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BRIEF COMMUNICATION

Ploidy instability of embryogenic cucumber (*Cucumis sativus* L.) callus culture

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Abstract

Embryogenic callus cultures were established from immature cucumber (*Cucumis sativus* L.) embryos on E20A (Dumas de Vaulx *et al.* 1981) or MS (Murashige and Skoog 1962) media supplemented with 6-benzylaminopurine (BAP), α-naphthylacetic acid (NAA) and/or 2,4-dichlorophenoxyacetic acid (2,4-D). Regeneration of plants was observed after a transfer to culture media either without growth regulators or supplemented with kinetin and NAA. Flow cytometry was employed to estimate DNA ploidy levels. Most of cell nuclei in young leaf tissues were found in G_1 phase with 2C DNA content. Callus cultures were mixoploid with DNA content ranging from 2C to 32C. The frequency of polyploid cells was increasing with the age of culture and the polyploidization was accompanied by a gradual loss of regeneration ability. Plants regenerated from callus cultures were classified as diploid (57 %), tetraploid (18 %), octoploid (4 %) and mixoploid (2n/4n, 4 %) and (4n/8n, 17 %). The results of this study confirmed a close link between the polyploidization and the loss of totipotency *in vitro*. Tetraploid plants obtained in this study have a potential to be used in interspecific crosses where their tetraploid status could help in overcoming existing breeding barriers due to differences in chromosome number.

Additional key words: flow cytometry, polyploidy, somatic embryogenesis, tissue culture.

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Abbreviations: BAP - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; DAPI - 4',6-diamidino-2-phenylindole; NAA - α-naphthylacetic acid.

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Most of genetic manipulation techniques rely on efficient and reproducible regeneration procedures from *in vitro* cultured cells and tissues. Somatic embryogenesis is the usually preferred mode of regeneration over organogenesis because of presumed single cell origin of regenerants and higher genetic stability of embryogenic cultures (Thorpe 1988, Peschke and Phillips 1992). In cucumber somatic embryogenesis was first described by Malepszy *et al.* (1982). Ziv and Gadasi (1986) and Chee and Tricoli (1988) improved the procedures for plant regeneration by the addition of activated charcoal to the media. Wroblewski and Malepszy (1992) established long-term embryogenic suspension culture and Burza *et al.* (1992) were successful in induction of direct somatic embryogenesis from cucumber protoplasts.

The frequency of regeneration of normal plants from cucumber tissue culture remains rather low (Lou and Kako 1994). In addition, regeneration of abnormal embryos, recalusing or vitrification have been often observed (Bergervoet *et al.* 1989, Ladyman and Girard 1992). It is interesting that very few studies were focused on karyological changes during *in vitro* culture and regeneration in cucumber (Sang-Gu *et al.* 1988). Malepszy *et al.* (1990) suggested that in addition to gene mutations, also polyploidization could be responsible for abnormal embryo and plant development. In a systematic study, Colijn-Hooymans *et al.* (1994) used flow cytometry to analyse nucler DNA content in cucumber cotyledons and plants regenerated from them *via* organogenesis and concluded that the decrease in regeneration competence corresponded with endopolyploidization of cotyledonary cells. Here we report on the induction of embryogenic callus in cucumber. Flow cytometry was used to estimate ploidy levels in callus cultures and plants regenerated from them.

Cucumber (*Cucumis sativus* L.) seeds of a SM 6022 line obtained from a breeding station (*Semo,* Smržice, Czech Republic) were used to obtain flowering plants and immature embryos after controlled pollination. Immature fruits (15 - 20 d after pollination) were surface sterilized with 5 % (m/v) *Chloramin B* (*Lachema*, Brno, Czech Republic), rinsed once with 70 % ethanol and 3 times with sterile water. Embryos extirpated from immature fruits were used to initiate embryogenic callus on MS (Murashige and Skoog 1962) or E20A (Dumas de Vaulx *et al.* 1981) media supplemented with 0.5 μM 6-benzyl-aminopurine (BAP) and 0.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 20 μM α-naphthylacetic acid (NAA). The explants were cultured in the dark at 28 ± 1 °C. After 1 month, the cultures were transfer onto fresh media supplemented with the same growth regulators and cultivated under a 16-photoperiod (irradiance 25 μmol m^{-2} s^{-1} provided by white fluorescent tubes) and temperature 25 ± 2 °C. Two months old calli were transferred to both basic media supplemented with 2.5 μM kinetin and 0.5 μM NAA or to the media without growth regulators. Regenerated plantlets were rooted *in vitro* on the media without growth regulators.

Flow cytometric analysis of nuclear DNA content was used to estimate DNA ploidy levels of callus cultures and plantlets regenerated from them. Briefly, cell nuclei were isolated by chopping callus or leaf tissues with a razor blade in LB01 buffer (Doležel *et al.* 1989) and supplemented with 2 μg cm^{-3} of 4',6-diamidino-2-

phenylindole (DAPI) in a glass Petri dish. The suspension of nuclei was filtered through a 50 μm nylon mesh to remove large tissue debris and the sample was kept on ice prior to analysis. The fluorescence of nuclei was analysed using a *Partec PAS II* flow cytometer (*Partec GmbH*, Münster, Germany) at a rate of 20 - 50 nuclei per second. Prior to analysis, the gain of the instrument was adjusted so that the peak corresponding to G_1/G_0 nuclei isolated from a diploid plant was located on channel 25. This calibration was checked periodically. In each sample, at least ten thousand nuclei were analysed.

Callus formation was observed from immature embryos approximately after one month in culture. The callus was soft and friable which is in contrast to the observation of Malepszy (1983), who found that only gel-like callus was embryogenic. No apparent differences were observed between callus cultures initiated on different media. After subculture onto basic media supplemented with 2.5 μM kinetin and 0.5 μM NAA or media without growth regulators, somatic embryos appeared. The embryo formation and development was intensive on E20A medium, which was used by Sauton and Dumas de Vaulx (1987) for induction of haploid plants of *Cucumis melo* L.

Regeneration capacity of calli decreased with their age, and after 4 - 6 months only malformed embryos were formed. Regeneration of abnormal embryos and plants (teratological changes, chlorophyll deficiency, callus formation, lack of roots) is common in cucumber tissue culture (Malepszy 1982, 1990). Plader *et al.* (1994) compared somaclonal variation in various regeneration systems in cucumber. No variation was found when micropropagation or direct regeneration from leaf explants was used. Largest variation was found after regeneration by direct somatic embryogenesis from protoplast.

Flow cytometric analysis showed that most of the nuclei isolated from young leaves of diploid plants obtained from seeds and grown in the greenhouse were in the G_1/G_0 phase of the cell cycle with 2C DNA content (Fig. 1A). Large part of plants regenerated from somatic embryos (57 %) had similar distributions of nuclear DNA content and thus were classified as diploid (Fig. 1B). A smaller fraction of regenerants (18 %) had G_1/G_0 nuclei with doubled DNA content and were classified as tetraploid (Fig. 1C). Still a smaller fraction of regenerants (4 %) were octoploids. In addition to these ploidy levels, some of the regenerated plants were mixoploid (4 % of regenerants were diploid/tetraploid while 17 % of plants were tetraploid/octoploid) (Fig. 1D).

The morphology of diploid and tetraploid regenerants was normal. However, some of them exhibited darker green leaves. On the other hand, octoploid and mixoploid regenerants were malformed and did not develop into normal plants. Similarly, Ezura and Oosawa (1994) observed formation of diploid, tetraploid and octoploid somatic embryos in *C. melo*. However, only diploid and tetraploid embryos gave rise to morphologically normal plants. Contrary to our results, these authors did not observe regeneration of mixoploid embryos and plants. If single cell origin of somatic embryos is assumed, polyploidization had to occur during the embryo development *i.e.* during the stage II in our system.

In an attempt to analyse the nature of the loss of regenerating ability of older

embryogenic calli, we have analysed their nuclear DNA content. It turned out that all of them were highly polyploid, with only a small fraction of diploid cells at G_1/G_0 phase (Fig.1E). In some calli, this fraction was completely absent (Fig. 1F). Typically, the callus consisted of 15 % cells with 2C DNA content, 45 % of cells with 4C, 35 % of cells with 8C and 5 % of cells with 16C or higher.

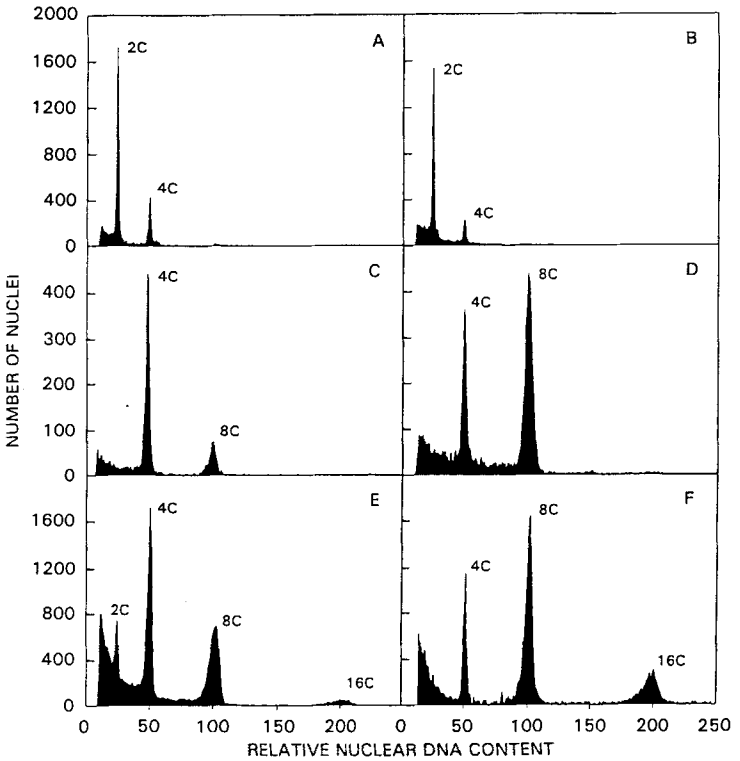

Fig. 1. Histograms of relative nuclear DNA content (in channel numbers) obtained after flow cytometric analysis of nuclei isolated from cucumber (*C. sativus* L.) leaves and calli: *A* - young leaf tissue of a diploid plant obtained from seed. Note that most of nuclei are in G_1/G_0 phase with 2C DNA content; *B, C, D* - young leaf tissue of plants regenerated from callus culture. Plants were classified as diploid (*B*), tetraploid (*C*) and mixoploid 4n/8n (*D*); *E, F* - six-month-old callus cultures. Note that diploid cells with 2C DNA content are absent in the sample *F*.

The occurrence of polyploid cells in callus explains the origin of polyploid regenerants. The strong tendency towards polyploidization of callus cells *in vitro* seems to be typical for the genus *Cucumis*. For instance Ezura *et al.* (1992) and Ezura and Oosawa (1994) reported regeneration of polyploid plants also in *C. melo*. Gilissen *et al.* (1993) described the occurrence of endopolyploid cells (polysomaty) in most of the cucumber plant organs and tissues. These endopolyploid cells could be induced to divide and may give rise to polyploid callus cells. However, in our experiment, embryogenic callus was initiated from immature embryo tissues

containing only diploid cells. Thus the polyploidization took place during the *in vitro* culture. It should be pointed out that the rate of polyploidization of callus cultures must have been relatively high considering the fact that 6 months after culture diploid cells were rare or even missing.

The loss of totipotency *in vitro* is often related to polyploidization (Colijn-Hooymans *et al.* 1994). Our results support this point of view. However, other interpretation should be considered. Physiological and/or epigenetic changes may lead to a switch from a differentiation programme to dedifferentiation and subsequently to endopolyploidization.

To conclude, the embryogenic callus culture established from immature cucumber embryos may be used to obtain normal, diploid regenerants in cucumber. However, the regenerating ability is limited to a relatively short period of culture. Tetraploid plants obtained *in vitro* have a potential to be used in interspecific crosses where their tetraploid status could help in overcoming existing breeding barriers due to differences in chromosome number.

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