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High Uniformity of plants regenerated from cytogenetically variable embryogenic suspension cultures of poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch)

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Summary. Shoot tip explants, callus and embryogenic cell suspensions of *Euphorbia pulcherrima* have been examined for quantitative variation in nuclear DNA content by means of cytophotometry. Increasing instability was found in calli and cell suspensions from Erlenmeyer flask and bioreactor culture. Nuclear DNA content ranged from 2C up to 32 C. Plants regenerated from embryogenic cell suspensions, however, were highly uniform with regard to phenotype and ploidy level indicating strongly impaired embryogenic potential of polyploid, aneuploid or other genetically altered cells.

Introduction

Genetic variation in cultured cells, tissues and regenerated plants has been reviewed by many authors (e.g. D'Amato 1985; Karp and Bright 1985; Lee and Phillips 1988; Phillips et al. 1990; Geier 1991). Apart from pre-existing variability in explant tissues, variant cell types have been shown to arise during in vitro cultivation, especially under conditions favouring unorganized growth. In addition to the more frequently discussed genetic changes, loss of trueness-to-type can also result from chimeral breakdown in cultivars of vegetatively propagated plants (Skirvin and Janick 1976; Preil and Engelhardt 1982; Skene and Barlass 1983), epigenetic alterations (Meins 1983) or physiological disorders (Varga et al. 1988) as well as from elimination of infectious agents (Cassells and Minas 1983).

Tissue culture-derived genetic variation is highly undesirable in commercial propagation. In order to minimize off-types arising from in vitro cultures, it has generally been recommended to use apical shoot meristems or bud explants as starting material followed by induction of axillary branching as means of multiplication. This type of propagation scheme requires labour-intensive division and transplanting operations and may be unsatisfactory in terms of multiplication rates. Somatic embryogenesis in liquid media offers advantages of automation of the in vitro propagation process, because freely suspended embryos need not to be separated manually. The possibility of cultivating embryogenic cell suspensions in bioreactors, both for a more precise control of culture conditions and for scaling-up the embryo yield, is being investigated (Ammirato 1989). However, since embryogenic cell suspensions are obtained through a callus stage, the risk of genetic variation has to be carefully assessed before such technology can be employed in clonal mass propagation.

A system of plant regeneration from embryogenic cell suspensions of *Euphorbia pulcherrima* has been developed and adapted to bioreactor cultivation (Preil 1991; Preil and Beck 1991). In the present paper we report on the examination of variability in this system, in terms of quantitative variation in nuclear DNA content in explant tissues, callus and cell suspensions as well as in phenotypic variation of regenerated plants.

Materials and methods.

In vitro culture, plant regeneration and evaluation of morphological characters. Callus and suspension culture

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followed the experimental protocol described by Preil and Beck (1991). Shoot tips (0.5 mm) of cv. 'Angelika' were placed for callus formation on MS agar medium supplemented with 0.2 mg/l 4-chlorophenoxyacetic acid (CPA) and 0.2 mg/l 6-benzylaminopurine (BAP). After 2-3 weeks, primary callus was transferred to Erlenmeyer flasks containing liquid medium of the same composition. The flasks were maintained at 90 rpm on a rotary shaker. Three weeks later, cell aggregates were either retransferred to MS-agar medium for production of secondary callus which retained its embryogenic potential or were used for the establishment of stock suspension cultures serving as bioreactor inoculum (bioreactor: Biostat M, Braun Diessel Biotech).

Somatic embryos formed spontaneously in liquid MS medium containing 0.2 mg/l CPA and 0.2 mg/l BAP. Improved results were achieved by using media supplemented with 0.1 mg/l CPA, 0.2 mg/l 1-naphthaleneacetic acid (NAA) and 0.1 mg/l (2-isopentenyl) adenine (2iP).

Globular embryos were removed from suspension culture by sieving and were placed on filter paper in Petri dishes containing auxin-free medium supplemented with 0.05 mg/l BAP. Three weeks later, regularly developed embryos had to be separated from various kinds of aberrant types and undifferentiated callus. All aberrant forms were discarded. The normal embryos placed again on MS agar medium of the same composition, germinated quickly. After development of radicles, plantlets of 20 - 25 mm size were transferred to greenhouse and planted in a peat-perlite substrate (2:1 v/v). 3000 flowering plants with fully developed bracts were examined for morphological variations and colour changes.

Cytophotometry. Explant tissues and callus pieces were fixed in FAA (5 parts of 37 - 40% formaldehyde, 5 parts of glacial acetic acid, 90 parts of 50% ethanol) for 14 days at room temperature. After washing with 50% ethanol, they were dehydrated in a graded series of ethanol, passed through xylene and finally were embedded in Histowax (Reichert-Jung; melting point 57 - 58°C). Cell suspensions were treated in the same manner exept that they were embedded in agar by mixing equal volumes of cell sediment and 50°C warm solution of agar (16 g/l). The mixture was immediately cast into a Petri dish and, after solidification, was cut into cubes which were then further processed like tissue and callus samples. Microtome sections 15 μm thick were cut, mounted on slides and, for blocking the aldehydes, were kept in 1% w/v dimedone in 70% ethanol overnight. The slides were then stained for DNA by the Feulgen procedure and after being made permanent they were analyzed within one week. DNA content was determined by measuring the extinction at 549 nm of individual Feulgen-stained nuclei with a Leitz MPV compact microscope photometer. Each nucleus was selected, by careful consideration through focussing, in order to ensure that only unsectioned nuclei were measured. Extinction readings were converted into C-values using the mean extinction of mitotic prophase nuclei in shoot apical meristems of diploid cuttings as 4C standard.

Results

Nuclear DNA Content of Explants

The upper histogram of fig. 1 shows the reference population of mitotic prophase nuclei of shoot apices which had been used to convert extinction readings into C-values. Standard deviation in this histogram can serve as an indication of the accuracy of measuring. Examination of epidermal and cortical cell nuclei 5 mm below the shoot apex (lower histogram of fig. 1) revealed on distinct peak at 2C, indicating that most of the cells were in stage G_1 of the cell cycle. As could be expected from the low mitotic activity still present in this tissue region, there is also a small proportion of cells in stages S and G_2, causing the asymmetrical shape of the histogram. Provascular and pith tissue of the same region yielded essentially similar nuclear DNA values. In no case, DNA content significantly exceeded 4C. Thus, it is obvious that somatic polyploidy does not occur in the shoot apex and adjoining tissues which were used as explant material.

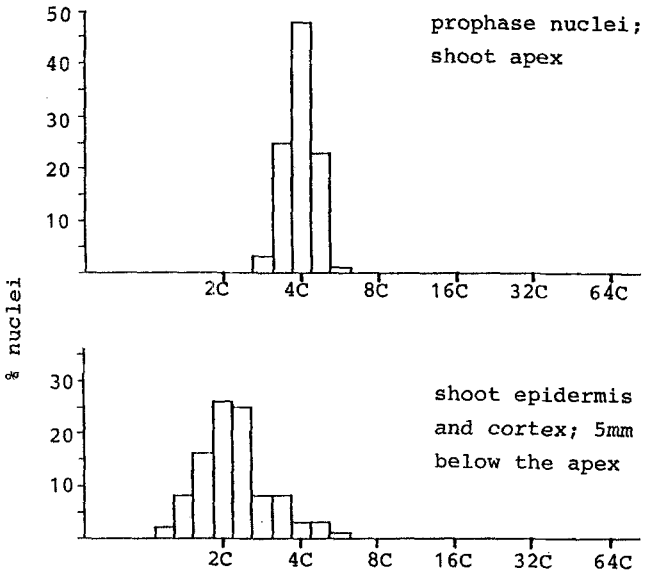

Fig.1. Nuclear DNA content in shoot tips of *Euphorbia pulcherrima*. 100 nuclei were measured for each histogram.

Nuclear DNA Content of Calli and Cell Suspensions

The frequency distributions of nuclear DNA content observed in calli and cell suspensions are shown in fig. 2. Compared with the explant tissue, primary and secondary calli exhibit a shift in nuclear DNA content from 2C towards 4C, and, in addition, show some nuclei with higher than 4C DNA content. A tremendous increase in variability was observed after the transition from callus to cell suspension cultures. In both, Erlenmeyer flask and bioreactor cultures nuclear DNA content ranged from 2C to 32C with an indistinct frequency maximum around 8C. The proportion of 2C and 4C nuclei appeared to be somewhat higher in the bioreactor than in the Erlenmeyer flask cultures. However, this difference may represent rather a trend than a statistically significant difference. It is important to note that in both types of cell suspensions single cells often had large nuclei with high DNA content, while 2C nuclei were found only in embryogenic clusters composed of small cells rich in cytoplasm.

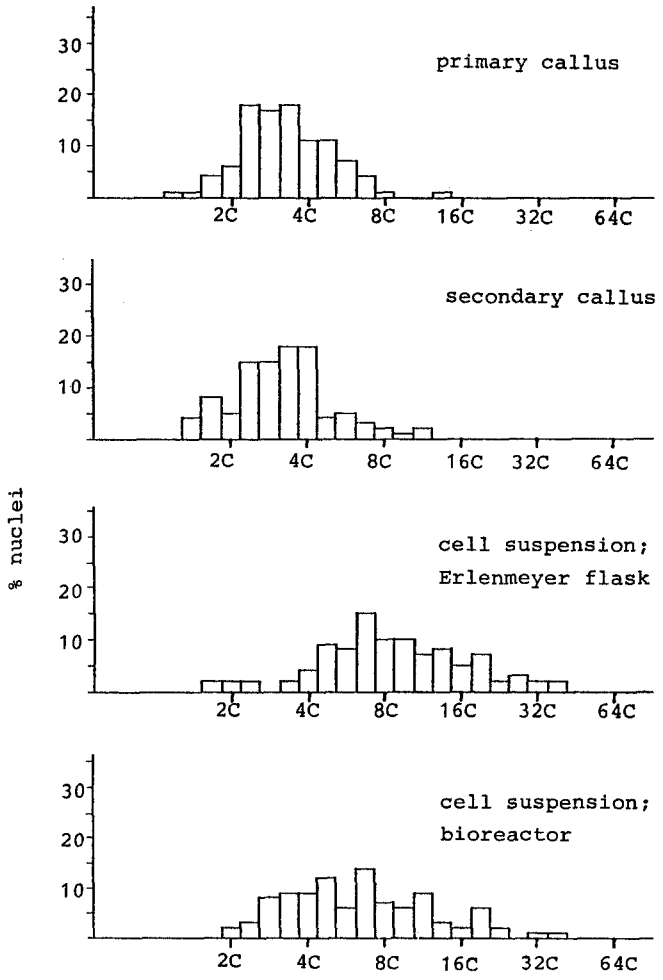

Fig.2. Nuclear DNA content in callus and embryogenic cell suspensions of *Euphorbia pulcherrima.* 100 nuclei were measured for each histogram.

Phenotype and Ploidy of Regenerated Plants

The investigated cv. 'Angelika' was chosen as model idiotype for studies on somaclonal variation because of its heterozygous anthocyanin locus WH. It is known that from WHwh idiotypes (with red bracts) whwh individuals (with white bracts) regularly occur in the course of conventional mass propagation by cuttings. Among the 3000 somatic embryo-derived plants no whwh idiotype was found. This was surprising because in previous experiments high percentages of whwh mutants could be induced by X-irradiation of embryogenic suspensions (Kleffel et al. 1986). Thus 4.2, 7.7 and 8.9% of whwh mutants were achieved after irradiation with 30, 40 and 60 Gy, respectively.

All plants investigated lost the free branching potential of the donor cultivar 'Angelika' completely. Additionally all leaves were lobed instead of the more rounded leaves of 'Angelika'. No deviation was found with regard to these two traits or any other morphological characters among the 3000 plants.

Tetraploid plants (2n = 4x = 56) were rarely observed. Only two tetraploids out of 198 plants originating from one Erlenmeyer flask culture could be identified. Regenerates from other cultures were all diploid. In previous experiments with poinsettia cv. 'Annemie' six tetraploids out of 295 plants (= 2%) were detected (Preil and Engelhardt 1982).

Discussion

The present study has revealed a high degree of quantitative variation in nuclear DNA of callus and cell suspension cultures of *Euphorbia pulcherrima.* A general feature is the lack of distinct peaks in the nuclear DNA histograms (fig. 2). This suggests that different processes, such as polyploidization, aneuploidy, endoreduplication and/or DNA amplification may contribute to the observed variation. As tetraploid plants have been regenerated at low frequencies

(2% maximum) from poinsettia cell suspensions in the course of this and a previous study (Preil and Engelhardt 1982), the occurrence of polyploidy has been demonstrated, but there is no definite evidence for the occurrence of any of the other possible causes of variation.

In view of the potential use of bioreactor technology in clonal mass propagation, the most important result of the present study is the high phenotypic uniformity of plants regenerated from embryogenic cell suspensions of poinsettia. It is generally accepted that the conditions of cultivation may both induce genetic changes and exert selective forces on the growth of the different cell variants (e.g. Bayliss 1977; Vanzulli et al. 1980; Binarová and Dolezel 1988). Usually, further selection takes place during plant regeneration by excluding those cells which have lost morphogenic ability. In some cases competition has also been shown to continue during organized growth within and among regenerates (Lupi et al. 1981; Fish and Karp 1986). In the present study it is not clear, to what extent a 'manual' selection has been effected by discarding malformed embryos during transplanting. However, since cells with normal nuclear DNA content were found only in proembryogenic cell clusters, there is an indication that a 'natural' selection may operate at an early stage of embryogenesis resulting in the exclusion from the morphogenetic process of polyploid, aneuploid or other genetically altered cells.

The uniformity of regenerated poinsettia plants seems to add to observations that might suggest a higher genetic stability of embryogenic cultures compared with organogenic ones (Swedlund and Vasil 1985; Rajasekaran et al. 1986). Such a generalization, however, is not admissible, since there are on the one hand examples of high variability in embryogenic cultures (e.g. Orton 1987; Cavallini et al. 1987), on the other hand, studies e.g. on soybean (Barwale and Widholm 1987) and maize (Armstrong and Phillips 1988) have shown no substantial differences, regarding genetic stability between plants regenerated through embryogenesis and shoot organogenesis, respectively. In the face of the highly complex interactions between genotype and culture conditions, it appears that the extent of variability in callus and suspension culture-derived regenerates can hardly be predicted. Therefore, every single case has to be carefully examined and undesirable variability minimized as far as possible, by adjusting the cultivation protocol.

Although the 3000 plants regenerated in the course of this study were highly uniform, all were different from the donor cultivar 'Angelika' in terms of branching potential and leaf morphology. In this connection it is important to note that in *Euphorbia pulcherrima* there are two groups of cultivars: (1) freely branching with rounded leaves, and (2) restricted-branching with lobed leaves. While the cultivar 'Angelika' used in this study belongs to the first group, the regenerated plants had the phenotypic characteristics of the second group. Branching potential and other morphological traits have been shown to be transmitted by grafting from type 1 to type 2 cultivars (Stimart 1983). Dole and Wilkins (1988) have suggested a virus as possible agent causing the graft transmitted changes, and further research on this possibility is in progress. According to this hypothesis, the changed morphology of the plants regenerated in this study via embryogenesis could well be due to the loss of the hypothetic virus during suspension culture. This seems to be the most likely explanation, since in embryo-derived plants branching ability could be restored by grafting on the original donor plants. This character remained stable in the course of conventional propagation by cuttings, however, was eliminated again via somatic embryogenesis (Preil, unpublished results).

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