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Full Length Research Paper

Effect of activated charcoal, abscisic acid and polyethylene glycol on maturation, germination and conversion of *Aesculus hippocastanum* androgenic embryos

Dušica Ćalić-Dragosavac[1]*, Snežana Zdravkovi-Korać[1], Borut Bohanec[2], Ljiljana Radojević[1], Branka Vinterhalter[1], Svetlana Stevović[3], Aleksandar Cingel[1] and Jelena Savić[1]

[1]Department of Plant Physiology, Institute for Biological Research, "Siniša Stanković", University of Belgrade, Republic of Serbia.
[2]Biotechnical Faculty, University of Ljubljana, Ljubljana, Slovenia.
[3]Department of Environmental Protection, Faculty of Ecology and Environmental Protection, University Union, University of Belgrade, Republic of Serbia.

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The influence of activated charcoal (AC), abscisic acid (ABA) and polyethylene glycol (PEG) on the maturation and conversion of horse chestnut (*Aesculus hippocastanum* L.) androgenic embryos were tested. Androgenic embryos originating from microspores and anther culture were maturated over 90 days. Androgenic embryos on media containing PEG (50 g l^{-1}), in combination with AC (1 g l^{-1}) showed a rapid development of embryos in the cotyledonary stage and lowered percentage of abnormal structures. The best results of androgenic microspore embryo germination were observed on media supplemented with AC alone (99%) and in combination with PEG (100%). Also, the greatest number of androgenic microspore plants (18%) and androgenic anther plants (12%) were formed on media enriched with 1 % AC. Lowest germination percentages of 37 and 39% in microspore culture and 33 and 38% in anther culture were obtained on maturation media with ABA 20 mg l^{-1} alone and in combination with AC 1g l^{-1}. Flow cytometric analysis showed that most of the androgenic embryos were haploid, corresponding to their microspore origin, while half of these became diploid after maturation for 90 days. All regenerants originating from microspore culture were haploid immediately after germination, but only 10% embryos retained haploidity after 3 years subculturing, while 10.5% were diploid, 73.5% tetraploid and 6% octaploid on hormone-free medium. Unlike those from anther culture, after 3 years of subculturing on hormone-free medium, there were no haploid regenerant from anther culture, while 8.5% were diploid, 81% tetraploid and 10.5% octaploid.

Key words: Anther culture, maturation, horse chestnut, suspension culture.

INTRODUCTION

Horse chestnut (*Aesculus hippocastanum* L.) is an endemic species with enormous horticultular important. Regeneration in the genus *Aesculus* via androgenesis has been demonstrated for *A. hippocastanum* (Radojević, 1978; Ćalić et al., 2003), *Aesculus carnea* (Radojević et al., 1989; Marinković and Radojević, 1992) and *Aesculus flava* (report in preparation). Androgenesis provides a large number of embryos at defined stages of development and allows alterations of the embryonic environment through manipulations of culture conditions. High quality somatic embryos of horse chestnut are commonly produced on media supplemented with abscisic acid (ABA) with decreased osmotic water potential compared to the proliferation medium (Attree and Fowke, 1993;

*Corresponding author. E-mail: calic@ibiss.bg.ac.rs.

Abbreviations: ABA, Abscisic acid; **AC**, activated charcoal; **2,4-D,** 2,4-dichlorophenoxyacetic acid; **KIN,** kinetin; **MS,** Murashige and Skoog's mineral solution (1962); **PEG,** polyethylene glycol.

Stasolla and Yeung, 2003). This decrease in water potential is mainly achieved by increasing the concentration of non-plasmolyzing osmoticum (polyethylene glycol, PEG) (Capuana and Deberg, 1997; Troch et al., 2009). Regeneration of this species via androgenesis provides a means of propagation and a model system for conducting physiological and biochemical studies. Although such embryos may appear "morphologically" mature, they do not perform well during postembryogenic growth without the imposition of a drying period. Improvement of embryo quality can be achieved through the application of osmotic stress, which is an important factor for directing embryo development and maturation both *in vivo* and *in vitro* (Capuana and Deberg, 1997; Troch et al., 2009). Generation of horse chestnut somatic embryos is commonly achieved by transferring embryogenic tissue onto an ABA, PEG and manitol-containing maturation media (Capuana and Deberg, 1997).

Activated charcoal is commonly used in tissue culture media to darken the immediate media surroundings and to absorb inhibitory or toxic substances and plant growth regulators (Moshkov et al., 2008). Charcoal has been used in all stages of somatic embryogenesis to increase initiation frequencies of *Pinus taeda* L. (Pullman and Jonson, 2002), induce embryogenic tissue on vegetative shoot apices of mature trees of *Pinus patula* (Malabadi and Van Staden, 2005), improve yield and quality of somatic embryos during maturation (Capuana and Deberg, 1997; Caraway and Merkle, 1997; Li et al., 1997, 1998; Groll et al., 2002; Pullman et al., 2005; Lelu-Walter et al., 2006) and most frequently during germination (Vooková and Kormuák, 2001; Salaj et al., 2004; Andrade and Merkle, 2005).

ABA is commonly used at various concentrations to improve somatic embryo development of *Abies* species prior to plantlet regeneration (Salajová et al., 1996; Vooková and Kormut'ák, 2001).

Abscisic acid has been found to stimulate the production of cotyledonary embryos and regulate the course of embryo maturation (Becwar et al., 1987; Boulay et al., 1988; Dunstan et al., 1988; von Arnold and Hakman 1988; Roberts et al., 1990a). The ability of ABA to inhibit precocious germination has facilitated the development of procedures for mass propagation of spuce emblings (Roberts et al., 1990b; Webster et al., 1990). Osmotic treatments have been used to influence embryo maturation in many gymnosperms and angiosperms (Raghavan, 1986; Capuana and Debergh, 1997).

A recent study, using scanning electron microscopy, has shown that desiccated embryos of *B. napus* L. cv Topas, which are not pretreated with ABA, exhibited a collapse of their tissue systems (Wakui et al., 1999). ABA induction of desiccation tolerance may be linked to accumulation of late embryogenesis–abundant proteins that may protect tissues (Dure et al., 1989; Dure et al., 1993). Indeed, mRNAs encoding these proteins have been shown to accumulate in desiccation-tolerant embryos of oilseed rape (Wakui et al., 1995).

The effect of PEG mimics the naturally occurring water stress on seeds during the late stages of maturation. Water stress caused by PEG and increased concentrations of ABA are essential for somatic embryo development to accumulation of storage compounds and inhibition of precocious germination (Strasolla and Yeung, 2003). PEG enhanced somatic embryo maturation in several species, including *P. glauca* (Attree et al., 1991, 1992, 1993, 1995), *Abies numidica* (Vooková and Kormut'ák, 2001) and *Abies hybrids* (Salaj and Salaj, 2003; Salaj et al., 2004).

The combined application of ABA and PEG, a non-plasmolyzing osmoticum, has become a routine method for stimulating embryo maturation (Attree and Fowke, 1993).

Variables as a carbon source, abscisic acid (ABA) and osmotic agents has been used to increase germination and conversion rates of somatic embryos of *P. glauca* (Attree et al., 1990, 1991, 1995), *A. hippocastanum* (Capuana and Deberg, 1997), *A. dentata* (Robichaud et al., 2004) and *A. cephalonica* (Krajňáková et al., 2009). Some treatments can be applied before or during culture in order to increase the success obtained from anther culture.

The aim of this research was to study the influence of AC, ABA and PEG on maturation of horse chestnut androgenic embryo from microspore suspension culture and anther culture and finally, flow cytometric analysis was used to verify the ploidy stability of the horse chestnut androgenesis process.

MATERIALS AND METHODS

Completely closed flower buds (4 - 5 mm long) with uninucleate microspores used in the experiments were obtained from 100 years old *A. hippocastanum* L. tree grown in the Botanical Garden "Jevremovac" of the Belgrade University. The selected buds were surface sterilized with 95% ethanol (2 - 3 min) and 70% ethanol (5 min), followed by three rinses in sterile distilled water. Basal medium (BM) which contained Murashige and Skoog (MS, 1962) mineral salts, 2% sucrose and was supplemented with the following (mg l^{-1}): panthotehenic acid (10), nicotinic acid (5), vitamin B_1 (2), adenine sulphate (2), myo-inositol (100) and casein-hydrolysate (200) was used. On induction, MS solid medium (MSS) with 0.7% agar was the established anther culture, while androgenic embryos were obtained by culturing uninuclear microspores in MS liquid medium (MSL). Both media contained BM with 2,4-D dichlorphenoxyacetic acid (2, 4-D) and kinetin (Kin), 1.0 mg l^{-1} of each. About 100 anthers with uninucleate microspores per Erlenmeyer flask with filter (200 µm) and 100 cm^3 MSL medium for androgenic induction were cultivated.

Isolation and culture of microspores

Microspores were isolated from anthers. The anthers were macerated in a glass Petri dish with scalpel through a 50-µm metal sieve in MSL induction medium. The microspores collected on the surface of the 50-µm sieve were carefully washed with same medium. The microspore suspension were subcultured every 30 days and refreshed with liquid MSL medium. After 60 days, a microspore suspension was plated by Bergmann technique (1960) on a solid medium with reduced concentration of 2,4-D (0.01 mg l^{-1})

and same concentration of Kin.

The suspension cultures were kept at 25 ± 1°C in the dark at a density of 1 – 3 x 10^5 microspores/ml. Also, microspore suspension cultures were grown on a horizontal shaker (85 rpm).

Isolation and culture of anthers

Six to seven anthers were inoculated in each culture tube containing 8 cm^3 of the MSS induction medium. Embryo development and multiplication of androgenic embryos from suspension and anther culture proceeded on medium with 2,4-D and Kin (1.0 mg l^{-1} each). After, medium for multiplication embryos were cultured on media for embryo maturation supplemented with AC, ABA and PEG 4000, 400 mg dm^{-3} filter sterilized glutamine (Glu) and various adjuvants were added to improve embryo maturation in later stages of their development.

Various concentrations (0.1, 0.5 and 1%) of activated charcoal (AC) were tested. ABA (2.5, 10.0 and 25.0 mg l^{-1}) alone, as well as in combination with 1% AC was investigated. Also, influence of PEG (5, 25 and 50 g l^{-1}) and combinations of PEG (5 and 50 g l^{-1}) with AC (1 g l^{-1}) on maturation of androgenic embryos were studied.

Effect of these different additives (AC, ABA and PEG) on phenomenon of secondary embriogenia were also investigated (Ćalić et al., 2005). We used 13 different maturation media which are presented in Tables 1 and 2. Chemicals used in all experiments were purchased from Sigma. Subculturing was done every 30 days and the maturation phase took 90 days. Cultures were monitored at the time of sub-culturing and the mature embryos were transferred to hormone and supplement free germination medium.

All media were sterilized by autoclaving at 0.9 x 10^5 KPa and 114°C for 25 min. Anther cultures as well as androgenic embryos originating from both types of cultures were grown at 25 ± 1°C and a 16-h photoperiod with irradiance of 33 - 45 µmol $m^{-2}s^{-1}$ produced by cool white fluorescent tubes.

Androgenic embryos on maturation media were investigated after 30, 60 and 90 days. Some of the characteristics evaluated are: shoot elongation, radicle development and conversion into the plants.

Determination of ploidy level

Nuclear suspension from androgenic embryos in the cotyledonary stage of development were 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, 15 mM DTE (Dithiothreitol) and 2 mg l^{-1} DAPI was used (modified by de Laat and Blaas, 1984). After maceration, the buffered mixture (ca. 2 ml), was passed through a nylon filter of 30 µm mesh size, stained with DAPI and analysed in a flow cytometer. Fluorescence levels were determined by a photomultiplier and coverted in voltage pulses that were processed with PC. Ploidy level of androgenic embryos was evaluated by flow citometry, 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

Influence of 13 different media on maturation and conversion of androgenic embryos were investigated during the 90 days. Sixty androgenic embryos per medium were analysed. Three repetitions were performed per each medium. The total number of pollen grains analyzed for each studied medium was 180.

The results were assessed using the variation analysis. Results were tested according to SNK (Student Newman Keuls) test (significance level α = 0.05) for determination of statistical significant differences among treatments.

RESULTS

Androgenesis of mononuclear *A. hippocastanum* microspores was induced in liquid and solid MS (Murashige and Skoog, 1962) medium with 2,4-D acid and Kin (1.0 mg l^{-1} of each). Light and scanning microscopy confirmed that androgenic embryos of horse chestnut grown in suspension had formed by direct division of microspores. Rapid differentiation of androgenic embryos were obtained in *in vitro* culture over the first several weeks, producing globular, heart, torpedo-like and embryos with different cotyledone numbers. After transfer to basal medium (BM), androgenic embryos showed asynchronous development (Figure 1A). Great numbers of these embryos were irregular, showed hypertrophy and had abnormal cotyledons or lacked a hypocotyl.

Percentage germination of androgenic embryos was done after 30, 60 and 90 days of growing on different maturation media. The best results of germination of horse chestnut androgenic embryos which maturated on media supplemented with AC (99%) alone and in combination with PEG (100%) were observed in microspore and anther culture (Tables 1 and 2).

With increasing concentration of activated charcoal in medium, the percentages of germination of embryos from 30 to 60% after 30 days, from 88 to 94% after 60 days and from 92 to 99% after 90 days in suspension cultures were increased. Similarly, in anther culture, with increasing concentration of AC, the percentage of germination of embryos from 60 to 89% after 30 days, from 69 to 95% after 60 days and from 78 to 99% after 90 days were increased.

Also, the greatest number of androgenic plants originating from microspore (18%) and anther (12%) culture were formed on the medium which was supplemented with 1% AC (Tables 1 and 2). Cultures on media containing 1% AC showed better maturation, germination, shoot elongation and conversion into plants (Figure 1C, Table 1).

However, the number of germinated embryos with increasing concentration of ABA (from 2.5 to 20 mg l^{-1}) in media was decreased (Table 1). Lowest germination percentages, 37 and 39% in microspore culture and 33 and 38% in anther culture were obtained on maturation media with ABA 20 mg l^{-1} alone and in combination with AC 1g l^{-1}. ABA concentration of 2.5 mg l^{-1} had a positive effect on shoot formation in microspore (23 %) and anther (24 %) culture. PEG in maturation media improved androgenic embryo germination and conversion into the plants. Androgenic embryos on media containing PEG (50 g l^{-1}), in combination with AC (1 g l^{-1}) showed rapid development of cotyledonary stage embryos and lowered percentage of abnormal structures.

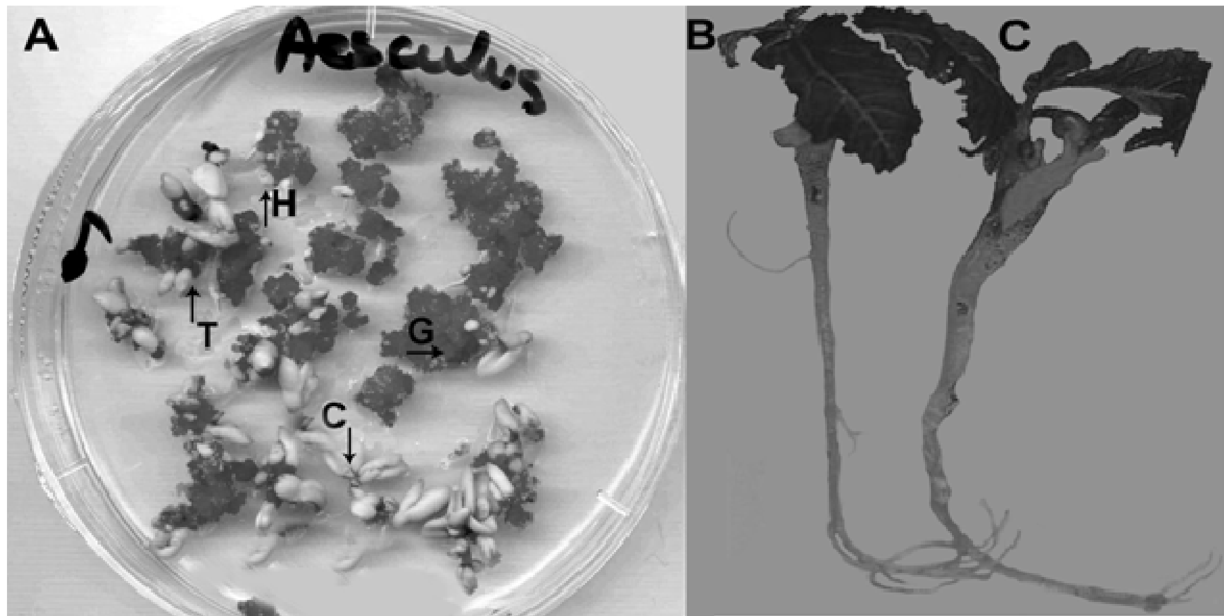

Figure 1. Androgenic embryos in different stage of development. (A) Globular (G), heart-like (H), torpedo (T) and cotyledonary on basal medium (BM). (B) Plantlet originating from microspore culture grown on medium with 5 g l^{-1} AC. (C) Plantlet originating from microspore culture grown on medium with 10 g l^{-1} AC.

Increasing PEG from 5 to 50 g l^{-1} in the maturation media improved germination of androgenic embryos originating from microspore culture from 62 to 87% and in anther culture from 60 to 81%. Concentration of AC 1 g l^{-1} in maturation medium with 5 and 50 g l^{-1} of PEG had a further beneficial effect on germination of embryos derived from microspore (79 - 100 %) and anther culture (72 - 100 %).

The addition of AC and PEG to the maturation medium significantly increased the percentage of androgenic embryos forming shoots and increased the conversion frequencies in microspore (5 - 13%; Table 1) and anther culture (6 - 18%; Table 2). Similar results of maturation treatment were observed in microspore and anther culture. Increasing duration of treatment from 60 to 90 had a positive impact on embryo maturation.

Cytogenetic analysis

Cytogenetic analysis of androgenic embryos originating from anther and microspore culture was done after a first generation of regenerants (after 90 days) and after 3 years of subculturing.

All androgenic embryos after 90 days of maturation treatments from microspore culture were haploid (Figure 2A), while 50% of the regenerants originating from anther culture were haploid and the other half diploid (Figure 2B). However, only 10% androgenic microspore embryos retained haploidity, while 10.5% were diploid, 73.5% tetraploid (Figure 2C) and 6% octaploid (Figure 2D) after 3 years subculturing. Similarly, there were no haploid regenerant from anther culture, while 8.5% were diploid, 81% tetraploid and 10.5% octaploid after 3 years of subculturing.

DISCUSSION

Great numbers of these embryos were irregular, hypertrophic and had abnormal cotyledons or lacked hypocotyls. This phenomenon is frequently observed in somatic embryos of trees (Pérez et al., 1986; Capuana and Deberg, 1997). Therefore, addition of different concentrations of AC, ABA or PEG in media with horse chestnut androgenic embryos overcame the problem. Our results about influence of activated charcoal on the *in vitro* androgenesis have a correlation with the results of Özkum Çiner and Tipirdamaz (2002).

The effect of activated charcoal has been attributed to the absorption of inhibitory substances (abscisic acid, phenolics) from the medium (Thomas, 2008). In the current study, the best results of germination, maturation and conversion of androgenic embryos into the plants were achieved on medium with 1% activated charcoal which is in accordance with the results of Pullman et al. (2005). Also, ABA and water stress may keep an embryo in a maturation state by encouraging development and preventing germination.

Abscisic acid has been found to stimulate the production of cotyledonary embryos with normal morphology and to regulate the course of horse chestnut somatic embryos (Capuana and Deberg, 1997; Troch et al., 2009) as well as American chestnut (Robichaud et al., 2004).

Table 1. Effect of AC, ABA and PEG on maturation of androgenic embryos originating from suspension culture, after 30, 60 and 90 days.

Additive	No. of embryos	Root (%)			Shoot (%)			Whole plant (%)		
		30 days	60 days	90 days	30 days	60 days	90 days	30 days	60 days	90 days
Control	300	18 c	55 b	70 b	3 cd	5 f	8 d	3 c	4 d	7 c
AC (g l^{-1})										
1	300	30 b	78 ab	84 ab	4 c	7 e	9 d	4 bc	6 c	9 bc
5	300	50 ab	89 a	93 a	6 b	14 b	15 bc	5 b	9 b	11 b
10	300	60 a	94 a	99 a	10 a	28 a	29 a	7 a	13 a	18 a
ABA (mg l^{-1})										
2.5	300	9 d	52 b	56 bc	-	7 e	18 b	-	-	-
10	300	7 de	39 c	50 bc	-	-	-	-	-	-
20	300	5 e	29 c	37 c	-	-	-	-	-	-
ABA (mg l^{-1}) + AC (g l^{-1})										
2.5 + 1	300	10 d	56 b	68 b	4 c	9 d	23 a	2 d	4 d	4 d
20 + 1	300	5 e	31 c	39 c	-	-	-	-	-	-
PEG (g l^{-1})										
5	300	18 c	54 b	62 b	-	2 g	5 e	-	1 e	1 f
25	300	24 bc	59 b	69 b	1 d	5 f	7 d	-	1 e	2 e
50	300	29 b	79 ab	87 ab	7 b	11 c	11 b	-	1 e	6 c
PEG (mg l^{-1}) + AC (g l^{-1})										
5 + 1	300	23 bc	62 b	79 ab	-	13 b	9 d	-	1 e	2 e
50 + 1	300	69 a	100 a	100 a	-	7 e	13 c	-	3 d	7 c

*The values of different letters are significantly different at the 0.05 probability level according to protected SNK test.

Table 2. Effect of AC, ABA and PEG on maturation of androgenic embryos originating from anther culture, after 30, 60 and 90 days.

Additive	No. of embryos	Root (%)			Shoot (%)			Whole plant (%)		
		30 days	60 days	90 days	30 days	60 days	90 days	30 days	60 days	90 days
Control	300	38 c	56 bc	58 bc	2 c	5 d	7 e	1 c	3 cd	5 cd
AC (g l^{-1})										
1	300	52 b	65 b	69 b	3 b	7 c	10 d	2 b	6 b	7 c
5	300	70 ab	80 ab	85 ab	4 ab	10 b	13 c	3 ab	7 b	9 b
10	300	89 a	95 a	99 a	5 a	14 a	15 bc	4 a	9 a	12 a
ABA (mg l^{-1})										
2.5	300	8 e	49 c	55 bc	2 c	4 d	7 e	-	-	-
10	300	6 ef	46 c	49 c	-	8 c	17 b	-	-	-

Table 2. Cont.

20	300	4 f	29 d	33 d	-	-	-	-	-	-
ABA (mg l^{-1}) + AC (g l^{-1})										
2.5 + 1	300	10 e	52 bc	66 b	4 ab	7 c	24 a	1 c	2 d	3 d
20 + 1	300	5 f	31 d	38 d	-	-	-	-	-	-
PEG (g l^{-1})										
5	300	18 de	57 bc	60 bc	-	3 e	6 e	-	1 e	2 e
25	300	23 d	66 b	71 b	-	6 cd	9 d	-	2 d	3 d
50	300	34 c	77 ab	81 ab	3 b	12 ab	13 c	-	3 cd	5 cd
PEG (mg l^{-1}) + AC (g l^{-1})										
5 + 1	300	26 d	61 bc	72 b	-	7 c	10 d	-	1	2 f
50 + 1	300	96 a	100 a	100 a	-	14 a	18 b	-	4 c	6 c

*The values of different letters are significantly different at the 0.05 probability level according to protected SNK test.

Significant accumulation of biomass and storage products in the cotyledons of chestnut embryos may not be critical for germination under *in vitro* conditions. In fact, Capuana and Deberg (1997) and Troch et al. (2009) noted that the highest conversion frequencies were obtained with embryos possessing thin cotyledons, while embryos bearing large, thick cotyledons, resembling those of mature zygotic embryos, did not regenerate plants at all. ABA also promotes the development of globular embryos in embryo-genic cultures of horse chestnut as well as in spruce (von Arnold and Hakman, 1988). PEG-derived horse chestnut embryos were also less aberrant than control embryos as well as somatic horse chestnut embryos (Troch et al., 2009) and *P. glauca* embryos (Attree et al., 1991).

Our results that PEG increased germination of horse chestnut androgenic embryos are in correlation with results of Attree et al. (1992) and Capuana and Deberg (1997). Attree et al. (1995) published that drying was essential for subsequent normal growth of *P. glauca* somatic embryos following maturation on PEG. We noticed that increasing duration of treatment from 60 to 90 days had a positive impact on embryo maturation.

The histograms of horse chestnut nuclei (Figures 2A - D) showed distinct G0/G1 peaks with coefficient of variation (CV) between 2.1 to 4.9%. 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 in very close agreement with the previously published data for horse chestnut (2C = 1.2 pg; Bennett and Leitch, 2005; Troch et al., 2009). The ploidy level of regenerated embryos and plantlets at different growth stages was determined using flow cytometry and chromosome counts, as presented in Figure 2, profiles A – D. These profiles clearly show the peaks corresponding to 1C (at an intensity of emitted 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).

Diploid, tetraploid and octaploid plants were present among the horse chestnut regenerants. This could be a result of spontaneous chromosome doubling occurring during androgenesis. These results suggested that ploidy level significantly increased during long-term cultures (Geier, 1991). In contrast to androgenic embryos, somatic embryos of horse chestnut did not show major genetic changes (Troch et al., 2009). However, further experiments by this author are necessary to determine whether horse chestnut somatic embryos are genetically stable during long-term cultures.

Conclusion

To the best of our knowledge, this is the first report of horse chestnut androgenic embryos, presenting influence of AC, ABA and PEG on

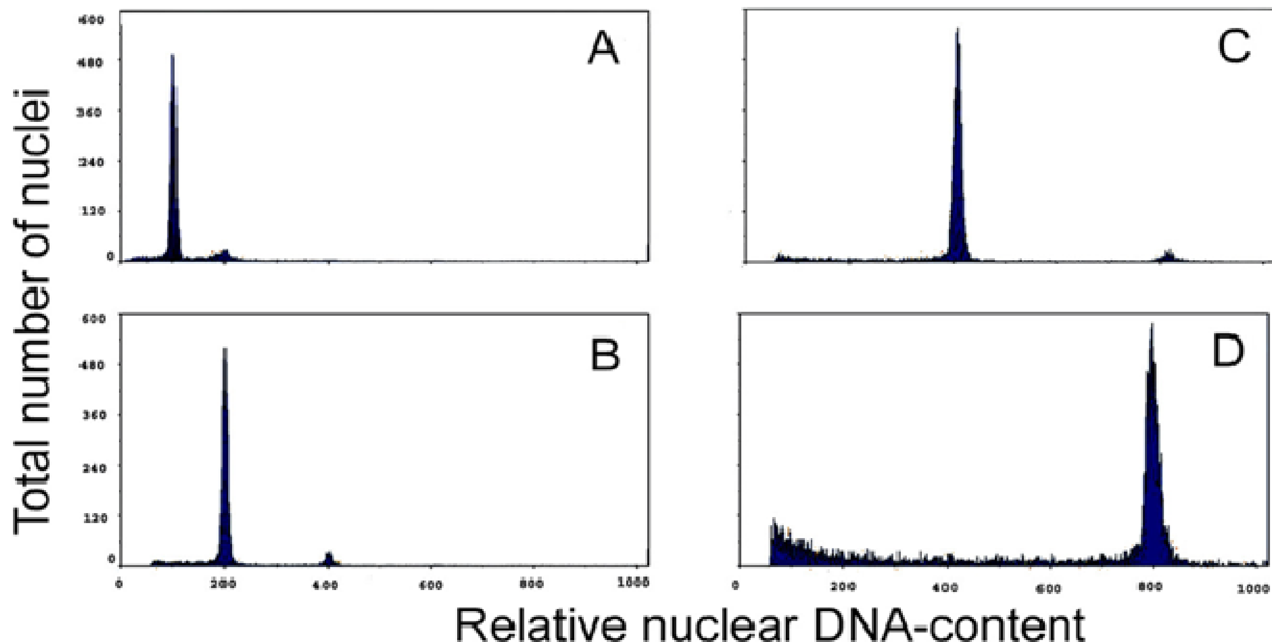

Figure 2. Flow cytometry histograms presenting some of the ploidy levels obtained after anther embryos analysis. (A) Haploid, (B) diploid, (C) tetraploid and octaploid (D) androgenic embryos.

maturation androgenic embryos and flow cytometry data. Our results have clearly shown that the 1% AC and the combination of AC and PEG to improve horse chestnut embryo quality, improved germination.

In conclusion, the use of 1% AC was found to have a greater effect than ABA and PEG on conversion of horse chestnut androgenic embryos into the plants.

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