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BRIEF COMMUNICATION

Efficient haploid induction in microspore suspension culture of *Aesculus hippocastanum* and karyotype analysis

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Abstract

Suspension culture was more efficient method for haploid production than anther culture. All analysed androgenic regenerants originating from embryogenic microspores in suspension culture of *Aesculus hippocastanum* L. had a haploid number of chromosomes (n=20), while 50 % of those derived from anther culture were diploids.

Additional key words: androgenesis, anther culture, horse chestnut.

Induction of androgenesis is very important for trees, on account of their highly heterozygous character and long developmental period before reaching the reproductive stage. Conventional breeding and cross-pollinating procedures are therefore both unpredictable and time-consuming. Haploids are desirable primarly in the production of homozygous dihaploids for controlled hybridisation to obtain reproducible heterosis (Bonga *et al.* 1988).

Although anther culture is much simpler in handling, microspore culture has several important advantages (Bonga *et al.* 1997, Radojević *et al.* 2002). Firstly, the formation of calli and embryos that often form on somatic tissues of the anther is avoided. Secondly, microspores are directly accessed, which speeds up the optimization of culture conditions. Comparative studies in *Brassica napus* and *Hordeum vulgare* (Siebel and Pauls 1989, Hoekstra *et al.* 1992) confirmed that microspore culture can be five to ten times more efficient than anther culture in embryo production.

Androgenesis of *A. hippocastanum* has a long history dating back to late 1970s (Radojević 1978). Androgenic embryos (Radojević 1995, Radojević *et al.* 1998), such as the somatic embryos of *A. hippocastanum* (Jörgensen 1989, 1991), can be rapidly produced but to overcome asynchronous maturation and low germination it is necessary to provide optimal nutritive and enviromental conditions (Capuana and Deberg 1997, Radojević *et al.* 1998).

In this paper we describe the protocol for mass production of haploids in suspension culture of *A. hippocastanum*, which has shown some advantages over the protocol for obtaining haploids from anther culture as embryos are this way sure not to originate from the filament, tapetum or anther wall (Radojević 1978).
Flower buds (4 mm long) were obtained from the 100-year-old *A. hippocastanum* tree growing in the Botanical Garden "Jevremovac," Belgrade University. The selected buds, completely closed and with uninucleate microspores, were surface sterilized with 95 % ethanol (3 - 5 min) and 70 % ethanol (5 min), followed by three rinses in sterile distilled water.

Basal medium (BM) contained Murashige and Skoog (1962; MS) mineral salts, 2 % sucrose, and was supplemented with the following [mg dm^{-3}]: pantothenic acid 10.0, nicotinic acid 5.0, vitamin B_1 2.0, adenine sulphate 2.0, myo-inositol 100 and casein-hydrolysate 200. Androgenic embryos were obtained by culturing uninucleate microspores in MS liquid medium (MSL) while anther culture was established on MS solid

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medium (MSS) with 0.7 % agar. Both media contained BM with 2,4-dichlorphenoxyacetic acid (2,4-D) and kinetin (Kin), 1.0 mg dm^{-3}, each. We cultured one hundred anthers with uninucleate microspores per Erlenmeyer flask with filter (200 μm) and 100 cm^3 MSL medium for androgenesis induction. The microspore suspension was subcultured every 4 weeks and refreshed with MSL medium of the same composition as the induction medium. The suspension was plated by Bergmann technique (1960) on a solid medium with reduced concentration of 2,4-D (MSS_1 = BM + 0.01 mg dm^{-3} 2,4-D + 1.0 mg dm^{-3} Kin). Six or seven anthers were cultured in each culture tube containing 8 cm^3 of the MSS induction medium. Embryo development and further multiplication of androgenic embryos from microspore suspension and anther culture proceeded on MSS_1. Embryo germination was achieved on a solid hormone-free medium enriched with glutamine 400 mg dm^{-3} (MSS_2). All media were sterilized by autoclaving at 0.9×10^5 Pa and 114 °C for 20 min. Suspension cultures were grown on a horizontal shaker (85 rpm) at temperature of 25 ± 1 °C for 4 weeks in the dark. All other cultures were grown at the same temperature and a 16-h photoperiod with irradiance of 33 - 45 μmol $m^{-2}s^{-1}$ produced by cool white fluorescent tubes.

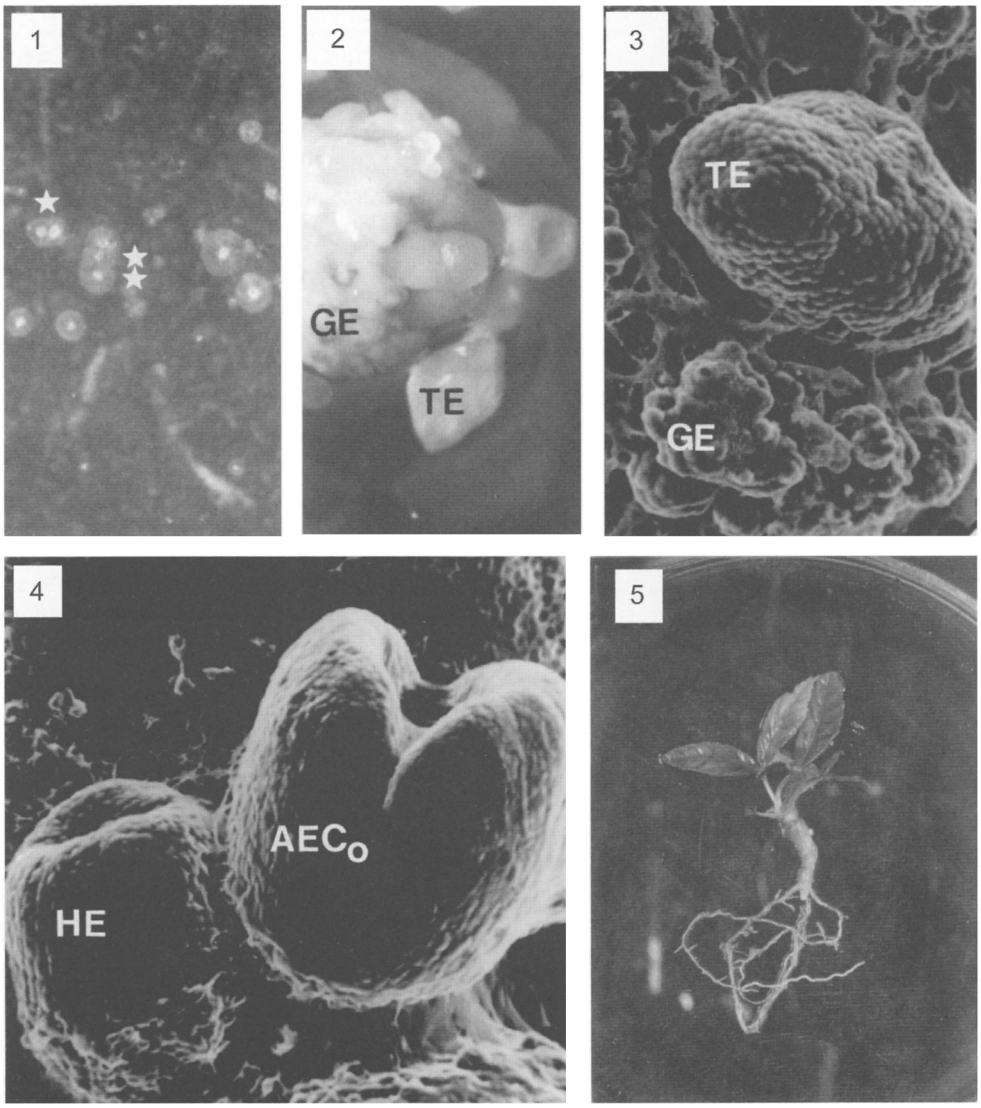

Fig. 1. Developing two (*asterisk*) and three (*two asterisks*) celled embryogenic pollen grains of *Aesculus hippocastanum* L. on the MSL medium with 1.0 mg dm^{-3} 2,4-D and 1.0 mg dm^{-3} Kin, after one week in culture (×62).

Fig. 2. Androgenic embryos of *A. hippocastanum* in the globular (GE) and torpedo-like (TE) developmental stage growing on the MSS_1 (0,01 mg dm^{-3} 2,4-D + 1.0 mg dm^{-3} Kin), after 8 weeks (×29).

Figs. 3 - 4. Scanning electron microscopy micrographs of embryogenic complex of *A. hippocastanum* with GE, HE, TE and AECo (androgenic embryo in the cotyledonary stage) on the MSS_1 medium after 8 weeks (×100).

Fig. 5. Androgenic plant of *A. hippocastanum* originating from suspension culture (×3).

For determination of chromosome numbers in regenerants, root tips squashes were prepared according to modified methods of Roberts and Short (1979). Prometaphase radicle cells of 60 regenerants orginating from microspore suspension and anther culture were analysed. Root tips were harvested and pre-treated with 0.002 M hydroxyquinoline overnight at room temperature, followed by fixation at 4 °C in Carnoy's fixative for 7 d. They were hydrolysed in 3.5 M HCl at 37 °C for 17 min. The samples were stained with 1 % aceto-orcein.

We used 4 mm-long flower buds as they contain most uninucleate microspores (Radojević 1991). The induction of androgenic embryos was achieved in MSL and MSS media in the presence of 2,4-D and Kin (1.0 mg dm^{-3}, each). 2,4-D and Kin had been found necessary for the formation of embryos and embryogenic calli of *Camellia japonica* (Pedroso and Pais 1994), *Populus nigra* and *P. deltoides* (Mofidabadi *et al.* 1995), *Malus domestica* and *M. zumi* (Höfer 1995). Differentiation and division of microspore were initiated over the first week in culture on the stated media and two- and three-celled pollen embryos were observed (Fig. 1). Androgenic embryos obtained from the microspore suspension culture have been formed by division of the vegetative nuclei. These results are in agreement with previous reports on the induction of androgenesis in anther culture of horse chestnut (Radojević 1978, 1980). After several weeks of growth on the MSS_1 medium, differentiation and development of androgenic embryos became more rapid. The development of androgenic embryos is asynchronous, so that embryos at their globular (Figs. 2, 3), heart-shaped (Fig. 2), torpedo-like (Fig. 3) and cotyledon (Fig. 4) stages were observed in the same culture. To enable further monitoring of differentiation and maturation, the embryos were grown on the MSS_1 medium and the medium for germination and convertion of embryos into plantlets (Fig. 5). The number of embryos and green regenerants in suspension culture (Table 1) were five times higher than in anther culture. The germinated plantlets were then used for the analyses of chromosome number in the root. We analysed 60 regenerants originating from uninucleate microspores and 60 from anther culture of horse chestnut. All regenerants acquired from suspension (Fig. 6) had a haploid number of chromosomes (n=20). Fifty percent of all analysed regenerants originating from anther culture were haploids, while the rest were diploids (2n=40; Fig. 7). The ratio between the long and short arms was used to determine the centromere position of each chromosome. The nomenclature system of Levan *et al.* (1964) was used to determine the chromosome types as: metacentric, submetacentric or acrocentric. An analysis of metaphasic chromosomes, involving conventional techniques, showed, by way of the centromeric position, that *A. hippocastanum* chromosomes belonged to one of three basic groups (A-C): 12 metacentric (A); 18 submetacentric (B) and 8 larger and 2 smaller acrocentric chromosomes (C), by decreasing size (Fig. 8).

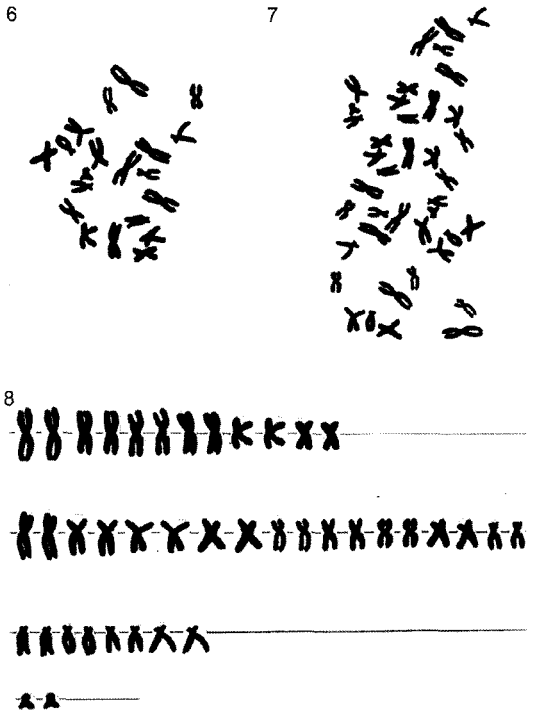

Fig. 6. Haploid (n=20) number of chromosome in androgenic regenerant of *A. hippocastanum* orginated from microspore suspension (×1500).
Fig. 7. Diploid (n=40) number of chromosome in androgenic regenerant derived from anther culture of horse chestnut (×1500).
Fig. 8. Karyogram of *A. hippocastanum*.

Table 1. Effect of different culture types on induction of androgenesis (total number of anthers and embryos, and number of green embryos per anther) of *A. hippocastanum*. Means ± SE.

Culture type	Number of anthers	embryos	Number of green regenerants
Anther culture	1680	5640	1.0 ± 0.05
Suspension culture	900	15300	5.0 ± 0.45

By applying a banding technique it will be possible to determine pairs of chromosomes. Techniques such as Restriction Fragment Length Polymorphism (RFLP) can bring considerable progress in the further definition of useful characters of *A. hippocastanum*.

Determination of chromosome numbers in woody plants is generally rather complicated. Although androgenic plantlets of horse chestnut originating from anther culture had been obtained quite a long time ago (Radojević 1978), we have only recently succeeded for the first time in getting a clear image of the dispersed

metaphasic chromosomes and do their karyogram. It means that microspore culture has an advantage over anther culture as we can be sure that all regenerants, being haploid, originate from a divided microspore.

Hitherto research has shown that spontaneous diploidization *in vitro* depends on the genotype, pre-treatment of donor plants prior to anther isolation, growth method of donor plants and environmental conditions existing over the growth period of anther culture or suspension of individual microspores (Germana *et al.* 1994). The anther culture of *A. hippocastanum* was based on indirect and direct embryogenesis. Indirect androgenesis puts an emphasis on the callus phase, so that it is possible to predict that all embryos will not be haploid. When the ploidy levels of anther and microspore cultures of horse chestnut are compared, it becomes evident that microspore culture with a haploid induction is a much better method than anther culture. This is the first reported successful karyotype analysis of horse chestnut regenerants. The described protocol for an efficient (100 %) haploid induction in microspore suspension culture of *A. hippocastanum* can be used in genetic manipulation relating to pathogene-resistance and secondary metabolism.

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