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Haploid induction from anther culture of stone fruits (Prunus spp.)*

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Abstract: Haploid induction from anther culture in sour cherry cvs Šumadinka and Čačanski Rubin, plum cv. Požegača and peach accessions BBGVJ with cold pretreatment at 4 - 5°C lasting 0, 5, 14 and 25 days, followed by culturing on Nitsch-Nitsch, Gamborgs'-B5 and two-stage MS media with a great number of differing combinations of IBA, BAP, GA3, glycine and cysteins was not achieved, although there was observed callus formation from the depth of anthers. The monitoring of the process of microgenesis aimed at the application of cold pretreatment and anther culture in the genotypes observed indicated the existence of certain specific traits which might reflect possible obstacles in the induction of embryogenic pollen haploids in these fruit species.

Key words: Haploid, anther, *Prunus*

Introduction

The development of homozygous lines through self-fertilization is practically impossible in most *Prunus* spp. due to self-incompatibility. Therefore, the induction of haploids remains for the present the only procedure with prospects of paving the way for obtaining homozygous lines.

* Experimental stage of the work was carried out in the Institute for Pomology and Viniculture in Sarajevo.

The work on anther culture in *Prunus* is highly aggravated by the fact that anthers can be collected only once a year over a very short period of 7-10 days, which resulted in few reports related to this problem. A successful regeneration of haploids from anther culture 'in vitro' has not been achieved so far in any *Prunus* species: *P.avium* - induced rhizogenic calli (Jordan, 1974); *P. amygdalus* - induced calli (Michellon et al., 1974); *P. armeniaca* - induced callus (Harn and Kim, 1972, cited after Bajaj, 1983); *P. cerasifera* - induced calli and adventitious buds on calli (Serilis et al., 1979); *P. cerasus* and *P. persica* - induced calli (Michellon et al., 1974; Seirlis et al., 1979; Ognjanov, 1989; Đulbić et al., 1990). The results of research conducted to date have not yielded evidence which could reliably set the trends of further work. The only general rule accepted so far was that haploid induction from anther culture was conditioned by a stress pretreatment (Sunderland, 1974; Sunderland and Dunwell, 1977; Huang and Sunderland, 1981).

Material and Methods

The anthers of five genotypes, viz. *P. cerasifera* Ehrh. and *P. persica* - accessions BBGVJ (Gene Bank of Fruit Crops of Yugoslavia), *P. domestica* cv. Požegača and *P. cerasus* cv. Šumadinka and cv. Čačanski Rubin were used for the induction of androgenesis over 1990-1991. Sampling of the fruit species and cultivars chosen for the „in vitro' culture was preceded by a thorough monitoring of the beginning and course of meiosis. Bud samples were collected at 2-day intervals from the moment of differentiation of archesporium till the release of microspores from tetrads and subjected to analysis using the following techniques:

1. Squash technique according to the method of Lespinasse and Salessis (1973);

2. Paraffin technique: fixation after Novashin, embedding and casting in paraffin, sectioning at 7-10 m, staining with Delafield hematoxilin, embedding in Canada balsam.

Twigs with flower buds of the species and cultivars chosen for „in vitro" anther culture were collected from the marked tres at the time when the tetrad stage was prevalent in the sporogenic tissue of anthers. Further procedure with the twigs, ie. the buds can be divided into three basic steps:

1. Cold pretreatment of plant material. Twigs taken from all genotypes were subjected to cold pretreatment in a growth chamber at 4-5^{0}C for 0, 7, 14 and 25 days.

2. Surface sterilization of buds with a part of wood and removed bark and scales was done in 75% of ethanol for 60 sec., in 5% of NaCl for 20min. with three rinses in sterile distilled water.

3. „In vitro" anther culture. The anthers were aseptically excised from flower primordia and placed on culture medium with stomium up. The following culture media were used for the induction of androgenesis:

a) Gamborg's-BP (1968) and Nitsch-Nitsch (1969) with three different combinations and growth regulator concentrations: 1) IBA + BAP + GA3 (0.7 +

0.1 + 0.1mg/l; 2) NAA + BAP (2.0 + 0.5mg/l); 3) 2,4-D + BAP (1.0 + 0.5mg/l). All combinations of culture media were made with 20g/l of sucrose, 0.6% of agar (Sigma) and pH value was adjusted to 5.8.

b) Two-stage MS medium with three differing combinations of phytohormones and amino acids: 1) 0.5mg/l BAP (glycine); 2) 0.5mg/l BAP + 0.5mg/l IBA (glycine + cysteine); 3) no phytohormones (glycine + cysteine).

For soil stage 0.65% of agar (Difco-Bacto), 30g/l of sucrose and activated charcoal (0.5%) were used.

Anthers were cultured in glass petri dishes (80mm in diameter) and incubated in the dark at 25-30°C until the appearance of calli which were then subcultured under a 16/8 (light/dark) photoperiod at 23/18°C. About 20,000 anthers were cultured annually.

The analysis of chromosome structure of the induced calli was done according to the method of Lespinasse and Salesses (1967, 1973), and histological analysis of calli by fixation after Novashin, embedding in paraffin, sectioning at 7-10 m, staining with Delafild hematoxilin and embedding in Canada balsam.

Results snd Discussion

None of the combinations of cold pretreatment and hormonal components applied in all genotypes studied in 1990 and 1991 resulted in the induction of haploid calli or haploid plantlets, although in some anthers dehiscence was observed as well as the formation of callus which seemed to have emerged from the depth of inner anther tissues (Fig. 1).

With some genotypes and combinations of hormonal components and to some extent of cold pretreatment, callusing response was up to 98%, whereas there was no callusing at all only in the combinations without cold pretreatment. Two distinct types of callus were induced: One was white - friable (soft) calli (Fig.1) and the other green - granular (compact) calli (Fig. 2). All calli were successivelly, according to the time of placing anthers on media, transferred to and subcultured on different regeneration media. However, regeneration was not achieved even after a year. Cytogenetic analysis showed that diploid and (only in some cases) polyploid calli were in question, but no haploid calli were observed.

Conclusion

The study of the process of microsporogenesis in the genotypes studied in order to determine the proper application of cold pretreatment (Fig. 3) indicated the following problems:

- The role of the tapetum in the isolation of sporogenic tissues and the interaction between its secretion and medium (Fig.4);
- The process of microsporogenesis in different anther locules is not syncchronized (Fig. 5);
- Occurrence of two nuclei in daughter cells at the end of the process of cytokinesis and immediately after the release of microspores from tetrads (Fig. 6);

- Formation of pollen grains with different number of nucleoli and nuclei (Fig. 7). This raises the question of defining a novel approach, or developing novel methods of obtaining embryogenic pollen and thereby the induction of haploids in the androgenesis of stone fruits.

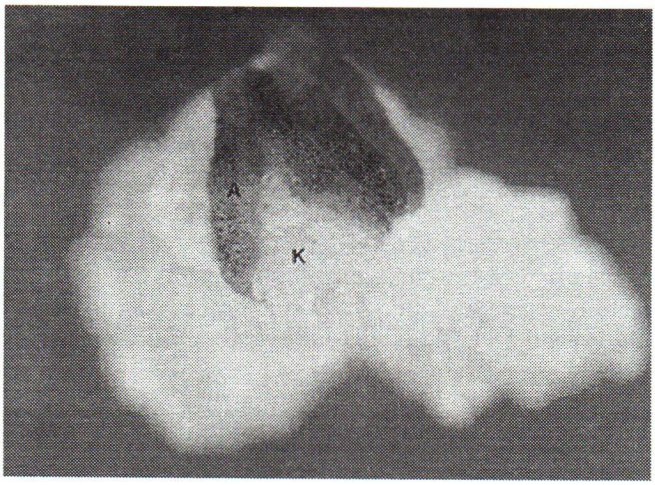

Fig. 1 White -friable (soft) calli developed from the depth of anthers in *Prunus cerasus* L.

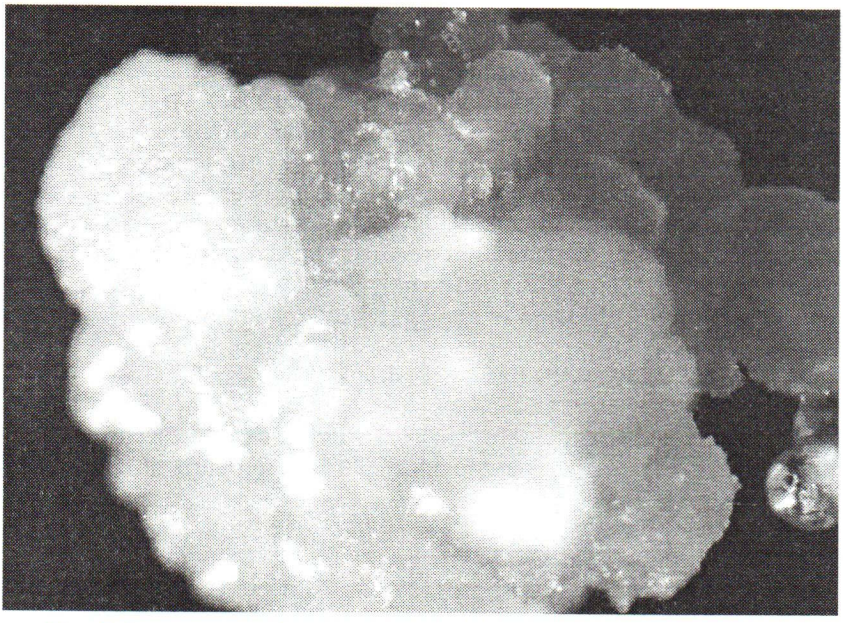

Fig. 2 Green -granular (compact) calli developed from the depth of anthers in *Prunus persica* L. accessions BBGVJ

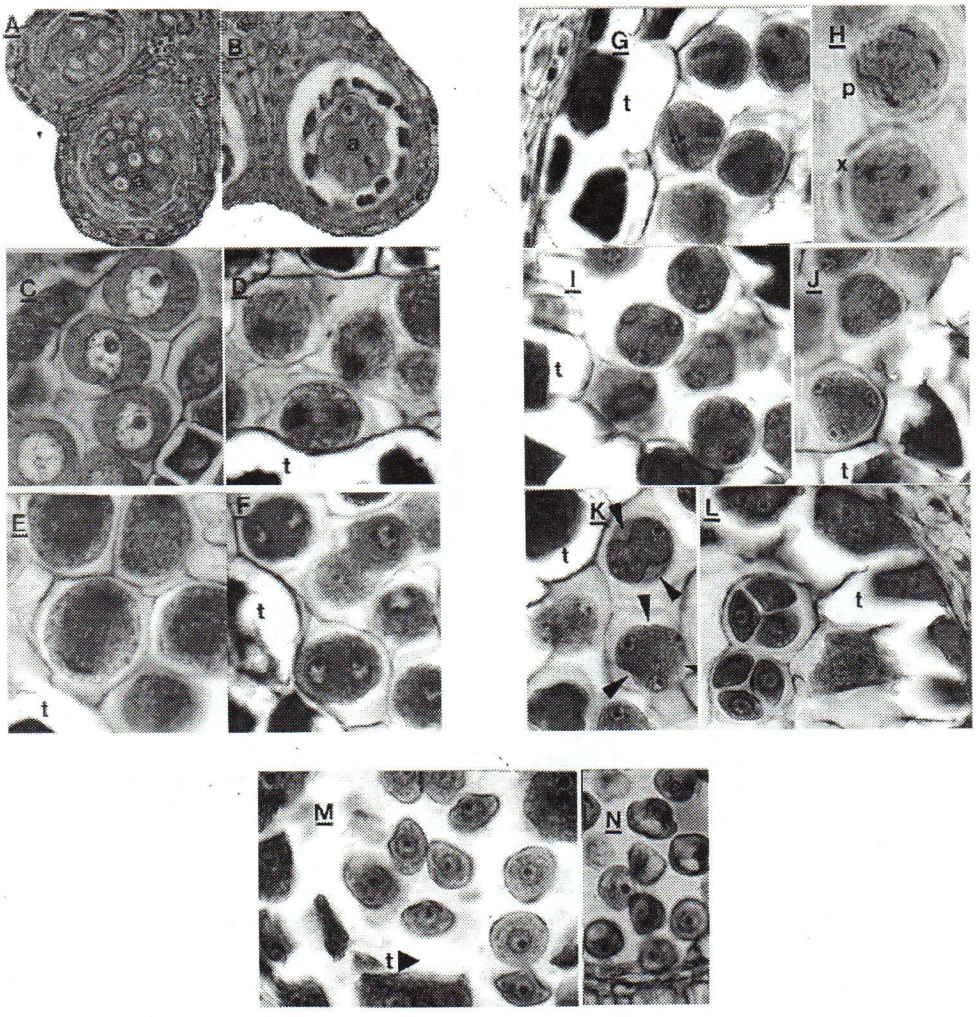

Fig. 3 The process of microsporogenesis in *Prunus domestica* L. cv. Požegača

The simultaneous process of meiotic division in which the first and second karyokinesis are completed first, and then cytokines by furrowing occurs, is also a characteristic of the other *Prunus* spp. observed.

A - formation of archespores (a) in the anther locule; B, C - differentiation of the tapetum (t) and isolation of pollen mother cells (mc) inside the locule; D - anaphase I; E - telophase I; F - interphase after the first karyokinesis (two nuclei - designated with arrow - are present in pollen mother cells); G - metaphase II; H - late anaphase II and telophase II (in pollen mother cells there are two division spindles of parallel (p) and cross (x) orientation; I - completion of second karyokinesis (there are 4 nuclei in pollen mother cells); J,K - after the completion of karyokinesis there occurs the process of cytokinesis by furrowing - designated

with arrow); L - four daughter cells - tetrads - have been formed by furrowing, which completes the meiotic division; M - the dehiscence of the callose membrane of tetrade and release of young microspores is preceded by the dehiscence of the callose membrane of the tapetum cells (t) and the release of the contents from their vacuoles into the anther locule; N-U: the tapetum is completely resorbed in the process of the differentiation of microspores.

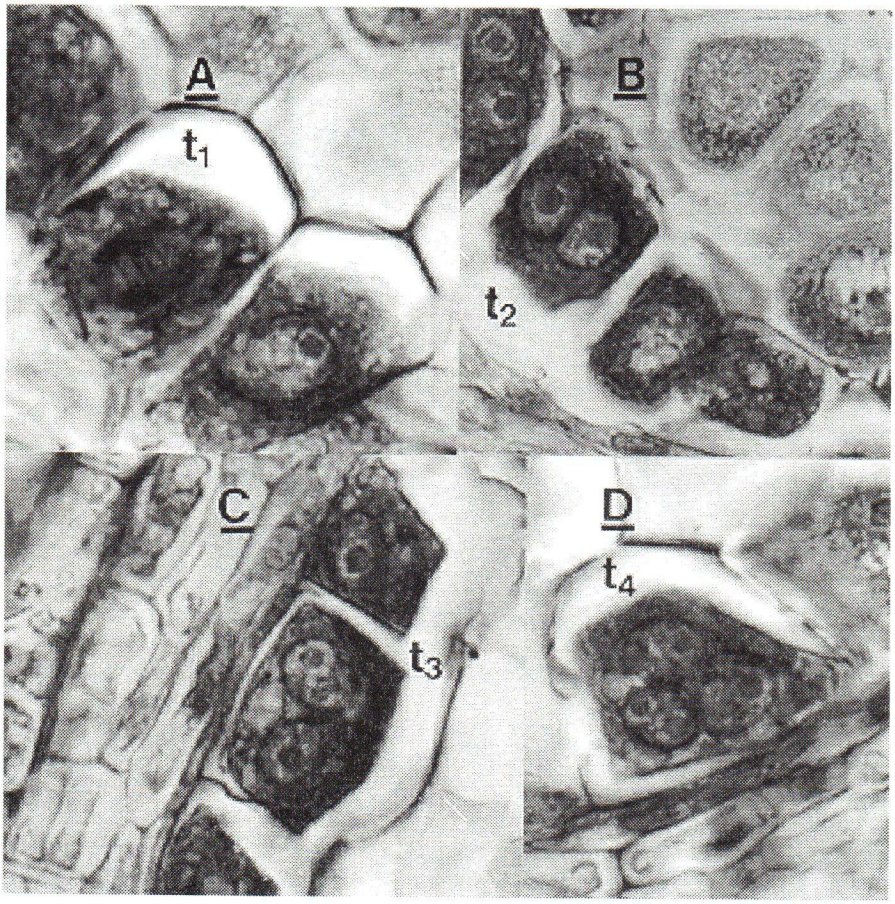

Fig. 4 The process of differentiation of tapetum cells: A - first endomitotic division (t_1); B - binucleate cells (t^2); C - threenucleate cells (t^3); D - fournucleate cells (t^4) . The increase of nuclei number is followed by the formation of large vacuoles towards the inner part of the anther locule.

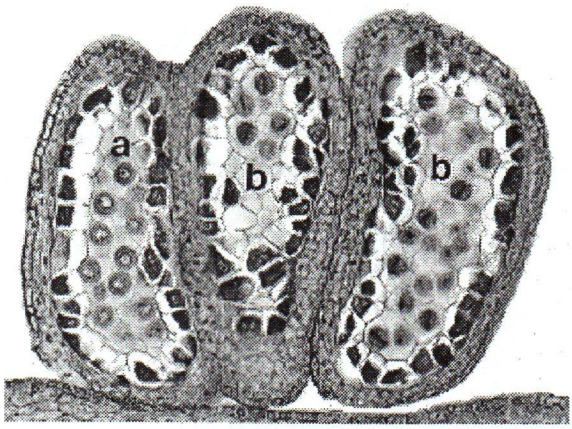

Fig. 5 Prophase I (a) and anaphase I (b) in different locules of the same anther.

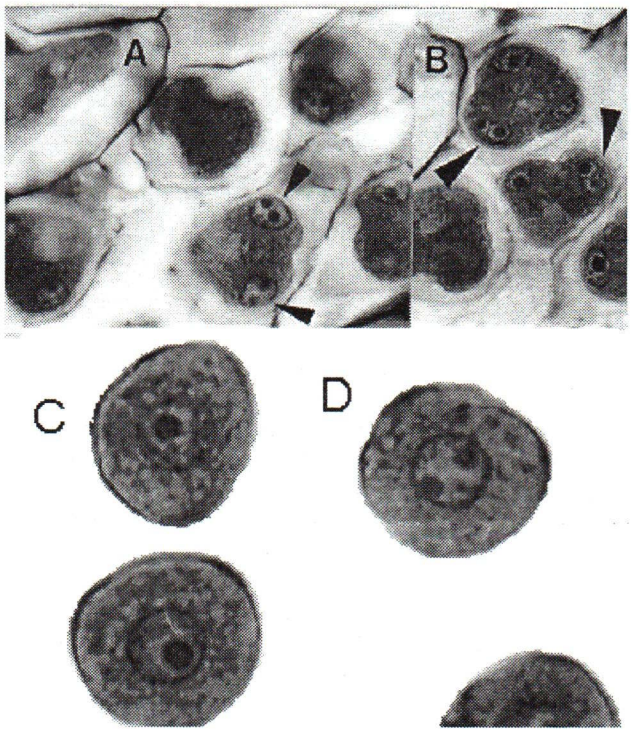

Fig. 6 After completion of karyokinensis and before completion of cytokinesis - tetrad formation - two or three nucleoles can be seen in daughter cells (A - designated with arrow) or separate pairs of nuclei (B - designated with arrow). The same phenomenon occurs in the young microspores immediately after their release from tetrads - C and D.

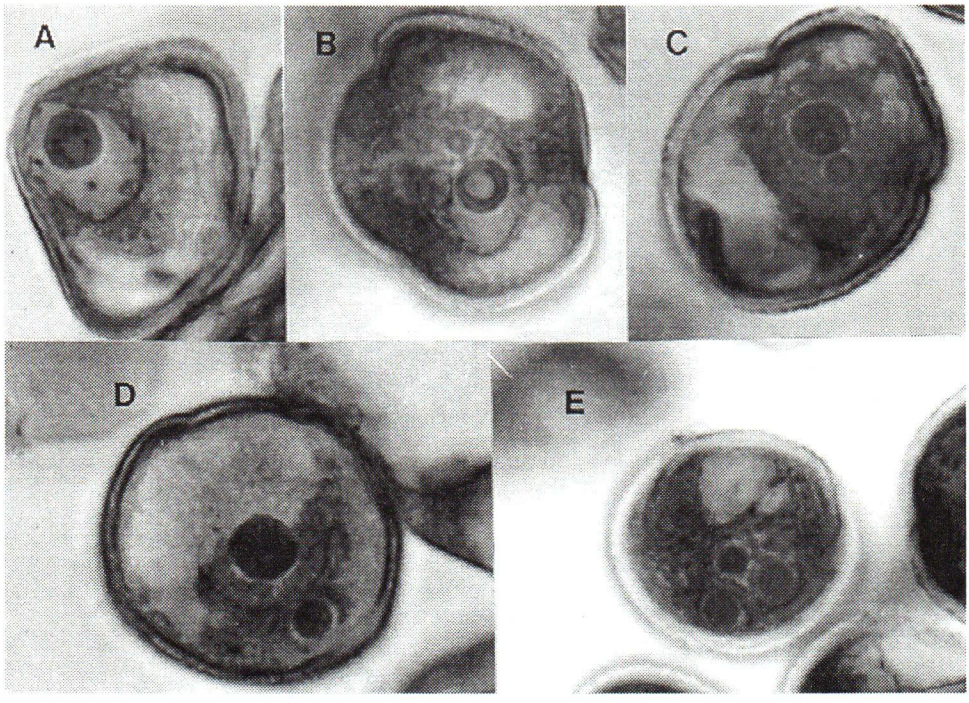

Fig. 7 Immediately after the formation, differing number of nuclei and nucleoli can be observed in pollen grains. Pollen grains with one (A), two (C) or three nucleoli (B) and with two (D) and three (E) nuclei.

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INDUKCIJA HAPLOIDA U KULTURI ANTERA KOŠTIČAVIH VOĆAKA *(Prunus sp.)*

- originalan naučni rad -

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Rezime

Rad na indukciji haploida u kulturi antera nekih genotipova iz roda *Prunus: P. cerasifera* Ehrh i *P. persica* (odabrani i izdvojeni accessions uvršteni u Banku biljnih gena voćaka Jugoslavije) ***P. domestica*** cv. Požegača i *P. cerasus* cv. Šumadinka i cv. Čačanski rubin pri različitim kombinacijama hladnog predtretmana i različitim medijima i odnosima hormonskih komponenti u njima, nije rezultirao indukcijom haploida.

Bez primljenog hladnog predtretmana u svim kombinacijama medija i hormona antere su uginule, dok je primena hladnog predtretmana imala za posledicu indukciju dva tipa kalusa: zeleni - granulirani (tvrdi kalusi) i beli - rastresiti (mekani kalusi).

Proučavanje procesa mikrosporogeneze u cilju analize uslova za primenu hladnog predtretmana pokazuje da do uspostavljanja dvojedarnog polena u posmatranih vrsta *Prunusa* dolazi relativno rano u odnosu na primenjeni predtretman što uslovljava drugačiji pristup u definisanju postupaka za dobijanje embriogenog polena kao osnove za indukciju haploida u lulturi „in vitro".