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An assay to measure the extent of variation in micropropagated plants of *Begonia* × *hiemalis*

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SUMMARY

The extent of somaclonal variation is usually assessed by determining the percentage of plants aberrant for a specific trait (e.g. phenotypical characteristics, chromosome number, electrophoresis pattern of proteins). We have assessed the extent of variation in micropropagated plants of *Begonia* × *hiemalis* cv. Schwabenland Red in another way. In three somaclonal populations the value of the standard deviation (SD) of a quantitative trait was determined, namely the SD of the natural logarithm of the ratio of the lengths of two leafribs (SD of ln c/a). Plants, regenerated from callus, induced and maintained at a high concentration of 2,4-dichlorophenoxyacetic acid (2,4-D), had a significantly larger SD, whereas the mean was not affected at all. The observed increase of the SD is likely to have been caused by somaclonal variation, but epigenetic variation cannot be ruled out. It is concluded that differences in the values of the SD may be used to assess differences in the extent of variation.

Key-words: Begonia hiemalis, micropropagation, somaclonal variation.

INTRODUCTION

Micropropagated plants of various ornamental crops show aberrant phenotypes. In The Netherlands at present, this causes losses of over US$1 million per year. It is not known whether the observed variation is somaclonal (i.e. genetic) or epigenetic (i.e. non-genetic), but in orchids, *Anthurium* and *Saintpaulia*, the variation is probably somaclonal because micropropagation occurs via adventitious buds and, in the case of *Anthurium*, with an extended callus interphase. Somaclonal variation is also a major obstacle in biotechnological breeding techniques, such as genetic engineering and the production of haploid plants.

Knowledge of the causes and mechanisms of somaclonal variation is very limited. One of the major obstacles for rapid progress of research is the absence of an accurate and easy assay to assess the extent of somaclonal variation (Orton 1983; De Klerk 1990). Usually, somaclonal variation is measured as the percentage of plants showing aberrations in phenotypical characteristics, chromosome number, chromosome structure, DNA sequences or protein/isozyme patterns. For such qualitative assays, at least 100 plants are

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required to obtain statistically reliable data. In addition, the assays are time-consuming and, in the case of judging the phenotype, dependent upon the eye of the observer.

In this communication we present the first results of our research aimed at the development of an easy and accurate quantitative assay for somaclonal variation. We have examined whether the value of the standard deviation (SD) of a quantitative trait can be used to assess the extent of somaclonal variation. We regenerated plantlets immediately on leaf explants or after a callus interphase on a low or a high concentration of 2,4-dichlorophenoxyacetic acid (2,4-D). As expected, these conditions will result in an increasing extent of somaclonal variation (cf. De Klerk 1990). The experiments were carried out with *Begonia* × *hiemalis* cv. Schwabenland Red. For this begonia, variation in micropropagated plants has been reported previously (Westerhof *et al.* 1984). In addition, micropropagation of this begonia has also been examined extensively (Hilding & Welander 1976; Mikkelsen & Sink 1978; Welander 1979; Takayama & Misawa 1982).

MATERIALS AND METHODS

Plants of *Begonia* × *hiemalis* (Fotsch.) cv. Schwabenland Red were purchased from local growers. The plants were used shortly before or during the early period of flowering. Leaves of 6–10 cm were surface-sterilized for 15 min in 1% (w/v) solution of NaOCl and rinsed three times with sterile water. Explants of 1 × 1 cm were cut and placed with the basal side down on 14 ml medium in 60-ml culture tubes. About half of the explant was submerged in the medium. The basal medium (modified from Hakkaart & Versluijs 1983) was composed of MS macro- and micronutrients (Murashige and Skoog 1962) at half stength, 100 mg l^{-1} myo-inositol, 0·4 mg l^{-1} thiamine–HCl, 10 g l^{-1} sucrose and 7 g l^{-1} agar (Difco Bacto). For induction, the basal medium was supplemented with (i) 0·1 mg l^{-1} α-naphthaleneacetic acid (NAA) + 0·2 mg l^{-1} 6-benzylaminopurine (BAP), (ii) 0·05 mg l^{-1} 2,4-D + 0·2 mg l^{-1} BAP, or (iii) 1 mg l^{-1} 2,4-D + 0·2 mg l^{-1} BAP. The culture temperature was 20°C and, unless indicated otherwise, the light intensity 30 μE m^{-2} s^{-1} (Philips TL 33) for 16 h per day.

After 10 days of induction in the dark on medium i, the explants were transferred to basal medium. Regenerated plantlets were transferred to rooting medium 2 months after the start of the culture. The rooting medium had the same composition as the basal medium except for the reduction of NH_4NO_3 to 82 mg l^{-1}. Explants cultured on medium ii or iii generated only callus. Three months after the start of the culture, pieces of callus of 3 × 3 × 3 mm were transferred to the NAA/BAP-medium. Two (medium ii) or 4 (medium iii) months after the transfer to NAA/BAP-medium, regenerated plantlets were transferred to rooting medium. The final number of plants was 21 (i), 16 (ii) or 31 (iii) respectively.

After 3 weeks on rooting medium, the rooted plantlets were transferred to soil in a greenhouse at 20–25°C and weaned. As a rule, this begonia is sprayed with 2-chloroethyltrimethylammonium chloride (CCC). However, because such spray may mask dwarf-mutants, the regenerated plants were not sprayed with CCC. After another 3 months, the plants flowered. They were evaluated for gross phenotypic aberrations and from each plant the shape of a leaf of approximately 15 cm was determined by measuring the lengths of ribs a and c (see Fig. 1). The leaf was located on one of the main stems at the third or fourth position from the apex. There were four or five more leaves on the stem below this leaf.

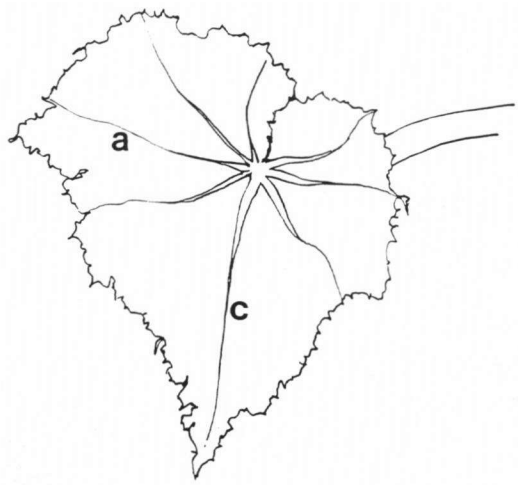

Fig. 1. A leaf of *Begonia x hiemalis* cv. Schwabenland Red. Ribs a and c are indicated.

RESULTS

Bud and callus induction

Explants cultured for 10 days on NAA/BAP-medium and subsequently transferred to basal medium showed the formation of only few buds (3–5 per explant) after 2 weeks.

Explants cultured on 0·1 mg l^{-1} 2,4-D + 0·2 mg l^{-1} BAP formed yellow–green, hard and fast growing callus. After transfer to NAA/BAP-medium, plantlets differentiated rapidly.

Explants cultured on 1 mg l^{-1} 2,4-D + 0·2 mg l^{-1} BAP formed yellow–brown, soft and slowly growing callus. After transfer to NAA/BAP-medium, plantlets differentiated slowly.

Gross phenotypic alterations

A small percentage of the plants (approximately 10%) showed obvious aberrations. Most notable were dwarf and non-flowering/late-flowering plants (Fig. 2). The percentages of aberrant plants of the three groups were not statistically different. It should be noted that the sample sizes were small.

Increased variation of the leaf shape

From each plant, the lengths of two ribs (a and c, see Fig. 1) of a leaf of approximately 15 cm were determined. Then, for each leaf ln c/a was calculated. Using the ratio c/a instead of ln c/a would result in an asymmetric treatment of a and c. Moreover, the analysis should be based on the parameter most normally distributed. In each group, the skewness of ln c/a was closer to zero than the skewness of c/a.

Figure 3 shows the mean values of c, a and ln c/a, and the SD of ln c/a for the three groups. The mean values of c and a demonstrate that in the three groups, leaves of the same size had been selected. The mean values of ln c/a were almost exactly the same for the three groups. However, the value of the SD of ln c/a in the sample of plants regenerated from callus grown in the presence of 1 mg l^{-1} 2,4-D differed significantly from the SD in the two other samples ($P < 0{\cdot}01$ and $P < 0{\cdot}05$).

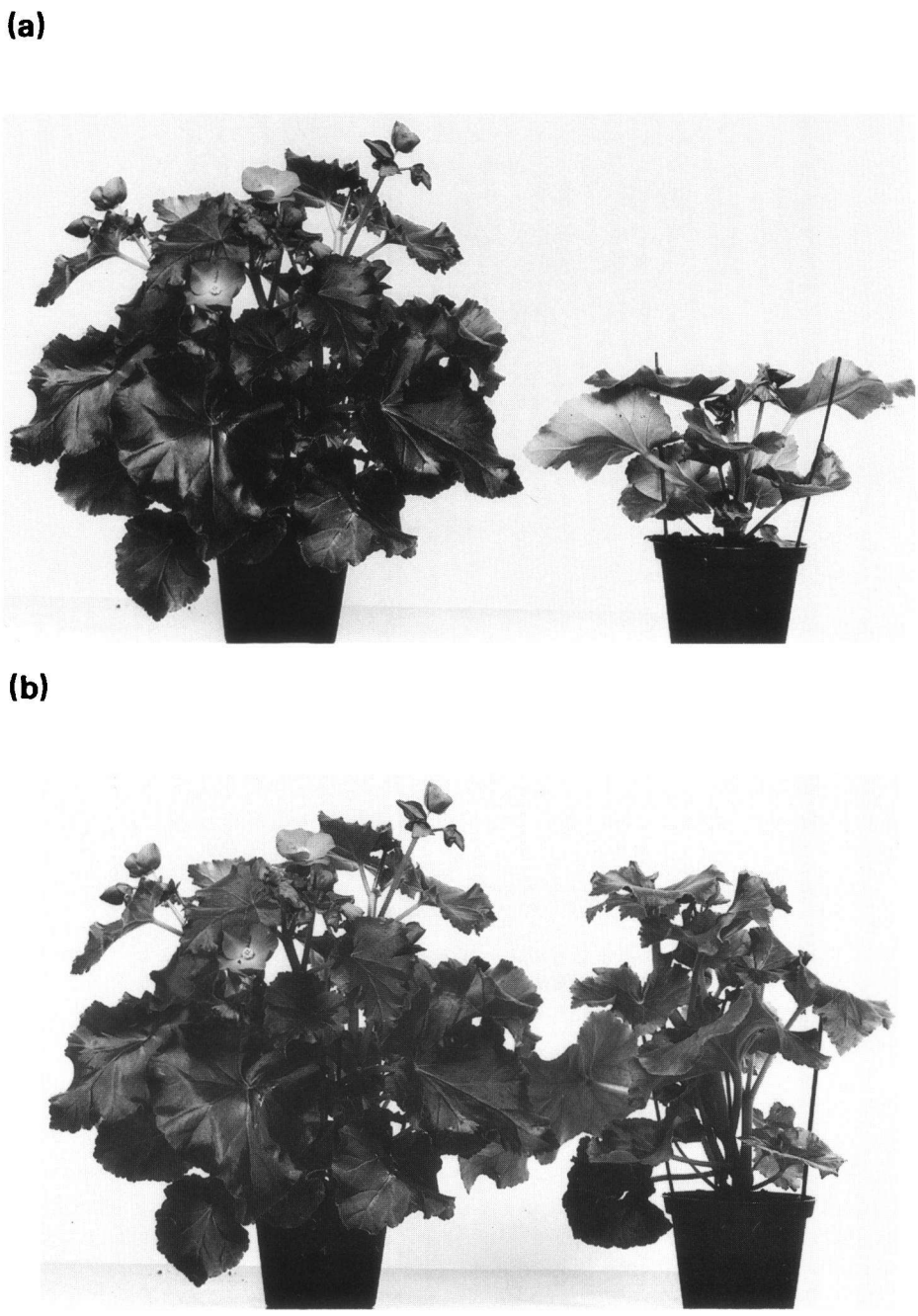

Fig. 2. Gross phenotypic aberrations in micropropagated plants of *Begonia x hiemalis* cv. Schwabenland Red. (a) Normal-looking plant (left) and dwarf plant (right). (b) Normal-looking plant and late-flowering (or non-flowering) plant.

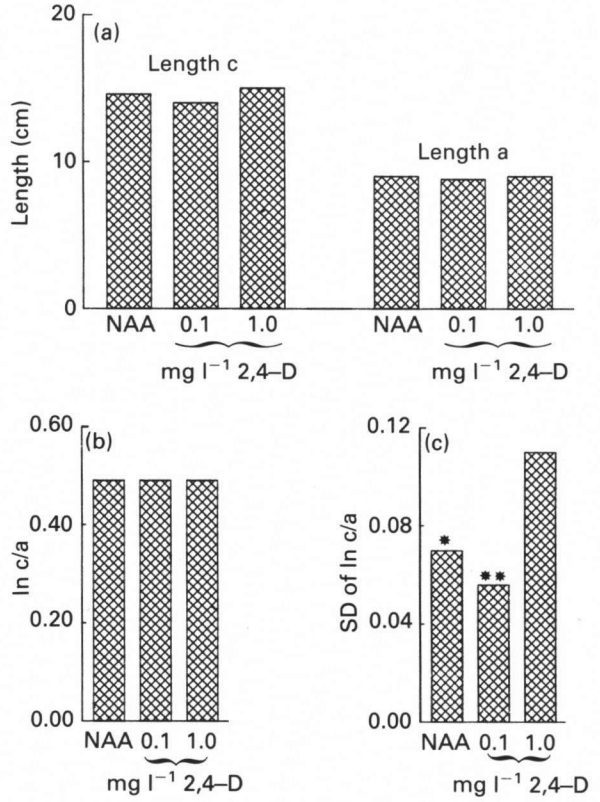

Fig. 3. Analysis of the leaf shape of three groups of plants, namely 21 plants regenerated directly on leaf explants on medium i (NAA), 16 plants regenerated after a callus interphase on medium ii (0·1 mg l^{-1} 2,4-D), and 31 plants regenerated after a callus interphase on medium iii (1·0 mg l^{-1} 2,4-D). (a) Mean values of leafribs a and c (see Fig. 1 for explanation); (b) mean values of ln c/a; (c) SD of ln c/a. The SD of plants from medium iii differs signficantly from the SDs of the other plants (*$P<0.05$; **$P<0.01$).

DISCUSSION

It has been noted that in somaclonal populations the spread of the values of quantitative traits is larger than in control populations (Karp & Bright 1985). To our knowledge the value of the SD has only been used in few papers to describe the extent of variation (see De Klerk 1990). Most notably, Jackson & Dale (1989) reported an increase of the coefficient of variation (i.e. the proportional SD) for various quantitative traits in the progeny of a regenerated plant after selfing relative to selfed control plants. They did not observe any changes on the chromosomal level and in isozyme patterns which demonstrates the sensitivity of an assay based on the SD. Similar results were obtained in rice by Zheng *et al.* (1989). In our experiments, we observed an increase of the SD of the leaf shape (measured as ln c/a) in plants regenerated from callus induced at a high concentration of 2,4-D. Only some of the plants in this group showed gross phenotypical changes and the percentage of these plants was not statistically different from the percentages of grossly aberrant plants in the other groups: because of the small numbers in each group, only large differences between percentages would have been statistically significant. This indicates the sensitivity of an assay based on the SD.

We do not know whether the observed increase of the SD of ln c/a is due to somaclonal or to epigenetic variation. Some lines of evidence indicate an epigenetic nature. First, it is known that the leaf shape is affected by applied plant growth regulators such as zeatin (Harte & Wallroth-Marmor 1988). We observed that the first leaves formed during tissue culture were round (ln c/a = $\pm$0). The increase of the SD could therefore be the result of an epigenetic effect of tissue culture factors still being strong in some plants but weak in others. Secondly, Wang & Holl (1988) reported that in red clover plants micropropagated through axillary buds, the SD of the length–width ratio was significantly larger than the SD in control plants. In plants generated from pre-existing meristems only epigenetic variations occur (De Klerk 1990).

Other evidence, though, suggests a genetic cause for the increase of the SD. First, epigenetic variation would probably also affect the value of the mean of ln c/a. As we found that this mean was almost the same for the three groups (Fig. 3b) and that only the SD was affected (Fig. 3c), high 2,4-D apparently had a random effect: the leaves were sometimes wider (decrease of ln c/a) and other times longer (increase of ln c/a) than the 'control' leaves. Such random effect strongly indicates the occurrence of genetic mutations (cf. De Klerk 1990). Secondly, it is known that leaf shape is under the control of genetic factors and that these factors have a considerably larger effect than added hormones (Harte & Wallroth-Marmor 1988). Finally, we have found that leaf shape is stable from the third to the fifth leaf from the apex and that the major factor causing variation is not the position of the leaf on the plant, but the individual plant (G. J. De Klerk unpublished observations). Together, these data strongly indicate that the observed increase of the SD is due to an increased extent of somaclonal variation. At present, we are examining this in more detail. As the begonia Schwabenland Red is triploid (Arends 1970), and consequently sterile, we cannot examine the sexual progeny.

In conclusion, we have shown that the value of the SD of the leaf shape (determined as ln c/a) can be used to assess the extent of variation. Although epigenetic variation cannot be ruled out, it is likely that the observed increase of the SD of ln c/a was caused by somaclonal variation.

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