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Original Article

Improvement of maturation and conversion of horse chestnut androgenic embryos

Dušica Ćalić-Dragosavac*, Ljiljana Radojević

Department of Plant Physiology, Institute for Biological Research, "Siniša Stanković", Bulevar despota Stefana 142, Belgrade, Serbia

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

***Abstract*:**
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Horse chestnut (*Aesculus hippocastanum* L., Hippocastanaceae) is a relict species of the tertiary flora and endemit of Balkan peninsula. It has enormous horticultular and medical important. Horse chestnut trees are native to the Balkan peninsula, but grow as ornamental trees in parks and avenues throughout the Northern Hemisphere. Because of the slow and difficult reproduction of great importance to be fast and cheap *in vitro* multiplication. Possible solution is regenerated by androgenesis. Microspore culture has been used in recent years as a tool for producing haploid plants in a varyety of higher plants, but the low frequencies of microspore-derived plants restrict the use of the technique in plant breeding.

Key words: *Aesculus hippocastanum*, anther culture, maturation, ploidy level, suspension culture

Introduction

Horse chestnut showed slow and difficult reproduction in nature conditions. Possible solution of this problem is regeneration by androgenesis *in vitro*.

Androgenesis *in vitro* has been used in recent years as a tool for producing haploid plants in a varyety of higher plants, but the low frequencies of microspore-derived plants restrict the use of the technique in plant breeding.

Regeneration in the genus *Aesculus via* androgenesis has been demonstrated for *A. hippocastanum* (Radojević, 1978; Ćalić et al., 2003), *A. carnea* (Radojević et al., 1989; Marinković & Radojević, 1992) and *A. flava* (Ćalić et al., 2005; Ćalić-Dragosavac et al., 2010). Androgenesis provides a large number of embryos at defined stages of development, and allows alterations of the embryonic environment through manipulations of culture conditions. 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 & Deberg, 1997; Troch et al., 2009). Generation of horse chestnut somatic embryos is commonly achieved by transferring embryogenic tissue onto an activated charcoal (AC), abscisic acid (ABA), polyethylene glycol (PEG) and manitol-containing maturation media (Capuana & 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 & Jonson, 2002), induce embryogenic tissue on vegetative shoot apices of mature trees of *Pinus patula* (Malabadi & Van Staden, 2005), improve yield and quality of somatic embryos during maturation (Capuana & Deberg, 1997; Caraway & 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 & Kormut'ák, 2001; Salaj et al., 2004; Andrade & 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 & 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 & 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 (Capuana & Debergh, 1997).

ABA induction of desiccation tolerance may be linked to accumulation of late embryogenesis – abundant proteins that may protect tissues (Dure et al., 1989).

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 inhibit precocious germination (Strasolla & Yeung, 2003). PEG enhanced somatic embryo maturation in several species, including *Picea glauca* (Attree et al., 1990, 1991, 1992, 1995), *Abies numidica* (Vooková & Kormut'ák, 2002) and *Abies hybrids* (Salaj & 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 & Fowke, 1993). The aim of this research was to study influence of activated charcoal (AC), abscisic acid (ABA) and polyethylene glycol (PEG) on the maturation and conversion of horse chestnut microspore-derived embryo for the diversity protection and conservation.

Material and methods

Closed flower buds (4-5 mm long) used in the experiments were obtained from 100 years old *Aesculus hippocastanum* L. tree growing in the Botanical Garden "Jevremovac" of the Belgrade University. The buds were surface sterilized with 95 % and 70 % ethanol. Basal medium (BM) contained Murashige and Skoog (1962; MS) 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). Uninuclear microspores culture was established on induction MS liquid (L) medium. Induction L medium 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 (50 μm) and 100 cm^3 L medium for androgenic induction were cultivated.

Isolation and culture of microspores

The anthers were macerated in a glass Petri dish with scalpel through a 50-μm metal sieve in L 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 L medium. After 2 months, 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.

After medium for multiplication embryos were cultured on media for embryo maturation supplemented with AC, ABA and PEG 4000.

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 PEG (5 and 50 g l^{-1}) with AC (1 g l^{-1}) on maturation of androgenic embryos were studied.

We used thirteen different maturation media which are presented in Figures 1-4. Subculturing was done every one month and the maturation phase took 3 months. 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 114^0C for 25 min. Androgenic embryos were grown at 25 ± 1 ^{0}C and a 16-h photoperiod with irradiance of 33 - 45 μmol $m^{-2}s^{-1}$ produced by cool white fluorescent tubes. Embryos on maturation media were investigated after 3 months.

Three characteristics were evaluated: 1) shoot elongation; 2) radicle development and 3) 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

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 SNK (Student Newman Keuls) test (significance level α= 0.05) for determination statistical significant differencesis among treatments.

Results

Androgenesis of horse chestnut micospores 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 formed by direct division of microspores. After transfer to basal medium (BM), androgenic embryos showed asynchronous development. Great numbers of these embryos were irregular, showed hypertrophy and had abnormal cotyledons or lacked a hypocotyl.

Percentage germination of androgenic embryos was followed after 3 months 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 culture (Fig. 1 and 4). With increasing concentration of AC increased the percentage of embryo germination from 92 to 99% after 3 months in suspension culture.

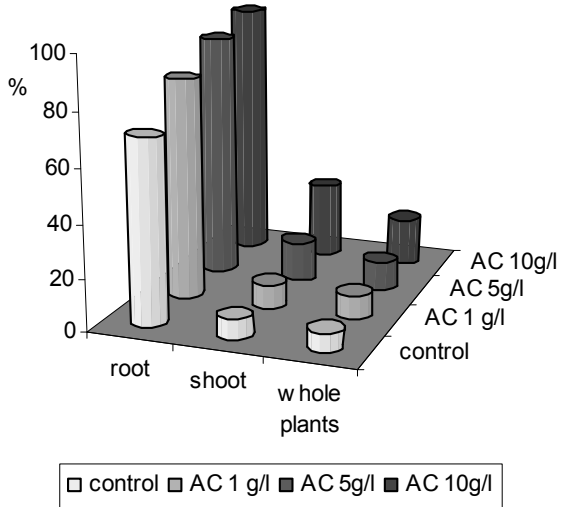

Fig. 1. Effect of AC on maturation microspore-derived embryos, after three months

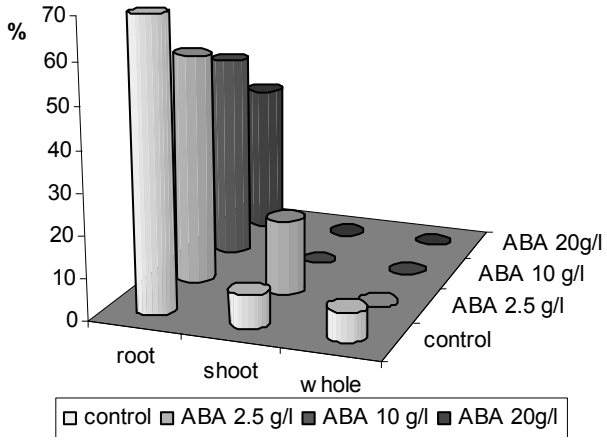

Figure 2. Effect of ABA on microspore-derived embryos, after three months

Also, the greatest number of androgenic plants originating from microspore (18 %) culture was formed on the medium with supplemented with

1 % AC (**Fig. 1**). Also, cultures on media containing 1 % AC showed better maturation, germination; shoot elongation and conversion into the plants (**Fig. 1**). However, the number of germinated embryos with increasing concentration of ABA (from 2.5 to 20 mg l^{-1}) in media was decreased (**Fig. 2**). Lowest germination percentages, 37 % and 39 % in microspore culture were obtained on maturation media with ABA 20 mg l^{-1} alone and in combination with AC 1g l^{-1}.

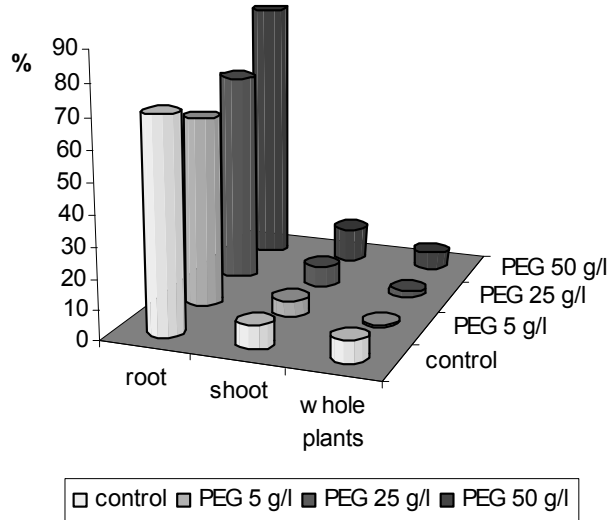

Figure 3. Effect of PEG on microspore-derived embryos, after three months

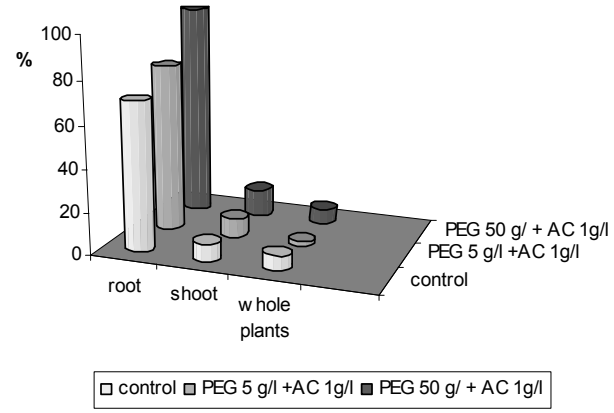

Figure 4. Effect of PEG and AC on maturation microspore-derived embryos, after three months

ABA in concentration of 2.5 mg L^{-1} had a positive effect on shoot (23 %) formation.

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.

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 %. 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 %) culture.

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 %; **Fig. 4**) culture.

Cytogenetic analysis of androgenic embryos originating from anther and microspore culture was done after a first generation of regenerants (after 3 months) and after 3 years of subculturing. All androgenic embryos after 3 months of maturation treatments from microspore culture were haploid. However, only 10 % androgenic microspore embryos retained haploidity, while 10.5 % were diploid, 73.5 % tetraploid and 6 % octaploid (**Fig. 5**) after 3 years of subculturing.

Discussion

Some of the horse chestnut androgenic embryos were irregular, hypertrophic and had abnormal cotyledons or lacked hypocotyls. This phenomenon is frequently observed in somatic embryos of trees (Perez et al., 1986; Capuana and Deberg, 1997).

Therefore, addition of different concentrations of AC, ABA or PEG in media with horse chestnut androgenic embryos has overcome this problem. Our results about influence of activated charcoal on the *in vitro* androgenesis in correlated 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 androgenic embryos into the plants were achieved on medium with 1 % activated charcoal which is in accordance with the results 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).

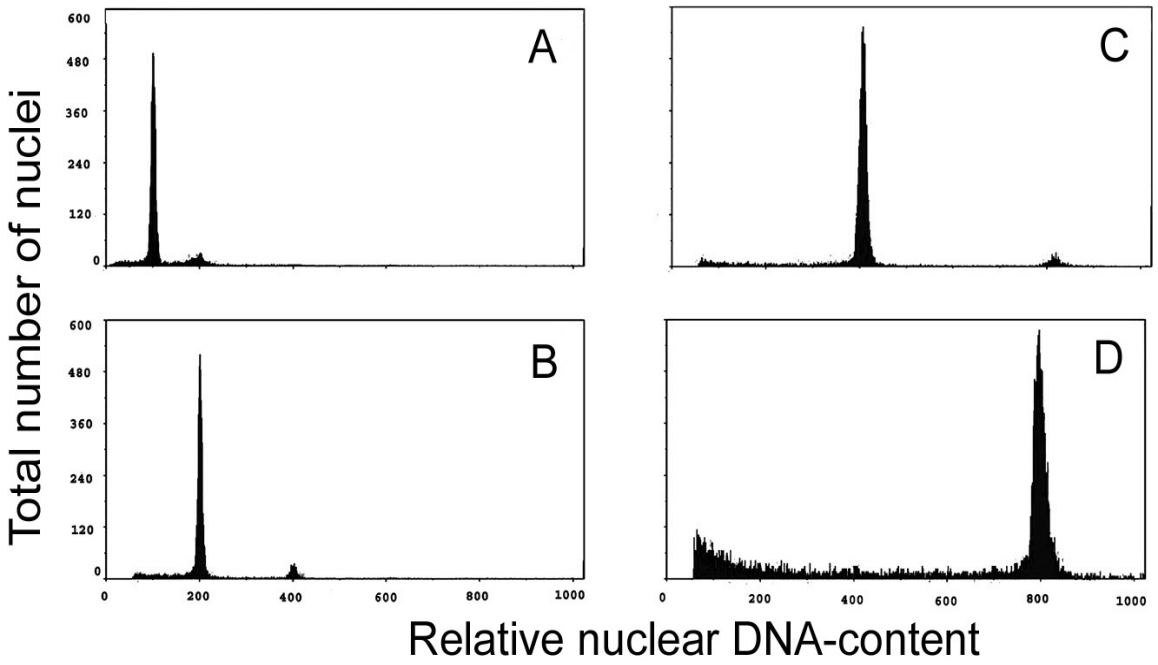

Figure 5. Flow cytometry histograms of haploid (A), diploid (B), tetraploid (C) and octaploid (D) androgenic embryos

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 all.

ABA also promotes the development of globular embryos in embryogenic 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 *Picea glauca* embryos (Attree et al., 1991).

Our results that PEG increased germination of horse chestnut androgenic embryos are in correlation with results Attree et al. (1992) and Capuana and Deberg (1997). Attree et al. (1995) published that drying was essential for subsequent normal growth of *Picea glauca* somatic embryos following maturation on PEG.

The ploidy level of regenerated embryos and plantlets at different growth stages was determined using flow cytometry and chromosome counts.

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 contrary of 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 about improvement of maturation and conversion of horse chestnut androgenic embryo for the diversity protection and conservation.

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