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Genesis of Microspore-derived Triploid Petunias

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Summary. A total of 61 microspore-derived plants of *Petunia parodii* were grown to maturity revealing a predominent population of triploids, 80.3%. Cytological investigations, together with the evidence from microfluorimetry, suggest that the origin of these triploids was due to the fusion of interphase nuclei in two different pathways. In the majority of embryogenic microspores, a vegetative nucleus of 1C DNA content fused with an endo-reduplicated 2C DNA generative nucleus at the binucleate stage and produced true triploid embryoids and plantlets (A pathway). Where this fusion failed, both the vegetative and the generative nuclei divided separately and in the multinucleate microspore two or more daughter nuclei fused to form a mixoploid embryoid. Such mixoploid embryoids produced a mixed population of plants with various ploidy levels as well as ploidy polymorphism within an individual. Since the triploids are morphologically superior with a faster growth rate than their diploids and related tetraploids, a predominent population of triploid plants was obtained from such mixoploid embryoids (B pathway). By low temperature treatment of the anther-donor buds, the embryogenic response of microspores was enhanced up to 5-fold.

Key words: *Petunia parodii* – Microspore-embryogenesis – Nuclear fusion – Triploidy – Horticulture

Introduction

The potentiality of microspores for organogenic differentiation in vitro has been well established. Apart from induction of haploidy in different species, androgenic diploids and polyploids have also been reported in several angiosperms (reviewed by McComb 1978). Cultured anthers of most of these species produced a mixed population of haploid, diploid and polyploid plants, whereas the almost exclusive production of triploid plants has been shown in a few cases (Raquin and Pilet 1972; Engvild 1973; George and Rao 1979).

Where seed is not the usable product and plants can be propagated vegetatively, especially in ornamental plants, the induction of triploidy is an important criterion for the improvement of plants because of their morphological gigas character which is normally superior to their diploids as well as to related tetraploids. The triploids are also useful for obtaining trisomic lines for genetic mapping. By conventional methods of plant breeding, triploid induction is laborious and normally only possible via chromosome doubling followed by tetraploid and diploid crossing (e.g. Straub 1973). In many cases such crosses may not be successful due to high sterility of autotetraploids and, therefore, some alternative methods are required for the induction of triploidy. In the present investigation, triploid plants of *Petunia parodii* have been raised from microspores in order to enhance the ornamental value of the species. At the same time it proved possible to determine the origins of these triploids by studying the DNA contents and behaviour of generative and vegetative nuclei during early ontogeny of the microspore embryoids.

Materials and Methods

Production of Microspore-derived Plants

Immature flower buds of greenhouse-grown plants ($2n = 2x = 14$) of *P. parodii* were harvested at weekly intervals during the first four weeks of flowering. The excised buds were subjected to cold treatments at 5 °C or 7–9 °C for 3, 6 and 12 days, and subsequently their anthers, possessing pre-mitotic, mitotic and post-mitotic stages of pollen development, were floated separately on shallow layer of liquid medium which comprises the basic constituents used by Nitsch and Nitsch (1969) supplemented with 100 mg l^{-1} L-serine, 800 mg l^{-1} L-glutamine, 2,500 mg l^{-1} myo-inositol and 1.0 mg l^{-1} Kinetin. The cultures were initially stored in the dark at 28 °C for 10

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days and later transferred to Grolux light (500 lx) for 12 h per day at 25 °C. Embryoids developed from microspores of these anthers were transferred to hormone-free nutrient agar of Murashige and Skoog (1962) for 4–6 weeks and finally brought into the greenhouse for growing to maturity.

Estimation of Nuclear DNA Content in in situ Microspores

The DNA content in vegetative and generative nuclei of the microspores was estimated by 4′-6-diamidino-2-phenylindole (DAPI) fluorimetry using a Leitz Wetzlar recording microscope fitted with an incident UV light source. The binucleate microspores taken from in situ anthers were squashed in the fluorochrome DAPI, which was made up as a stock solution of 1 mg ml^{-1} in Tris buffer (pH 7.5), 100 mM NaCl and 10 mM EDTA. Preparations were squashed until the exine was fractured, allowing nuclei to be isolated from the thick starchy exine which suppressed the staining of nuclei. The fluorescence intensities of DAPI and concentration of DNA in interphase nuclei were determined with excitation at 355 nm and emission at 450 nm. Under identical conditions *Allium cepa* (Suttons A_1) was incorporated as a standard in each replicate. For each replicate at least 50 microspore nuclei of a plant were observed and different plants were used for different replicates.

Cytology of Cultured Microspores and their Derived Plants

The anthers were removed from culture vessels at 2-day intervals up to 20 days, and their microspores were taken out and fixed overnight at room temperature in an acetic acid: chloroform: ethanol (1 : 1 : 3 v/v) mixture. The fixed microspores were hydrolysed for 15 min in 1 N HCl at 60 °C, stained in leuco-basic fuchsin at least 2 h and squashed in either 45% acetic acid or 1% acetocarmine.

The chromosome numbers of microspore-derived plants were determined from their juvenile leaves and/or microspore mother cells. A pretreatment of 2 mM 8-hydroxyquinoline for 3 h at room temperature was applied on the excised leaves. Carnoy's fluid fixation and usual standard acetocarmine staining techniques were used for both mitotic and meiotic preparations.

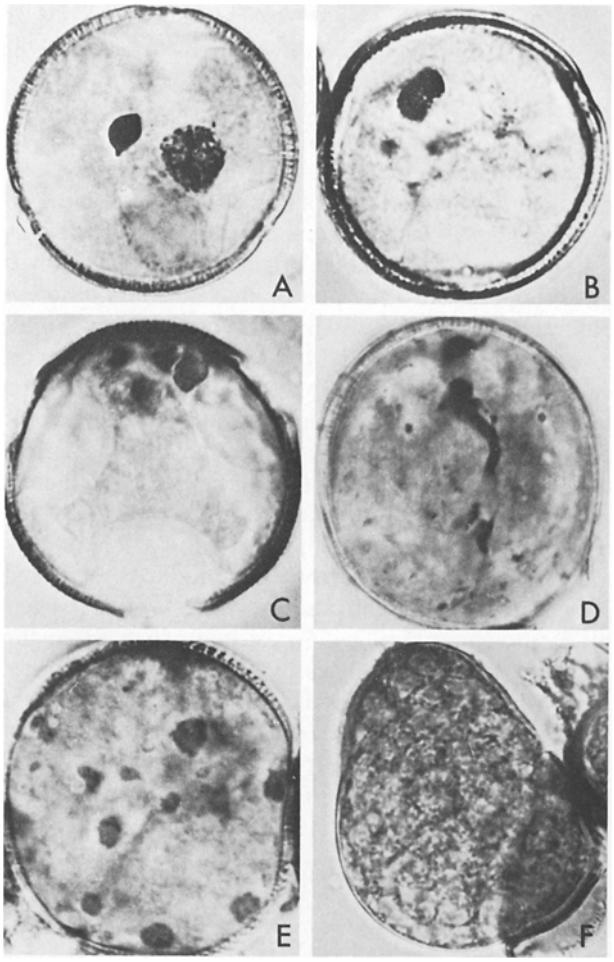
Results

Organization of DNA Synthesis in Microspore Nuclei

Although the first mitotic division of the microspores was equal, the resulting interphase nuclei were found to differ. When these nuclei were stained with leuco-basic fuchsin, the vegetative nucleus appeared as a diffused chromatin body whereas the generative nucleus was relatively small with condensed chromatin. The amounts of DNA in these phenotypically different vegetative and generative nuclei, estimated by 4′-6-Diamidino-2-Phenylindole fluorimetery, are presented in Table 1. The nuclear DNA values showed that the generative nucleus had almost double amount of DNA (17.84 picograms) in comparison to the vegetative nucleus (9.21 picograms). No further substantial synthesis of DNA was found in the generative nucleus during the

Table 1. Nuclear DNA content in binucleate microspores of *Petunia parodii*

Microspore nuclei	Replicates			$\bar{x}$ DNA (pg.)
	R1	R2	R3	
Vegetative	9.03	9.19	9.41	9.21
Generative	17.61	18.37	17.55	17.84



Figs. 1A–F. Stages of embryogenic microspores in *Petunia parodii*. **A,** Immature binucleate microspore possessing a 1C DNA vegetative nucleus (diffuse chromatin body) and endo-reduplicated 2C DNA generative nucleus (condensed chromatin body); **B,** Fusion of vegetative (IC DNA) and generative (2C DNA) nuclei on second day of culture; **C,** Division of unfused vegetative and generative nuclei resulting in a 4-nucleate microspore after 6 days of culture; **D,** Fusion of three nuclei of 1C DNA at 6-nucleate stage of microspore after eight days of culture; **E,** Multinucleate microspore showing a mixed population of x, 2 x and 3 x nuclei after ten days of culture; **F,** Multicellular structure (embryoid) developed from a multinucleate microspore after thirteen days of culture. **A–E** X1500 and **F** X650

second microspore mitosis. The completion of essential synthesis of DNA prior to the second pollen mitosis confirms the endo-reduplicated 2C DNA status of the generative nucleus.

Pathways of Triploid Embryogenesis

Embryogenesis from microspores of the cultured anthers was found only at the binucleate stage of development. The first embryogenic division started as early as 72 to 96 hours after culturing. Interestingly, nuclear division in the microspores was not followed by the cell wall formation during the first few divisions. Therefore, the resulting nuclei were in a free state within the pollen sac. When the microspores reached the 16–32 nucleate stage after 8–12 days of culture, cell walls formed rapidly and eventually a multicellular structure, or embryoid, formed from the multinucleate microspore.

During early ontogeny of the microspore-embryoids, two remarkable embryogenic events have been observed. The first embryogenic event appeared in the majority of productive microspores where 1C DNA (x) vegetative nucleus fused with endo-reduplicated 2C DNA (2 x) generative nucleus (Fig. 1 B). The fusion of these nuclei in the binucleate microspores took place soon after the cultures were started and continued for 72 hours. Embryoids developed from such microspores were unfailingly triploid (A pathway).

The second embryogenic event appeared in nearly one-third of the total productive microspores. Both the vegetative and the generative nuclei divided separately once or more (Fig. 1 C), and subsequently some of these nuclei fused together and formed diploid and/or polyploid nuclei (Fig. 1 D). Such fused nuclei and the remaining haploid nuclei of the microspores eventually produced a mixoploid embryoid (Figs. 1 E and F). Plants developed from such embryoids would have various ploidy levels as well as ploidy polymorphism within an individual. However, since triploids are vigorous and have a faster growth rate than their diploids and related tetraploids, a population of predominantly triploid plants could be obtained from these mixoploid embryoids (B pathway).

Sixty-one plants, regenerated directly from the microspore-embryoids, were grown to maturity. Chromosome counts of these functional plants revealed 80.3% (49 plants) triploids, 11.5% (7 plants) tetraploids and 8.2% (5 plants) diploids. Meiotic pairing in these microspore-derived plants showed a true homologous nature of the chromosomes.

Stimulation of Microspore-embryogenesis

Low temperature treatment of anther-donor buds significantly influenced the embryogenic response of the microspores (Table 2). Without any pretreatment the anthers did show embryoid and plantlet formations, but both the frequency of embryogenic anthers and the number of plantlets derived from an embryogenic anther were very low: only 2.35% of the total cultured anthers showed embryogenesis, and an individual embryogenic anther produced one or rarely two plantlets. When pre-excised anthers were subjected to cold treatment at 5 °C for 3 and 6 days or at 7–9 °C for 6 and 12 days at the stage just before or during first pollen mitosis, the embryogenesis was stimulated significantly. The most effective pretreatment, in this study, was found to be 7–9 °C for 6 days which produced an average 2.60 plantlets (range: 1–6 plantlets) from an embryogenic anther. The frequency of embryogenic anthers also increased remarkably and reached 5.75%.

Discussion

It is obvious from the microfluorimetric studies that binucleate microspores of *Petunia parodii* possess dif-

Table 2. The response of excised bud-pretreatment on microspore-embryogenesis of *Petunia parodii*

Pretreatment		No. of anthers cultured	Frequency of productive anthers (%)	No. of plantlets per productive anther		Ploidy variation in surviving plants		
Temperature (°C)	Duration (days)			Range	Mean	2×	3×	4×
Control (28 – 28)	Freshly excised	170	2.35	1 – 2	1.25	1	2	–
5	3	290	4.48	1 – 4	2	1	7	–
5	6	324	6.79	1 – 4	2.15	3	13	2
5	12	345	2.86	1 – 3	1.33	–	2	–
7 – 9	3	260	3.08	1 – 4	1.65	1	5	–
7 – 9	6	336	5.75	1 – 6	2.60	1	14	2
7 – 9	12	350	1.71	1 – 2	1.87	–	6	1

ferent DNA contents in their vegetative and generative nuclei. The generative nucleus contained almost twice as much DNA as the vegetative nucleus because of the completion of essential DNA replication prior to the second microspore mitosis of gamete formation. This present finding does not support the earlier proposals of Hesemann (1971) that *P. hybrida* microspores have 1C DNA in both their vegetative and generative nuclei at the time of anthesis, and that extensive DNA synthesis takes place during germination. In contrary to his findings, Jackson and Linskens (1978) have also shown by inhibitor studies, along with the labelling experiments, in *P. hybrida* that no substantial DNA synthesis occurs in the generative nucleus during the second microspore mitosis.

Evidence presented here suggests that the production of triploid plants from microspores of *P. parodii* is due to a nuclear fusion which started soon after the culture at the binucleate stage and continued for a week up to the multinucleate stage. It may be assumed that the fusion of nuclei in the cultured microspores was not spontaneous, but was influenced by one or more chemical constituents of the medium since no fusion of vegetative and generative nuclei was found in in situ binucleate microspores of *P. parodii.*

For enhancing the embryogenic response of microspores in cultured anthers of *P. parodii,* cold treatment does seem a very important factor as originally discovered in *Datura innoxia* by Nitsch and Norreel (1973). In the present findings, both the frequency of embryogenic anthers and the number of plantlets derived from an embryogenic anther increased remarkably (nearly 5-fold) by treating anthers just before or during first pollen mitosis at 7–9 °C for 6 days. The frequency of triploids also increased by increasing the microspore-embryogenesis, but their proportion in the heterogeneous population of microspore-derived plants does not change significantly by cold treatment.

Under optimal conditions, 6.79% of cultured anthers produce plantlets. The number of plantlets per productive anther ranges from 1 to 6 (mean: 2.60). These data of anther productivity, found in this study, are much higher than those in earlier reports by Engvild (1973) in *P. axillaris,* Wagner and Hess (1974) in *P. hybrida* and Raquin and Pilet (1972) in *P. axillaris* × *P. hybrida,* where productive anthers were only about 1% and the number of plantlets per productive anther was one or (rarely) two.

Triploid plants, raised from microspores of *P. parodii,* are more vigorous and much more ornamental than their parental anther-donor diploids (Fig. 2). The propagation of these triploid plants is possible by conventional vegetative methods since sexual sterility leads to complete seedlessness. When the triploid flowers are fertilized artificially by microspores of the parental diploids, a high seed set is achieved. From these seeds, the production of haploid and various aneuploid lines of *P. parodii* is in progress. Attempts are also being made to enhance the ornamental value of other flowering plants by producting homozygous triploids from microspores through artificial fusion of vegetative and generative nuclei in cultures.

Fig. 2. Anther-donor diploid (left) and microspore-derived triploid (right) plants of *P. parodii*

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