU or indirectly from callus. For each concentration, 25 plants were regenerated. Each regenerated plant was propagated vegetatively in vitro via adventitious regeneration from leaf explants. Per originally regenerated plant, 1 plant was taken at random. From these new populations the coefficients of variation were determined. The significance of the difference form the control (direct regeneration) was evaluated in an *F*-test

| | Regenerated plants (R) | Plants directly regenerated from R |
|---|---|---|
| Direct (control) | 11.5 | 10.5 |
| Direct, 0.2 m*M* NMU | 14.2 | 13.4 |
| Direct, 2 m*M* NMU | 19.4** | 18.1** |
| Direct, 10 m*M* NMU | 22.1*** | 21.8*** |
| Via callus, 5 µ*M* 2,4-D | 21.2*** | 13.9* |

* $P<0.05$, ** $P<0.01$, *** $P<0.001$

20–50 instead of 100–1,000), but the difference was significant even at the lowest concentration of NMU. When a new plant was produced from each NMU-plant in a second direct regeneration step, the coefficient of variation only showed a small (statistically non-significant) decrease (Table 3).

For analysis on the DNA level we used RAPDs. We first tested the method by compiling RAPD patterns of several *Begonia elatior* cultivars from the Schwabenland group and from more distant relatives from other *elatior* groups. Primers were selected for providing a reproducible pattern with many bands. A number of primers (A1, A10, A15, A17, A18, A19, B5, B20, C5, C10, D7, D8, D20, E1, E2, F12, G16, H1) was chosen that could discriminate between the groups (Fig. 1a) and also within the Schwabenland group (data not shown). The number of bands represented by the primers tested was approximately 200. Using this set of primers, we subjected leaf samples of regenerated plants to RAPD analysis. RAPD analysis of plants regenerated from explants after NMU treatment also never revealed reproducible differences compared to the normal pattern (Fig. 1b). We tested 20 normal and 20 phenotypically deviant plants with the primers mentioned above. These strongly aberrant plants also had the same pattern as the parent plant.

## Variation induced by an intermediate callus phase

The culture of leaf explants on medium with 0.5 µ*M* 2,4-D resulted in callus with many nodules (buds from which outgrowth had been arrested by the presence of 2,4-D). These structures became less abundant after a number of subcultures on the same medium, and non-nodular callus became predominant. At 5 µ*M* 2,4-D non-nodular callus was formed directly from the leaf explants. The phenotypic changes in plants regenerated from callus were similar to the NMU-induced variation with three exceptions: pink flowers (3 out of the 83 plants that showed mutations) and liverwort-like plantlets (2 out of 83) were only observed after NMU treatment, and juvenile plants (these are discussed below) were found only after a callus phase. Regeneration from both types of callus gave a higher percentage of plants displaying somaclonal variation than direct regeneration. The highest incidence of somaclonal variation was obtained in plants regenerated from non-nodular callus (Table 1). The callus phase also resulted in an increase in physiological variation (23.8% instead of 11.2%). In a few experiments, callus-inducing media supplemented with 2,4,5-T and picloram were used. A small number of plants were regenerated, and hardly any severe aberrations were found (data not shown).

In the quantitative phenotypic assay, the extent of somaclonal variation was also dependent on the type of callus. Plants regenerated from nodular callus formed during culture on 0.5 µ*M* 2,4-D showed a small increase in the variation of 'ln r' (Table 2). The maximum variation was reached in plants regenerated from high-2,4-D callus. Table 2 also shows that the use of other synthetic auxins (picloram and 2,4,5-T) resulted in less variation than the use of 2,4-D. When callus was subcultured for several cycles on 0.5 µ*M* 2,4-D, somaclonal variation increased (Table 2). There was no further increase in the variation of 'ln r' when the callus was maintained for a longer time (until 4 years), even though both the time required for regeneration and the number of callus fragments that did not show regeneration increased significantly.

Whereas NMU-mutated plants kept the same high coefficient of variation in a second regeneration step (see above), there was a strong decrease in the variation of 'ln r' in callus-derived plants after a second regeneration step (Table 3). However, the coefficient of variation was still significantly higher than in the control ($P<0.05$).

Individual plants from 2,4-D callus were submitted to RAPD analysis in the same way as the NMU-mutated plants. Only 2 plants (out of 40) gave a reproducible change in the electrophoresis pattern (one new band).

**Fig. 2** *Begonia elatior* cv. Schwabenland Red. *Left* A normal plant, *right* a juvenile-type of plant regenerated from callus (see text). *Bar*: 5 cm

Both were normal looking plants obtained from long-term callus. Aberrant plants did not show different RAPD patterns when tested with the primers mentioned before.

We frequently observed 'juvenile' plants following the regeneration of plants from callus (Fig. 2). These plants showed the characteristic round leaf shape of very young begonia plants. The size of the leaf varied greatly among plants, but the leaves of each plant all had a similar size, i.e. plants with uniformly large and small leaves, respectively, occurred. These plants also showed another juvenile trait: they were unable to flower, even after a period of more than 8 years in the glasshouse. Direct plant induction on leaf fragments yielded once again juvenile plants. We found, however, an indication that this variation is not an irreversible mutation. After regeneration in the presence of azacytidine, 2 out of 42 regenerated plants were normal-looking with an adult leaf shape and an ability to flower.

## Discussion

### Comparison of the three assays in NMU-treated regenerated plants

For research into the backgrounds of somaclonal variation and for the practical use of biotechnological breeding and propagation techniques, assessment of the extent of somaclonal variation is required (De Klerk 1990). We examined three putative assays in begonia plants regenerated from explants treated with the chemical mutagen NMU.

The first assay is on the DNA level. We did not find reproducible differences in RAPD-patterns, neither in normal plants from NMU treatments nor in the phenotypically strongly aberrant ones. This might be expected since even use of 100 primers covers only a very minor area of the genome ($5 \cdot 10^{-4}$% of the genome). Allowed mispairing in the base pairs of the primers and overlap (although not probable) reduces this area even more. Mulcahy et al. (1993) made similar calculations for the analysis of apple genotypes. Consequently, RAPD analysis is not sufficiently sensitive to detect NMU-induced mutation pressure. It should be noted that NMU induces point mutations, whereas an intermediate callus phase may induce not only point mutations but also DNA rearrangements, chromosome losses and activation of transposons, which are easier to detect by RAPD analysis.

In the second, qualitative phenotypic assay, i.e. scoring of the percentage of plants with a deviant phenotype, we found a significant increase in the percentage of variation at 0.2 m*M* NMU. We used more than 1,000 plants for the experiment in which plants regenerated without NMU treatment and approximately 100 plants for the NMU-treatment. From the $\chi^2$-value ($P<0.05$), it can be calculated that two samples of 85 plants or more (so a total of at least 170 plants) should be taken to obtain a statistically significant increase from 1.5% to 7.8%. However, in the third, quantitative phenotypic assay, in which the variation of 'ln r' was used as an indication of the occurrence of genetic variation, a total of 65 plants (control+treatment) was sufficient to show a significant difference. It should also be noted that in the quantitative assay the values are underestimations since severely mutated plants (scored as somaclonal variation in Table 1) had been left out. Another advantage of the quantitative assay is that observations do not require skilled, careful, time-consuming judgement.

An important observation was that all plant populations had the same mean value of 'ln r'. This means that the changes after the NMU-treatment occurred in two directions: in some plants the leaves became wider and in others longer. A bi-directional effect is unlikely with respect to physiological changes (e.g. after tissue culture plants may become more bushy but it has never been observed that after tissue culture some plants become more bushy and others less bushy than the original plants). Table 3 demonstrates that the increase in variation after NMU treatment of the explants only shows a slight decrease after a second regeneration step using leaves of the NMU-derived plants as explants. This indicates that the increase is truly based on genetic changes.

### Measurements of somaclonal variation in callus-derived plants

With RAPD analysis we observed only incidental changes in callus-derived plants. Thus, this assessment appeared to be insensitive. Smulders et al. (1995) observed no differences in RAPD patterns in tomato; Fourre et al. (1997) failed to find any differences in RAPD patterns of *Picea* somatic embryo-derived plants despite major chromosomal aberrations. Other authors also observed only occasional differences amongst RAPD patterns of regenerated plants after prolonged callus culture or in plants regenerated from protoplasts (Isabel et al. 1996, Hashmi et al. 1997). Only Brown et al. (1993) found major differences in wheat plants derived from protoplasts, and Piccioni et al. (1997) found new RAPD markers in somatic embryos derived from alfalfa callus. These authors did not show correlations with the phenotype. Therefore,

any validation of true-to-typeness of regenerants by absence of differences in RAPD patterns (e.g. Valles et al. 1993; Isabel et al. 1993; Shoyama et al. 1997; Gallego et al. 1997) is not justified. Nevertheless, an assessment of genetic variation with RAPD analysis may be achieved if DNA regions occur with an increased instability (as suggested by Al-Zahim et al. 1999 for garlic), and primers are developed specifically for these regions.

In callus-induced variation as revealed by the variation in 'ln r', a physiological component was present: The increased variation after an intermediate callus phase largely disappeared after a second regeneration step (Table 3). In tomato, Smulders et al. (1995) reported similar results in variation patterns for polysomaty, i.e. the first generation of seedlings of callus regenerants shows a high degree of variation in polysomaty, whereas the second-generation progeny does not show this variation. The data presented in Table 3 demonstrate the limitations of the quantitative, phenotypic assay. It should be noted, however, that since the assay detects long-term aberrations, it can be applied to evaluate vegetative propagation methods. We assume (see below) that the callus-induced variation is at least partly related to long-term changes in, for example, DNA methylation. It has been postulated that such changes are the cause of genetic mutations in callus-derived plants (Phillips et al. 1994; Gonzalgo and Jones 1997). Thus, the quantitative assay may also be useful in determining the mutation-inducing properties of tissue-culture methods, albeit in an indirect way.

As shown in Tables 1 and 2, the type of callus affects the occurrence of variation. Regeneration from non-nodular callus resulted in a much higher degree of variation than did regeneration from callus with nodules. From Table 2 it is clear that 2,4,5-T and picloram are not as potent in inducing variation as 2,4-D. At the same time, this coincides with the frequency of nodules in callus induced by these growth substances.

### Juvenile plants

Regeneration from 2,4-D-callus cultures always resulted in low percentages of juvenile plants (Table 1). This change was stable in a second regeneration step using leaf explants excised from the aberrant plants but could be reversed occasionally (2 out of 42) by the addition of azacytidine during regeneration. Because azacytidine influences the methylation state of the DNA, it may be that these juvenile plants were partly blocked in their ontogenetic development, thereby staying juvenile, by methylation events that are not easily reversible. In the regulation of development of organisms (de)methylation of DNA is a well-known mechanism (e.g. Holliday 1990) for the regulation of gene expression. In callus culture, other methylation states are present than in differentiated tissue, and during callus growth the methylation state changes (Arnholdt-Schmitt 1993; Smulders et al. 1995).

### Conclusions

The phenotypic, quantitative assay that determines the level of variation by determining the variation in a somaclonal population has proven to be more valuable than the other two assays. Even though there are apparent limitations, the assay has been successfully applied by Jain (1993) in S*aintpaulia* and begonia, and by Cassells et al. (1993, 1997) in carnation and *Pelargonium* using image analysis to determine the leaf shape. The phenotypic qualitative assay requires high numbers of plants and careful but nevertheless subjective observation. RAPD analysis for the detection of somaclonal variation is not sufficiently sensitive.

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