



EFFECT OF DIFFERENT GENOTYPE ON PRODUCTION OF HORSE CHESTNUT ANDROGENIC EMBRYOS

Dušica ČALIĆ-DRAGOSAVAC^{1*}, Snežana ZDRAVKOVIĆ-KORAĆ¹,
Jelena MILOJEVIĆ, Ljiljana RADOJEVIĆ¹

¹Institute for biological research "Siniša Stanković", Belgrade, Serbia.

SYNOPSIS

Key words:

Aesculus hippocastanum, androgenic embryos, genetic stability, genotype, ploidy level.

Effect of five horse chestnut genotypes on induction of androgenesis was studied. Efficiency of *in vitro* androgenesis *via* anther and microspore culture were investigated using anthers from the same closed flower bud material. Androgenic response of different genotypes was measured and compared. Anther induction rates were from 13.6 % to 37.6 % depending of genotype. Under optimal conditions, the number of embryos per isolated anther varied between 0.5 to 5.0 embryos in anther culture, while in microspore culture varied between 3.0 to 27 embryos, depending of genotype. A microspore culture was more efficient method for androgenic embryos production than anther culture.

However, flow cytometric analysis of embryos showed that a half of androgenic embryos retained haploidy, while a half of these were diploid, after 6 months in culture.

INTRODUCTION

Aesculus hippocastanum L. (*Hippocastanaceae*, common name horse chestnut) is a relict and endemic species of Balkan Peninsula. The development of anthers with uninuclear pollen grains leading to androgenesis and haploid plants originating from one genotype has been described for *Aesculus hippocastanum* (Čalić et al., 2003). Anther culture has been used in recent years as a tool for producing haploid plants in a variety of higher plants, but the low frequencies of microspore-derived plants restrict the use of the technique in plant breeding (Bueno et al., 2004). There are several factors affecting androgenesis in many species, such as genotypes, growth of donor plants, pretreatments of anthers, composition of

medium and culture conditions (Assani et al., 2003; Hofer, 2004). Androgenic response is genetically controlled and is affected by environmental factors. Genotype is the most crucial factor for androgenic response *in vitro* androgenesis in apple (Höfer, 2004; Höfer et al., 2008). Also the developmental stage of microspores within anthers is an important factor for success in anther cultures (Perera et al., 2008, 2009). The anthers containing microspores at the uninuclear stage and the first pollen mitosis are determined to be optimal for the induction androgenesis for many woody plant species (Marinković & Radojević, 1992; Assani et al., 2003; Germana et al., 2000; Germana & Chiancone, 2003; Hofer, 2004; Peixe et al., 2004; Pintos et al., 2007). Radojević (1978, 1991) determined that horse chestnut anthers taken from buds at the size between 3 and 7 mm containing microspores at the uninuclear stage and gave good results. Stress treatments play a major role in androgenesis. The influence of temperature and nutrition in different crop species is well documented (Touraev et al., 1996; Smykal, 2000; Shariatpanahi et al., 2006).

The production of haploids offers new possibilities for genetic studies and breeding (Hofer, 2005). Anther culture is a widely used method to generate genetic variability. Therefore, through this method, it is possible to regenerate gametic or somatic embryos with many applications for plant breeding (Assani et al., 2003). Production of haploid plants from anther cultures is specially useful for regeneration and breeding of forest trees, since the long regeneration time and strong inbreeding depression of these species makes the traditional breeding methods impractical. Woody species in general, and forest trees in particular, have shown to be extremely recalcitrant in anther cultures, and only few examples of successful regeneration of plantlets, from confirmed microspore origin have been reported (Chen, 1986; Höfer & Lesinasse, 1996; Höfer et al., 2002; Germana, 2006).

Analysis of ploidy level was used to verify the genetic stability of the horse chestnut androgenic embryos.

MATERIALS AND METHODS

PLANTS MATERIALS

Inflorescences were harvested from five horse chestnut (110-years-old) trees growing in the Botanical garden "Jevremovac" of Belgrade University. Anthers were excised from closed flower buds (size 4-5 mm). In fact, a high correlation has been shown between flower length and phase of pollen development. In buds of 4-5 mm microspores were at the uninucleate stage of development.

ANTHER AND MICROSPORE CULTURE

The selected closed buds with uninucleate microspores were surface sterilized with 95 % ethanol (3 min) and 70 % ethanol (5 min) and three rinses in sterilized

water. Basal medium (BM) contained MS mineral salts (Murashige & Skoog, 1962), 2% sucrose, 0.7 % agar, 100 mg l⁻¹ myo-inositol, 200 mg l⁻¹ casein-hydrolysisate, 2 mg l⁻¹ vitamin B₁, 10 mg l⁻¹ pantothenic acid, 5 mg l⁻¹ nicotinic acid and 2 mg l⁻¹ adenine sulphate. Uninucleate microspores cultured in MS liquid medium (MSL) while anther culture establish on same medium (MSS) with 0.7 % agar. Liquid (MSL) and solid (MSS) media contained BM and 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin) about 1 mg l⁻¹. A twenty dissected anthers with uninucleate microspores per Erlenmeyer flask with filter (100 µm) and 20 ml MSL medium for androgenesis induction. The microspore suspension was refreshed every 4 weeks with MSL medium. After 8 weeks, the suspension was plated by Bergmann technique (1959) on a solid MSS₁ medium reduced concentration of 2,4-D 0.01 mg l⁻¹ and Kin 1 mg l⁻¹. Anthers were cultured on petri dish containing about 30 ml MSS induction medium. Development and multiplication of androgenic embryos originating from anther and microspore culture proceeded on MSS₁ medium. All media were sterilized by autoclaving at 0.9 x 10⁵ Pa and 114 °C for 25 min. Suspension cultures were grown on a horizontal shaker (85 rpm) at temperature of 23 ± 1 °C for one month in the dark. All other cultures were grown at the same temperature with irradiance of 33-45 µmol m⁻²s⁻¹ produced by cool white fluorescent tubes. Androgenic embryos were growth on MS hormone-free medium a 16/8 h light/dark photoperiod.

DETERMINATION OF PLOIDY LEVEL

Nuclear suspension from androgenic embryos in cotyledonary stage of development was prepared. Young leaf material of horse chestnut was used as control. Plant material was macerated with a sharp razor blade in a ice-cold neutral buffer, and placed in plastic Petri dishes. Neutral DNA buffer (pH 7) with 15 mM Hepes, 1 mM EDTA, 80 mM KCl, 20 mM NaCl, 0,5 mM spermine, 300 mM sucrose, 0.2% Triton X-100, 15m MDTE (Dithiothreitol) and 2 mg l⁻¹ DAPI was used. After maceration, the buffered mixture (ca. 2 ml), was passed through a nylon filter of 40 µm mesh size, stained with DAPI, and analysed in a flow cytometer. Fluorescence levels were determined by a photomultiplier and converted in voltage pulses that were processed with PC. Ploidy level of androgenic embryos was evaluated by flow cytometry, using a PAS II cytometer (Partec GmbH), equipped with a high pressure mercury lamp (OSRAM HBO 100 W/2) and using the excitation filters UG-1, BG-31, KG-1 and TK-420 and emission filters TK560 and GG435.

STATISTICS AND REPETITION

Effect of five horse chestnut genotypes on androgenic embryo induction was investigated during one year. The sample size was about 200 embryos for each of five genotypes. The number of obtained embryos per one anther and percentage of responding anthers were used as indicators of the efficiency of androgenesis. The

results were assessed using the variation analysis. The means were compared by the least significant difference (LSD) test (significance level $\alpha = 0.05$).

Three repetitions had been performed per each genotype.

RESULTS AND DISCUSSION

EFFECT OF GENOTYPE

Genotype is important factors for androgenesis control. Androgenic responses were recorded as: a) the total number of embryos and b) the number of embryos per isolated anther.

All horse chestnut genotypes had a high, but different androgenic response. Anther induction rates were from 13.6 % to 37.6 % depending of genotype (Table 1). Genotypes T₁ - T₅ were 110 years old. Anthers originating from T₃ and T₅ genotypes produced the less number of androgenic embryos (1.0 and 0.5 embryos/anther; respectively), then anthers from T₁, T₂ and T₄ genotypes, see Table 2. Results shown in Table 2, confirmed that microspore culture was about 5-6 times efficient method for androgenesis induction than anther culture for same genotype. The number of embryos per isolated anther varied between 0.5 to 5.0 embryos in anther culture, while in microspore culture varied between 3.0 to 27 embryos, depending of genotype (Table 2).

Table 1: Effect of T₁-T₅ genotype on induction of horse chestnut embryogenic anthers on MS₁ medium.

Genotype	N° of anthers	Embriogenic anthers	
		N°	%
T ₁	208	47	22.6
T ₂	210	79	37.6
T ₃	210	68	32.4
T ₄	205	75	36.6
T ₅	206	28	13.6

*values in each column marked by different letters are significantly different at 0.05 using the LSD t

Our results that the genotype of the donor plant is the most crucial factor for horse chestnut embryogenic capacity in are correlated with results on citrus (Germana et al., 2000), apple (Höfer, 2004; Höfer et al., 2008) and banana (Assani et al., 2003). According to HASSAWI et al. (2005), the genotype dependency of anther culture response is the major limitation to a wider exploitation of anther culture in breeding. However, genotype difference can be overcome by crossing a

highly responsive genotype to a non-responsive genotype (Zamani et al., 2003; Hassawi et al., 2005).

Table 2: Effect of T₁-T₅ genotype on the induction of horse chestnut androgenic embryos using anther and microspore culture on MS₁ medium.

ANTHER CULTURE		MICROSPORE CULTURE	
Genotype	N ^o of anthers	N ^o anthers	embryos/ embryogenic anthers
T ₁	208	~ 200	27.0 ^a
T ₂	210		11.0 ^b
T ₃	210		6.0 ^{bc}
T ₄	205		9.0 ^b
T ₅	206		3.0 ^c

*values in each column marked by different letters are significantly different at 0.05 using the LSD test.

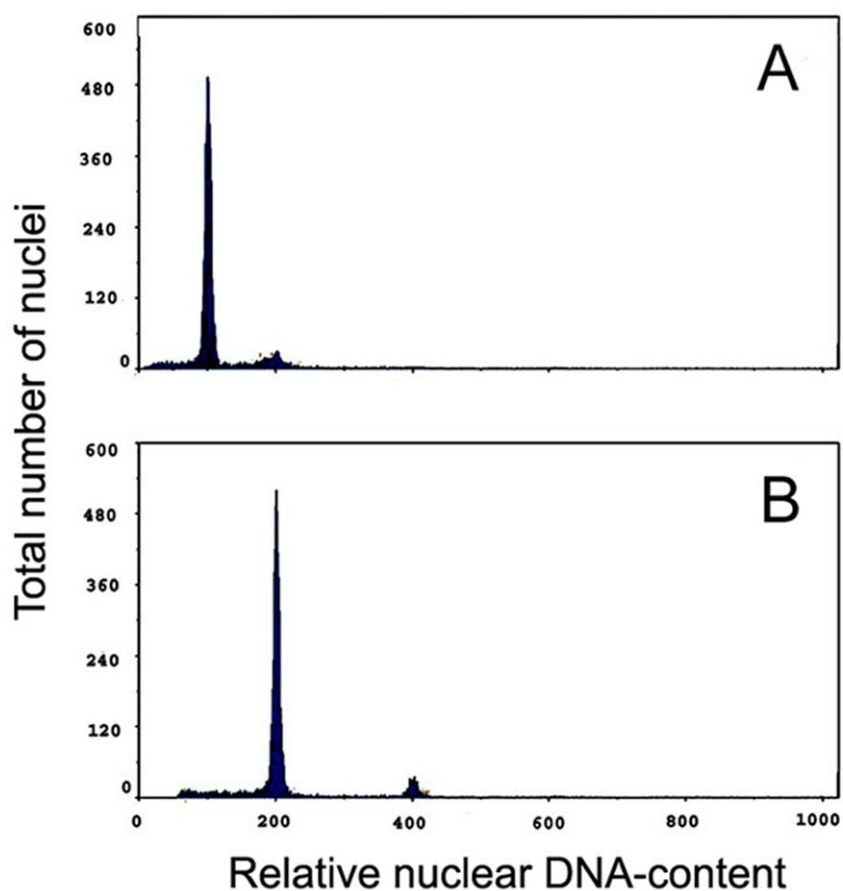


Figure 1: Flow cytometry histograms A. Haploid and diploid (B) androgenic embryos.

CYTOGENETIC ANALYSIS

Cytogenetic analysis of androgenic embryos originating from anther and microspore culture was done after a first generation of regenerants. All androgenic embryos the first generation from microspore culture were haploid (Figure 1A). Immediately after germination, 50 % of the regenerants originating from anther culture were haploid, and the other half diploid (Figure 1B).

CONCLUSION

To the best of our knowledge, this was also the first report on horse chestnut androgenic embryos, presenting influence of old genotype on induction androgenesis. Our results show that the androgenic embryo induction and frequency of haploid plant regeneration was strongly genotype-dependent. Diploid plants were present among the androgenic regenerants, this could be a result of spontaneous chromosome doubling occurring during androgenesis. The haploids may be important role for the improvement of horse chestnut through breeding program.

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SINOPSIS

UTICAJ RAZLIČITIH GENOTIPOVA NA PRODUKCIJU ANDROGENIH EMBRIONA DIVLJEG KESTENA

Dušica ĆALIĆ-DRAGOSAVAC, Snežana ZDRAVKOVIĆ-KORAĆ, Jelena MILOJEVIĆ, Ljiljana RADOJEVIĆ

Proučavan je uticaj pet različitih genotipova na indukciju androgeneze kod divljeg kestena. Efikasnost *in vitro* androgeneze u kulturi antera i mikrospora je ispitivana korišćenjem antera poreklom iz istih zatvorenih cvetnih pupoljaka. Androgeni odgovori različitih genotipova su mereni i upoređivani. Indukcija antera je bila 13.6 % to 37.6 % u zavisnosti od genotipa. Pod optimalnim uslovima, broj embriona po izolovanoj anteri varira izmedju 0.5 do 5.0 embriona u kulturi antera, dok u kulturi mikrospora se kreće izmedju 3.0 do 27 embrina, u zavisnosti od genotipa. Kultura mikrospora je mnogo efikasniji metod za produkciju androgenih embriona od kulture antera.

Flow citometrijska analiza je pokazala da polovina androgenih embriona zadržava haploidnost, dok polovina embriona je diploidna, posle 6 meseci u kulturi.

Ključne reči: *Aesculus hippocastanum*, androgeni embrioni, genetička stabilnost, genotip, nivoploidije.

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