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Cellular behaviour in root meristem cells of *Zinnia elegans in vivo* and in tissue culture systems

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ABSTRACT *Zinnia elegans* is an attractive ornamental plant imported from overseas. Tissue culture of this species is beneficial for mass propagation, especially for commercial purposes. In this paper, we report on cellular studies of this species which is rarely done. Cellular parameters such as Mitotic Index (MI), chromosomes counts, ploidy level/ nuclear DNA content, cell doubling time (Cdt) and measurement of mean cell and nuclear areas were recorded from root meristem cells grown *in vivo* and roots cultured *in vitro*. In this study, we have found that *in vitro* root under short term culture, showed an increase in the MI value, the chromosome number remained stable, the mean cell and nuclear areas decreased, the polyploid cells increased slightly, more cells entering S phase of the cell cycle and the duration of Cdt shortened *in vitro*. These behaviour are consistent with the readiness of this species to regenerate *in vitro* and the long term culture confirmed this observation and the regenerants obtained were true to type.

ABSTRAK *Zinnia elegans* adalah sejenis tumbuhan hiasan yang menarik dan diimport dari luar negeri. Kajian kultur tisu adalah penting bagi spesies ini untuk dipropagasi secara besar-besaran terutama untuk tujuan komersial. Dalam kajian ini dilaporkan mengenai aspek aktiviti sel yang jarang sekali dilakukan. Parameter sel seperti Indeks Mitosis (MI), bilangan kromosom, tahap ploidi/ kandungan DNA nukleus, masa penggandaan sel (Cdt) dan pengukuran luas sel dan nukleus telah dijalankan menggunakan sel meristem akar yang ditanam secara *in vivo* dan juga menggunakan akar yang terhasil dari kultur tisu. Hasil keputusan menunjukkan, dibawah keadaan kultur jangka pendek, nilai MI bertambah dalam akar *in vitro*, bilangan kromosom menjadi stabil, purata luas sel dan nukleus berkurangan, jumlah sel poliploid bertambah, kebanyakan sel memasuki fasa S kitaran sel dan tempoh Cdt menjadi pendek bagi sel-sel akar *in vitro*. Kelakuan sel yang ditunjukkan ini konsisten dengan kesediaan spesies ini untuk diregenerasi secara *in vitro* dan kajian jangka panjang telah membuktikan regeneran hasil kultur adalah serupa dengan induk.

(tissue culture, cellular behaviour *Zinnia elegans*, cell cycle)

INTRODUCTION

Zinnia elegans is an attractive imported ornamental plant. One of our aims in this study is to mass propagate this plant using tissue culture system. It is hoped that by culturing this species *in vitro*, various vegetative parts can be used for propagation instead of using seeds which are normally imported. This species is quite popular as an ornamental plant in Malaysia. Therefore, establishing an efficient regeneration system for this plant is quite useful and it is hoped to be commercially viable. In order to check for early occurrence of somaclonal variation, some cellular activities of the root meristems were investigated such as chromosome number, Mitotic index, nuclear DNA content, ploidy level, cell and nuclear areas and cell doubling times. The root meristem cells of *Z. elegans* both cultured *in vivo* and *in vitro* were used for the determination of the above parameters. The characteristics of the cells grown *in vivo* and *in vitro* were then compared and contrasted. The relationship between cellular behaviour and regeneration potential could then be established.

Genetic stability of selected plant species which are regenerated from tissue culture is of vital importance. There are numerous reports stating that plantlets regenerated from *in vitro* systems

undergo genetic changes (Partanen, 1965). Changes at morphological level needs a long time to be seen. However, in tissue culture systems, changes that occur at cellular level can be associated with the changes that may occur at morphological level. For instance, one can predict the nature of the cells or tissues based on their cellular behaviour, since tissues that have potential to regenerate possess different characteristics from the ones that are unable to regenerate. In this paper we report the attempt to determine the early cellular changes that occur when cells are transferred from *in vivo* to *in vitro* environment.

MATERIALS AND METHODS

The standard tissue culture methods were used. The seeds were sterilised and cultured on MS medium without hormone to get axenic explant sources. For *in vivo* determination of cellular parameters, the seeds were grown on moist cotton · wool in petri dishes under sterile conditions. The standard root length and age of the seedlings were determined and permanent squashed preparations were made. The root segments were then cultured on MS supplemented with various hormones to get new roots. The new roots formed from root segments and roots of the regenerated plantlets were also made into squashed preparations and the same parameters [Mitotic index, chromosome counts, ploidy level/ nuclear DNA content, mean cell and nuclear areas and cell doubling times (Cdt)] were measured from the permanent slides.

Regeneration was achieved from this species and the cellular behaviour *in vivo* and *in vitro* were examined.

Determination of Mitotic Index (MI)

Mitotic index (cells at division) was scored from permanent slides that were stained with Feulgen. A total of 1,500 cells from three slides were counted.

Chromosome Counts

Chromosome counts were made of suitable chromosome spreads and at least 15 metaphase plates from root squashes were counted. The slides used were the same as those examined for MI.

Determination of Cell and Nuclear Areas

The slides which were stained with Feulgen and counter-stained with light green were used for this purpose. The slides were then examined under a light microscope (Zeiss Axioscope) connected to a system comprising a computer, TV video camera and a graphics tablet (image analysis system). Nuclear and cell areas were recorded from prophase cells. The perimeter of the cells and nucleas were traced using a mouse attachment and an image of the tracing appeared as a series of pixel points on the TV. The perimeter data were analysed using a computer programme. By calibrating the monitor screen in μm^2, the perimeter measurement was converted into cell or nuclear areas in μm^2 by the computer programme. 150 prophase cells were measured.

Determination of ploidy level/ nuclear DNA content using VIDAS 21 image analysis system
Feulgen-stained squash preparations were analysed with a light microscope (Zeiss Axioskop) attached to VIDAS 21 image analysis system by Kontron Elektronik. This image analysis system uses a software package for DNA measurements.

The DNA package allows DNA measurement and evaluation of measurement data. The underlying principle is densitometric using the integrated optical density (IOD) of data nuclei calibrated with reference nuclei. The amount of DNA in the nucleus is proportional to the IOD value provided that quantitative staining such as the Feulgen –Schift reaction is used. The 2C value is determined by calibrating with the IOD of internal reference cell nuclei. Fifty interphase nuclei per slide and three slides (150 nuclei) per sampling time were scored. Different classes of C values were used as indicators of the component phases of the cell cycle where 1C is defined as the amount of the nuclear DNA in the unreplicated genome of a gamete. The proportions of nuclei with 0-2.2C (G_1), 2.2-3.6C (S-phase), 3.6-4.8C (G_2) and > 4.8C were calculated for each ploidy histogram. The normal distribution of G_2 nuclei is from 3.6 to 4.8C. Therefore, in this work, nuclei with densities > 4.8C are classified as polyploid.

Determination of cell doubling time (Cdt)

Five-day-old seedlings with standard root length and 7-day-old cultures were exposed to 0.025%

(w/v) colchicine solution for 5 hours. At hourly intervals the roots were fixed in 3:1 (v/v) absolute ethanol: glacial acetic acid and permanent Feulgen-stained squash preparations were made. At 0 hour, the primary roots and *in vitro* roots were also fixed as controls. The Mitotic index at 0 hour was scored and the percentage frequency of prophases and metaphases were scored at each sampling time. The initial linear relationship between colchicine induced metaphase accumulation and time was used to calculate mean Cdt using the formula of Clowes (1961)[modification of the methods of Evans, Neary and Tonkinson (1957)]. The formula is:

$$Cdt = \ln 2/m$$

whereby m = the rate of cells at metaphase, which is equal to the rate of entry of cells into mitosis over time following the start of exposure to colchicine when no cells are escaping from metaphase and when the number of cells in prophase remains constant. $\ln 2 = 0.693$.

RESULTS AND DISCUSSION

From tissue culture studies, it was found that *Zinnia elegens* was very responsive in culture. However, in this paper we only focused on the investigations of the cellular behaviour *in vivo* and *in vitro* for the short term culture. Any cellular changes that occur showed that the cells were not stable *in vitro* and there is possibility that the instability will be affected at morphological level. Tissues which are regenerative normally possess different characteristics compared with the non-regenerative ones.

Table 1 shows the mean MI, chromosome counts and duration of Cdt in cells grown *in vivo* and *in vitro*. The value of MI *in vitro* was slightly higher, indicating that cell division occurs more actively in culture, probably due to the suitable medium and hormone added. This is in agreement with Abdullah (1998) working on *Petunia hybrida*. Both species could regenerate readily in culture. In species such as *Psophocarpus tetragonolobus* where the MI was lower *in vitro*, regeneration was found to be difficult (Abu Shah and Taha, 1994). In some cases the lower MI might be due to organogenesis whereby the dividing cells are restricted to certain areas such as nodular meristematic bodies (Yeoman et.al.,1965).

Present study, also observed that the highest percentage of cells *in vitro* were arrested at G2 phase of the cell cycle (41.62%) and percentage of cells which become polyploid was 38.38%. The percentage of cells arrested in G1 was 0% and 20% in S phase of the cell cycle. The values of the different parameters obtained were more or less the same as those from *in vivo* values (0% in G1, 14.37% in S, 55.62% in G2 and 33.75 % polyploid). Polyploid cells were observed both *in vivo* and *in vitro,* implying that polyploid cells were carried on from the explants (Table 2). Yeoman and Mitchell (1970) obtained similar results using *Helianthus tuberosus*. Polyploidy had no effect for this species since regeneration could occur easily. In some species, mitotic activities may stop but not DNA synthesis, therefore cells increased their DNA and become polyploid. The advantages of being polyploid are cells are more resistant to radiation, cells are bigger and produced more vigorous plants.

Minocha (1979) stated that when hormones were added to the media, more cells would enter the S phase and this is in agreement with the present work. It was found that the chromosome number was stable under both conditions for this species. The Cdt was significantly ($P<0.05$) shorter *in vitro* (96 h) compared to *in vivo* (133 h). This is in line with the high ability of this species to regenerate under long term culture.

The mean cell and nuclear areas *in vitro* were smaller compared to *in vivo* values. (Table 3). This was also observed by Abdullah (1998) working on *Petunia hybrida*. According to Jordan et.al. (1987), nuclear and cell sizes are controlled by DNA content and can change independently of each other. In this case both parameters changed together, i.e. when cell size decreased the nuclear size also decreased *in vitro*. Decrease in cell size may be related to formation of organs, in this case root formation. The ratio of nuclear to cell area was not stable, probably due to the shock effect; moreover, the culture was only short term.

Table 1. The mean MI, chromosome counts and cell doubling time of root cells of *Zinnia elegans* grown *in vivo* and *in vitro*.

(In Vivo)	**Mean MI ± SE (%) Range**	**Chromosome counts Mean**	**Cell doubling time {(Cdt) (hour)}**
13.47 ± 0.46	18-28	22.9	133
(In Vitro) 15.60± 0.20	18-28	23	96

Table 2. The proportion of nuclei at G1, S, G2 and polyploid in the root cells of *Zinnia elegans* grown *in vivo* and *in vitro*.

G1 (%)	**S (%)**	**G2 (%)**	**polyploid (%)**
(In Vivo) 0	14.37	55.62	33.75
(In Vitro) 0	20	41.62	38.38

Table 3. The mean cell and nuclear areas and the ratio of nuclear area: cell area in the root cells of *Zinnia elegans* grown *in vivo* and *in vitro*.

Mean Cell Area (μm^2)	**Mean Nuclear Area (μm^2)**	**Ratio nuclear: cell**
(In vivo) 113.55 ± 4.44	40.58 ± 0.95	0.39
(In vitro) 92.40 ± 5.70	36.86±1.44	0.48

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