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Changes in cellular behaviour during *in vitro* flowering of *Petunia hybrida* Vilm

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ABSTRACT Cellular activities, including Mitotic Index (MI), chromosome counts, cell doubling time (Cdt), nuclear DNA content, mean cell and nuclear areas were examined in root meristem cells of flowering plantlets of *Petunia hybrida* Vilm to evaluate changes occuring during flowering phase of the plantlets in tissue culture system. The main objective was to investigate the relationship between cellular activities and *in vitro* flowering. Leaf explants were cultured on MS medium supplemented with 1.0 mg/l IAA with combination of 1.0 mg/l zeatin, while pedicel explants were cultured on MS medium supplemented with 1.0 mg/l kinetin for flowering to occur. We found that regeneration and *in vitro* flowering could only be achieved when leaf explants (from 1-month - old seedlings) and pedicels (from 1-month-old intact plants) of *Petunia hybrida* Vilm were used. The Mitotic Index and chromosome counts increased during flowering as compared to *in vivo* values (determined from 5 day old seedlings after standardisation of root growth being done). The cell doubling times were shorter ($P<0.05$), no polyploid cells were detected and cell and nuclear sizes were bigger ($P<0.05$) in root meristem cells of flowering plantlets as compared to root meristem cells of 5-day-old seedlings.

ABSTRAK Aktiviti sel seperti Indeks Mitosis (MI), bilangan kromosom, masa penggandaan sel (Cdt), kandungan DNA nukleus, purata luas sel dan nukleus telah dikaji dalam sel meristem akar tumbuhan *Petunia hybrida* Vilm yang menghasilkan bunga secara *in vitro* untuk melihat perubahan yang berlaku semasa pembungaan dalam sistem kultur tisu. Tujuan utama adalah untuk menghubungkait antara aktiviti sel dan pembungaan *in vitro*. Eksplan daun dikultur di atas media MS yang ditambah dengan 1.0 mg/l IAA dan 1.0 mg/l zeatin, sementara pedisel dikultur di atas media MS yang ditambah dengan 1.0 mg/l kinetin untuk pembungaan *in vitro*. Regenerasi dan pembungaan *in vitro* didapati hanya boleh berlaku apabila eksplan daun yang berasal dari anak cambah aseptik yang berumur 1 bulan dan juga pedisel yang berumur 1 bulan dari tumbuhan "intact" digunakan. Berdasarkan kajian aktiviti sel yang telah dijalankan, didapati MI dan bilangan kromosom bertambah semasa pembungaan berbanding dengan sel akar tumbuhan "intact" yang berusia 5 hari yang digunakan sebagai data rujukan. Masa penggandaan sel telah menjadi singkat, tiada sel poliploid diperhatikan dan purata luas sel dan nukleus lebih besar dalam sel akar tumbuhan yang berbunga dalam sistem *in vitro* berbanding dengan anak cambah 5 hari.

(*Petunia hybrida*, cellular behaviour, tissue culture, *in vitro* flowering)

INTRODUCTION

Studies concerning *in vitro* flowering were reported quite extensively, for eg. in *Lolium* (McDaniel *et.al.*,1991), *Citrus* (Garcia-Luis *et al.*,1989), *Bambusa* (Nadgauda *et al., 1990;* John and Nadgauda, 1999), *Murraya paniculata* (Taha, 1997), tobacco (Smulders, *et.al.,* 1990), soybean (Julian and Wyndaele,1992), *Melia azedarach* (Handro and Floh, 2001) etc. Change in phase from vegetative to flowering is usually accompanied by change in cellular activities (Gifford and Nitsch, 1967) which includes increase in DNA synthesis, mitotic activities and cell sizes. The significance in the study of *in vitro* flowering is usually for breeding improvement programmes, for understanding of the physiology underlying flowering processes and also for biochemical and molecular approaches to understand the transition from juvenile to the mature state. All these changes are known to occur in intact plants but there are few reports

dealing with the association of cytological parameters with the flowering process, especially *in vitro* flowering.

Many factors are involved in *in vitro* flowering. For instance, the interactions of phytohormones, light, day length, addition of sugars, e.g. (sucrose and glucose), minerals and phenolic compounds are often associated with flowering (Tanimoto and Harada, 1981a; 1981b). Several authors have reported the correlation between *in vitro* flowering and sugar concentration in the culture media and also the interaction between nitrogen and sugars which can determine the organogenetic pathway *in vitro*. Therefore, in the present study, our objective was to investigate the changes in cellular activities that may take place during *in vitro* flowering. Tissue culture system was used, since it is easier to manipulate cells or plants cultured under controlled environment.

MATERIALS AND METHODS

In these experiments, explants used were leaves (from 1 month-old- aseptic seedlings) and pedicels from intact plants of the same age of *Petunia hybrida* Vilm. The medium used was MS (Murashige and Skoog, 1962) supplemented with 1.0 mg/l IAA (Indole acetic acid) and 1.0 mg/l zeatin for regeneration and *in vitro* flowering of the leaf explants, whilst, for pedicel explants, the medium used was MS supplemented with 1.0 mg/l kinetin. All cultures were maintained at 25°C with 16h light and 8 h dark. After 2 months, multiple shoots were formed and each shoot was transferred individually to the same media in a bigger container for rooting and flowering. One month later, root meristem cells from flowering plantlets were then squashed and made into permanent slides which were stained with Feulgen (Conger and Fairchild, 1953). At least 5 slides were used for determination of MI and a total of 5000 cells were examined. For cell and nuclear areas measurement, slides were counter-stained with light or fast green and 150 prophase cells were measured. The chromosome counts were determined from 15 well spread metaphase cells and cell doubling times (Cdt) were calculated following the methods of Clowes (1961), modification of Evans et. al. (1957). Image analyser system (VIDAS 21) was used for determination of nuclear DNA content and mean cell and nuclear areas (Abu Shah and Taha, 1994).

RESULTS AND DISCUSSION

Generally, this species was very responsive in tissue culture system. Multiple shoots were easily obtained from leaf explants. Even pedicels taken from intact flowers could form complete plants. *In vitro* flowering was achieved on MS supplemented with 1.0 mg/l IAA together with 1.0 mg/l zeatin for leaf explants and on MS supplemented with 1.0 mg/l kinetin for pedicel explants. After 3 months, the plantlets flowered and cytological studies were done on root meristem cells of the plantlets. The value of cytological parameters obtained were then compared with the *in vivo* values determined from 5-day-old seedlings as obtained from previous study (Abdullah, 1998). From our experiments, we found that the Mitotic Index (MI) increased significantly from 11.63 ± 0.26 % (*in vivo*) to 22.06 ± 2.87 % ($P<0.05$) in the plantlets derived from leaf explants. In plantlets derived from pedicels, the MI value also increased significantly to 24.36 ± 2.98 % ($P<0.05$). The mean and range of chromosome number in plantlets derived from leaf explants was 15, and 13-21 respectively, whereas in plantlets derived from pedicels, the mean and range of chromosome number was 19 and 13-28 respectively. The normal diploid chromosome number in this species is 2n=14. The increase in chromosome number could be associated with culture conditions and period of culture Usually, the longer the culture period, the more abnormalities (polyploidy and aneuploidy) in chromosome count become apparent (Torrey, 1967).

The duration of the cell doubling times was 32 h in intact plant cells but the values decreased to 21 h and 20 h in both leaf - and pedicel- derived flowering plantlets, respectively. The shorter duration of the cell doubling times in flowering plantlets indicate that less time is required for the cells to double its populations. The cell doubling time may be used as a rough estimate for the cell cycle duration. Unorganised growth such as in callus cells, normally has long cell cycles (Bayliss, 1985) with G1 phase being the longest. These increases may be related to the increased in cell volumes (Gould, 1984).

No polyploid cells were observed in all cases for this species and this may be related with the nature of this species which was easily manipulated and regenerated *in vitro*. For

instance, *Vicia faba* which was quite difficult to regenerate was shown to have very high occurrence of polyploidy compared to *Petunia hybrida* (Taha and Francis, 1990). It was observed that most of the cells were in S and G2 phase of the cell cycle during flower initiation, whereas in non-flowering plantlets, most of the cells were arrested in G1 phase of the cell cycle. This is in agreement with Bayliss's finding (1985), that unorganised cells stayed longer in G1.

Both mean cell and nuclear areas increased significantly ($P<0.05$) in flowering plantlets. The mean cell area in intact plant was 113.72 ± 3.82 μm^2, whereas the mean cell in flowering plantlets derived from leaf explant and pedicel-derived plantlets was $181.99 \pm 5.57 \mu m^2$ and $219.10 \pm 11.61 \mu m^2$ respectively. The nuclear to cell area ratios *in vitro* were unchanged (0.37 and 0.39) and this may be associated with the cells stability in culture, which could easily give rise to plant regeneration and subsequent flowering.

CONCLUSION

The events that occurred at cellular level during flower initiation of *Petunia hybrida* were increased in MI and the chromosome counts. The cell doubling times were faster *in vitro* indicating that the cells were more active in culture, no polyploid cells were detected, most cells in S and G2 phase of the cell cycle, bigger mean cell and nuclear areas and finally the constant ratios of nuclear to cell areas indicating stability and well adapted to culture conditions and this is revealed at morphological level by the fact that this species is amenable to culture conditions.

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