

Full Length Research Paper

Impact of genotype, age of tree and environmental temperature on androgenesis induction of *Aesculus hippocastanum* L.

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Influence of ten different genotypes, age of trees and environmental temperature on induction of androgenesis and appearance albino horse chestnut embryos were studied. Efficiency of *in vitro* androgenesis via anther and microspore culture had been investigated. Microspores and anthers were used from the same closed flower bud. Androgenic response of different genotypes was measured and compared. Anther induction rates were from 5 to 37.6%, depending on 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 on genotype. A microspore culture was 5 - 6 times efficient than anther culture for same genotype. Age of the trees had no influence on androgenesis induction. Temperature of about 4 - 5 °C was optimal for androgenic embryo induction. Albino horse chestnut embryos phenomenon depended on genotype. The number of albino appearing in anther was higher than in microspore culture. The same correlation of appearing albino was observed between short and long day. Flow 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, while 50% of the regenerants originating from anther culture were haploid, and the other half diploid.

Key words: Horse chestnut, genotype, environmental impact, androgenic embryos, ploidy stability.

INTRODUCTION

Horse chestnut (*Aesculus hippocastanum* L., *Hippocastanaceae*) represent a relict species of the tertiary flora and endemite of Balkan peninsula. Also, horse chestnut is an important horticultural and medical plant. 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 and Radojević, 1992; Assani et al., 2003; Germana, 2000; 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 gave good results. Besides green embryos, albino embryos were also obtained, but their development in culture was slow. Although the occurrence of albino plants is a general phenomenon,

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extensive and systematic studies are presently lacking on this subject. The problem is particularly significant for plant breeding. 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).

Most trees are characterized by a long reproductive cycle with several years of a juvenile phase, a tendency to allogamy and a large tree size. They are generally highly heterozygous, outbreeding species, which are asexually propagated. For this reasons, their genetic improvement by conventional methods is time-consuming and limited by space for field experiments. The production of haploids offers new possibilities for genetic studies and breeding (Hofer, 2005). Biotechnological methods can improve the efficiency and increase the speed of breeding. 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). Gametic embryogenesis by anther and isolated microspore culture, allows the single-step development of complete homozygous lines from heterozygous parents (Germana et al., 2006). 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 and Lesinasse, 1996; Höfer et al., 2002).

Androgenesis induction from only one genotype has been previously described for *A. hippocastanum* (Čalić et al., 2003). The fact that this research and results were limited to one genotype opens a lot of questions for further investigation. The objective of the study presented in this paper was to prove the induction of androgenesis and appearance albino horse chestnut embryos depending on genotypes, age of trees and environmental temperature.

MATERIALS AND METHODS

Plants materials

Inflorescences were harvested from five genotypes (20 years old) and five genotypes (100 years old) of *A. hippocastanum* L. trees growing in the botanical garden "Jevremovac" of Belgrade University. Anthers were excised from closed flower buds (size 5 mm).

Cytological examination

Anthers from closed flower buds (size 5 mm) were resected and free microspores were stained. Dimorphysam determined with 1%

aceto-carmin while microspore viability with fluorescein diacetate (FDA). Aceto-carmin solutions were prepared in 45% acetic acid. Fresh microspores were treated with FDA (Heslop-Harrison, 1970). FDA (2 mg l^{-1}) dissolved in acetone was diluted (1:1) with 0.5 M sucrose solution. Determining the correct developmental stage of the microspores, were performed with 0.1 mg l^{-1} 4',6-diamidino-2-phenylindole (DAPI) according to procedure Coleman and Goff (1985). The content of anthers was squeezed out and stained with DAPI ($1 \mu\text{g ml}^{-1}$) solution prepared in distilled water. Microspores treated with FDA observed under UV fluorescence microscope (DMLB, Leica), while microspores coloured with DAPI were analysed with a Carl Zeiss Jenalumar fluorescence microscope with an ultraviolet exciter filter.

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 and Skoog, 1962), 2% sucrose, 0.7% agar, 100 mg l^{-1} myo-inositol, 200 mg l^{-1} casein-hydrolysis, 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 ($100 \mu\text{m}$) 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 MSS, 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 MSS, medium. However, maturation and germination of androgenic embryos was induced on MS hormone free medium (MSHF). All media were sterilized by autoclaving at $0.9 \times 10^5 \text{ Pa}$ and 114 C for 25 min. Suspension cultures were grown on a horizontal shaker (85 rpm) at temperature of $23 \pm 1^\circ\text{C}$ for one month in the dark. All other cultures were grown at the same temperature with irradiance of $33 - 45 \mu\text{mol m}^{-2}\text{s}^{-1}$ produced by cool white fluorescent tubes. Androgenic embryos were grown on MS hormone-free medium a 16/8 h light/dark (long day; LD) and 8/16 h light/dark (short day; SD) 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 an 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, 15 mM DTE (Dithiothreitol) and 2 mg l^{-1} DAPI was used. After maceration, the buffered mixture (ca. 2 ml), was passed through a nylon filter of $40 \mu\text{m}$ 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.

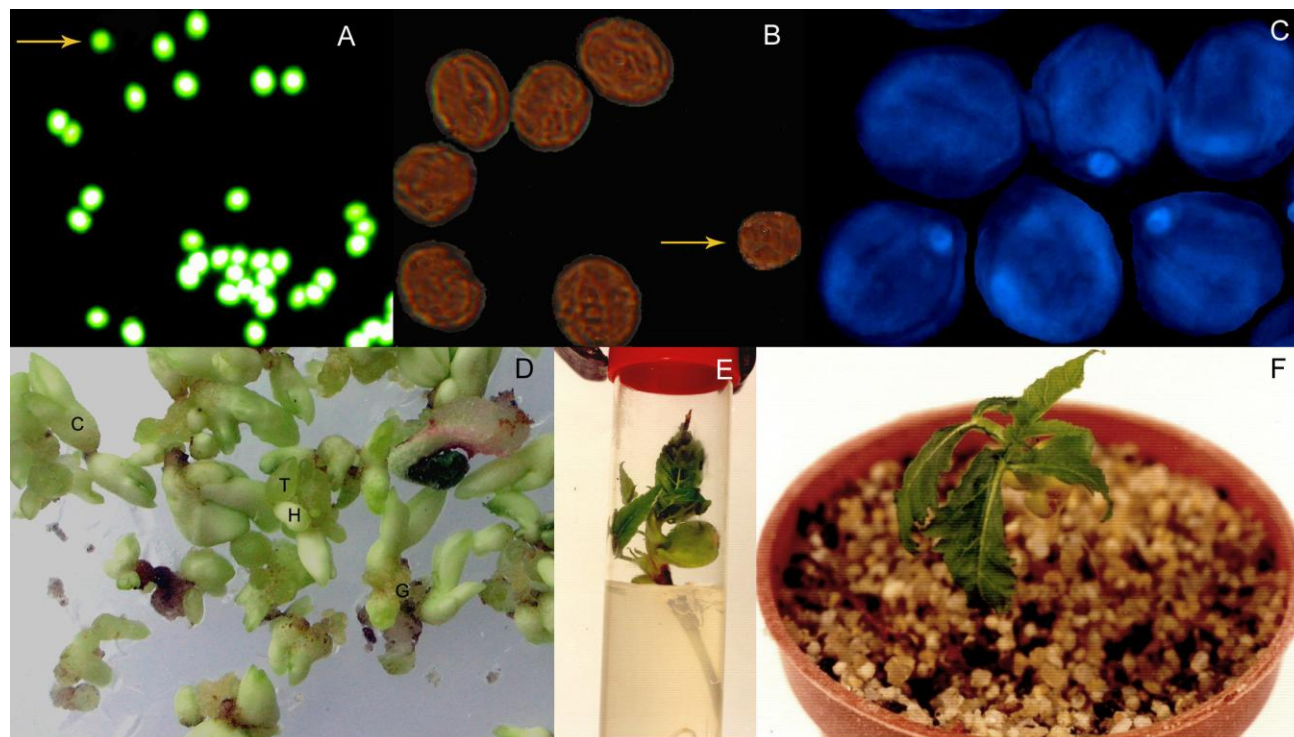


Figure 1. Androgenic microspore, embryos and plant of horse chestnut. A. Microspores stained with FDA (smaller microspore-arrow; x 200), aceto-carmine (B; x 400) and DAPI (C; x 1000). D. Embryos in globular (G) heart-like (H), torpedo (T) and cotyledonary (C) stage of development. E. Androgenic embryo converting into plant. F. Acclimatized androgenic plant.

Statistics and repetition

Influence of ten horse chestnut genotypes on androgenic embryo induction was investigated during one year. The sample size was about 201 - 210 embryos for each of ten genotypes. Environmental temperature influence on androgenesis induction was tested on two genotypes (20 and 100 years old) from 1997 to 2003 years.

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 genotypes.

RESULTS

Cytological analysis

The effectiveness of plant regeneration by androgenesis from anther culture and suspension of single-nucleus microspores of ten horse chestnut genotypes was examined. The most isolated fluorescein-treated microspores shows viability (Figure 1A). Aceto-carmine treated microspore (Figure 1B) showed differences in size and uninuclear developmental stage after staining with DAPI (Figure 1C). Uninuclear microspores of *A. hippocastanum* can be grouped in two classes: small, lightly staining and large, densely staining with aceto-carmine (Figure 1B).

Androgenesis induction

Rapid differentiation of androgenic embryos were obtained in microspore suspension and anther culture after 7 - 8 weeks, producing globular, heart, torpedo-like and embryos with different cotyledon numbers (Figure 1D). Development of androgenic embryos was asynchronous in same culture. Irregular embryos with abnormal cotyledon shapes, single, more than two cotyledons, cone shaped cotyledons or absence hypocotyls were observed.

Maturation and germination of androgenic embryos

Premature embryos at the heart like (Figure 1D) and torpedo stage (Figure 1D) were moved to hormone free medium, resulting in complete plantlet regeneration with both root and shoot (Figure 1E). Conversion androgenic embryos in whole plants occurred only occasionally (2%). A few plants were acclimatized (Figure 1F).

Effect of genotype

Genotype, the microspore stage, growth regulators and relative environment temperature several days before harvested inflorescence are important factors for

Table 1. Effect of different genotype (YJ₁-JT₅ and OT₁-OT₅) and age of tree on the induction of androgenic embryos in horse chestnut using anther and microspore culture on MS₁ medium.

Genotype	Age of trees (years)	Anther culture				Microspore culture	
		Total no. of anthers	Embryogenic anthers		No. of embryos/ isolated anthers	No. of anthers	Embryos/embryogenic anthers
			No.	%			
YT ₁	~ 20	206	35	17.0	3.0 ^b	100	16.0 ^b
YT ₂		210	27	12.9	1.0 ^c		4.5 ^d
YT ₃		203	12	5.9	2.7 ^b		9.0 ^d
YT ₄		209	19	9.1	0.9 ^c		5.0 ^e
YT ₅		201	10	5.0	1.6 ^{bc}		7.0 ^{de}
OT ₁	~ 100	208	47	22.6	5.0 ^a		27.0 ^a
OT ₂		210	79	37.6	2.0 ^{bc}		11.0 ^c
OT ₃		210	68	32.4	1.0 ^c		6.0 ^{de}
OT ₄		205	75	36.6	1.7 ^{bc}		9.0 ^d
OT ₅		206	28	13.6	0.5 ^c		3.0 ^e

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

androgenesis control.

Androgenic responses was recorded as: 1) the total number of embryos 2) the number of embryos per isolated anther and 3) the number of embryos per one anther after 60 days of anther culture and microspore culture on induction MSS medium with 2,4-D 1.0 and kin 1.0 mg l⁻¹.

Genotypes JT₁ - JT₅ were 20 years old, while genotypes OT₁ - OT₅ were 100 years old. Anthers originating from young YT₁ and YT₃ genotypes had higher embryogenic potential (3.0 and 2.7 embryos/anther) than those originating from genotypes YT₂, YT₄ and YT₅ (1.0; 0.9 and 1.6 embryos/anther, respectively), Table 1. However, anthers originated from old genotype OT₁ produced the largest number (5.0 embryos/anther), while anthers isolated from genotypes OT₃ and OT₅ produced the less number of androgenic embryos (1.0 and 0.5 embryos/anther; respectively), (Table 1).

All horse chestnut genotypes used in these experiments were having a high, but significantly different androgenic response. Anther induction rates were from 5 to 37.6% depending on genotype (Table 1).

Results shown in Table 1, confirmed that microspore culture was 5 - 6 times an efficient method for androgenesis induction than anther culture for same investigated 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 on genotype (Table 1).

In all horse chestnut genotypes examined, the number of embryos formed in the induction media was around five times higher in the suspension of single-nucleus microspores. The frequency of albino androgenic embryos in hormone-free medium was also monitored. Similarly, the number of albino embryos produced per 210 anthers ranged between 4 - 14 % (LD) and 15 - 25%

(SD) in anther culture, while percent of albino embryos in microspore culture was between 1 - 11% (LD) and 11 - 24 % (SD), depending on genotype (Table 2). It was also observed that albino androgenic embryos formation is significantly increased in a short, in comparison to a long day.

Number of albino horse chestnut embryos depended on genotype, type of culture (anther or microspore) and length of photoperiod.

The largest number of androgenic embryos of normal morphology and the minimum number of albino embryos was derived from an inflorescence that were exposed to relative air temperature of about 4 - 5°C, 7 days before harvesting (Table 3). In our study, cold treatment of inflorescences at 4 - 5°C for 7 days was also beneficial for horse chestnut androgenesis induction. It was also evident that the induction of microspore embryogenesis is provoked by low temperature as a shock or stress treatment given to the microspore.

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 2A). Immediately after germination, 50% of the regenerants originating from anther culture were haploid, and the other half diploid (Figure 2B).

The histograms of horse chestnut nuclei (Figures 2A - B) showed distinct G0/G1 peaks with coefficient of variation (CV) between 2.4 to 4.9%. DNA content values in cotyledonary androgenic embryos were 2C = 1.2 pg. The ploidy level of regenerated embryos and plantlets at different growth stages was determined using flow

Table 2. Influence of horse chestnut genotypes (YT₁-YT₅ and OT₁-OT₅) and age of tree on appear of albinism in anther and microspore cultures on MS₄ medium without hormone, on long day (LD) and short day (SD).

Genotype	Age of tree (years)	Medium	Albino embryos (%)			
			Anther culture		Microspore culture	
			LD	SD	LD	SD
YT ₁	~ 20	MS ₄ - without hormone	11.0 ^b	23.0 ^b	9.0 ^b	20.0 ^b
YT ₂			9.0 ^b	19.0 ^c	7.0 ^b	17.0 ^c
YT ₃			7.0 ^c	17.0 ^c	5.0 ^c	15.0 ^c
YT ₄			6.0 ^c	15.0 ^d	3.0 ^d	12.0 ^d
YT ₅			14.0 ^a	27.0 ^a	11.0 ^a	24.0 ^a
OT ₁	~100		4.0 ^d	15.0 ^d	1.0 ^e	11.0 ^e
OT ₂			9.0 ^b	22.0 ^b	6.0 ^{bc}	17.0 ^c
OT ₃			7.0 ^c	19.0 ^c	5.0 ^c	15.0 ^c
OT ₄			5.0 ^d	17.0 ^c	3.0 ^d	13.0 ^d
OT ₅			11.0 ^b	25.0 ^a	8.0 ^b	19.0 ^b

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

Table 3. Influence of temperature on embryo forming in horse chestnut anther and microspore culture of genotypes YT₂ and OT₁, on MS₂ induction medium.

Year of isolation	Genotype	Relative air temp. (°C)	Anther culture					Microspore culture		
			Total no. of anthers	Embryogenic anthers		No. of embryos/ anther	Albino embryos (%)	Total no. of anthers	No. of embryos / anther	Albino embryos (%)
				No.	%					
1997	YT ₂ ~ 20 years	4	210	27	12.9	3.0 ^a	9.0 ^b	100	16.0 ^b	7.0 ^b
1998		10		24	11.4	1.5 ^b	12.0 ^a		8.6 ^c	9.0 ^a
1999		7		26	12.4	1.5 ^b	12.0 ^a		9.22 ^c	9.0 ^a
2000		17		17	8.1	1.0 ^b	13.0 ^a		7.67 ^c	10.0 ^a
2001		16		20	9.5	1.2 ^b	12.0 ^a		8.11 ^c	9.0 ^a
2002		5		35	16.7	2.7 ^a	9.0 ^b		15.8 ^b	6.0 ^b
2003		5		32	15.2	2.8 ^a	10.0 ^b		15.0 ^b	7.0 ^b
1997	OT ₁ ~ 20 years	4		79	37.6	5.0 ^a	4.0 ^c		21.43 ^a	1.0 ^d
1998		10		52	24.8	2.0 ^b	8.0 ^b		15.12 ^b	4.0 ^c
1999		7		57	27.1	2.5 ^b	8.0 ^b		16.54 ^b	4.0 ^c
2000		17		45	21.4	1.6 ^b	10.0 ^b		14.17 ^b	5.0 ^c
2001		16		48	22.8	1.8 ^b	9.0 ^b		14.98 ^b	5.0 ^c
2002		5		75	35.7	3.8 ^a	4.0 ^c		20.15 ^a	2.0 ^d
2003		5		70	33.3	3.5 ^a	5.0 ^c		19.85 ^a	2.0 ^d

*Values in each column marked by different letters are significantly different at 0.05 using the LSD test. RT = Relative air temperature

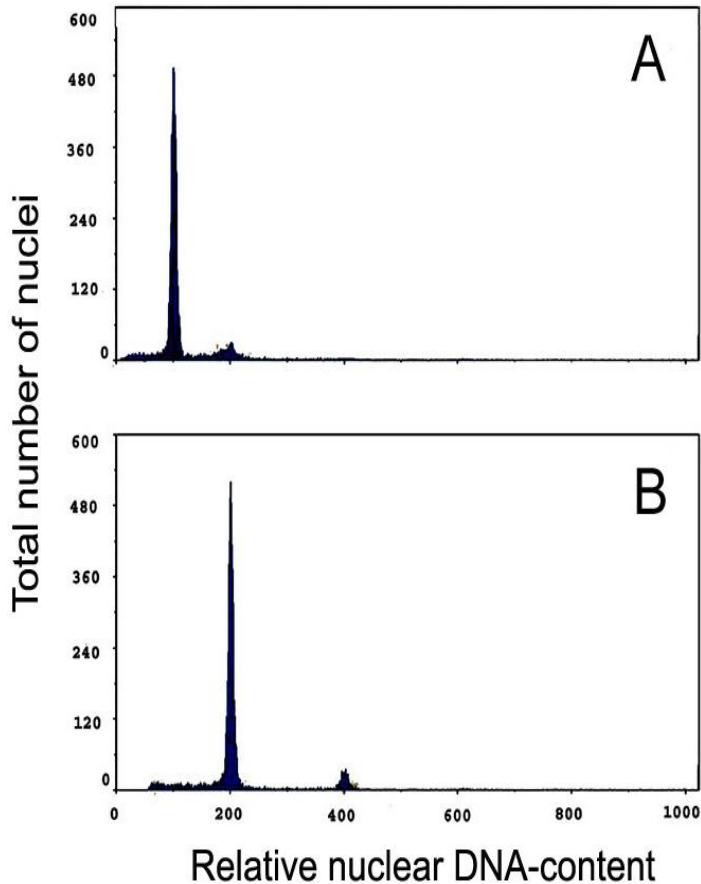


Figure 2. Flow cytometry histograms presenting some of the ploidy levels obtained after anther embryos analysis. (A) Haploid and diploid (B) androgenic embryos.

cytometry and chromosome counts, as presented in Figure 2, profiles A – B. These profiles clearly show the peaks corresponding to 1C (at an intensity emitted of epifluorescence below 100) and 2C (at around 200 intensity of emitted epifluorescence) relative DNA nuclear content. In this respect, the DNA profiles observed from embryos during early growth phases revealed haploid profiles (Figure 2A).

DISCUSSION

Uninuclear microspores of *A. hippocastanum* like microspores of *A. flava* (Ćalić-Dragosavac et al., 2008) can be grouped in two classes: small, lightly staining and large, densely staining with aceto-carmin. Significant determination of small microspores in uninucleate stage is known to be crucial for androgenesis induction (Zheng, 2003; Ćalić et al., 2003).

2,4-D and Kin had been found necessary for the androgenic embryo formation of *A. hippocastanum* (Radojević, 1978, 1991; Ćalić et al., 2003) as well as *Populus* (Mofidabadi et al., 1995) and *Malus* (Höfer,

1996). Androgenic horse chestnut embryos like somatic embryos (Capuana and Deberg, 1997; Troch et al., 2009), as well as other trees (Bueno et al., 2003; Assani et al., 2003; Germana, 2003) had asynchronous development and maturation and low germination. It is necessary to provide optimal nutritive and environmental conditions (Capuana and Deberg, 1997; Ćalić et al., 2005; Troch et al., 2009).

Our results about genotype depended on frequency of anther induction in correlation with result (Assani et al., 2003; Garcia et al., 2009).

Also, our results that the genotype of the donor plant is the most crucial factor for horse chestnut embryogenic capacity 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).

Number of albino horse chestnut embryos depended on genotype, type of culture (anther or microspore) and length of photoperiod. Our results about albino appearing in horse chestnut cultures are in agreement with results on other plant species (Kiviharju and Pehu, 1998; Shimada, 1981; Jacquard et al., 2006).

Temperature of about 4 - 5°C was optimal for horse chestnut androgenic embryo induction which correlated with the results of Höfer et al. (1999) and Garcia et al. (2009). Also, cold treatment slows the degradation process in anther tissue, thus protecting microspores from toxic compounds released in decaying anthers and so assures the survival of a greater portion of embryogenic pollen grains as compared to heat treatment (Duncan and Heberle, 1976). Low temperature also increases the frequency of endo-reduplication leading to an increase of spontaneously doubled-haploid plants (Amssa et al., 1980) as well as induces many morphological, physiological and hormonal changes in plant cells.

Our results about distinct coefficient of variation (CV) of G0/G1 peaks with correlated with results Galbraith et al. (2002). Galbraith et al. (2002) suggested a CV value of less than 5% as the acceptance criterion that reflects the quality of the applied methodology. DNA content values in cotyledonary androgenic embryos are very close to the previously published data for horse chestnut (Bennett and Leitch, 2005; Troch et al., 2009).

The results of the flow cytometry investigation reported that haploid horse chestnut plants can be produced efficiently in agreement with results on banana (Assani et al., 2003) and oak (Bueno et al., 2003).

Number of albino horse chestnut embryos depended on genotype. Also, the number of albino appearing was higher in anther than microspore culture, as well as in short in comparison to a long day.

There are some conclusions on the literature before (Čalić et al., 2003) saying that microspore culture is a more efficient model than anther culture. Detailed analysis of those investigation showed that the number of androgenic embryos, originating from only one genotype, was much lower than in this research. Therefore these conclusions about horse chestnut androgenesis, from only one genotype, could carry hidden mistakes.

The objective of the study, presented in this paper, was to absolutely exclude possible mistakes in conclusions, related to the question of embryo induction and conversion and to find the best genotype for embryos production.

Conclusion

To the best of our knowledge, this was also the first report on horse chestnut androgenic embryos, presenting influence of ten different genotype, age of trees and environmental temperature on induction of androgenesis and albino embryos appearance.

Haploid plant production can be achieved in horse chestnut. Our results show that: (1) the androgenic embryo induction and frequency of haploid plant regeneration was strongly genotype-dependent, (2) microspore culture was a more efficient model system than anther culture, (3) diploid plants were present among the regenerants; this could be a result of spontaneous chromosome doubling occurring during androgenesis, (4) number of albino horse chestnut embryos depended on genotype, (5) also, the number of albino appearing was higher in anther than microspore culture, as well as in short in comparison to a long day.

The haploid plants may be important for the improvement of horse chestnut through horticultural breeding program and future research.

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