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MARCH 9-10TH, 2010
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A COMPARISON BETWEEN ANTHER CULTURE AND MICROSPORE SUSPENSION CULTURE OF *AESCULUS FLAVA*

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Abstract: *Anthers of A. flava were isolated from disinfected flower buds and cultivated on solid callus induction medium (CIM), containing Murashige and Skoog's (MS) mineral solution with 1 mg/l 2,4 dichlorophenoxyacetic acid (2,4 D) and 1 mg/l 6 furfurylaminopurine (Kin). A half of anthers was kept on solid CIM medium and the other half was macerated 3-5 days later, to create microspore suspension cultures. The microspores released from anthers were suspended in liquid CIM medium, sieved through a 50 μm mesh and shaken on a platform shaker. Eight weeks later, anthers were transferred to regeneration induction medium (RIM) containing MS salts, 0.01 mg/l 2,4 D and 1 mg/l Kin. Microspore suspensions were mixed with equal volumes of cooled RIM and the mixture was dispensed in Petri dishes. Embryo emergence from both types of cultures had been monitored over a 2-month period. The difference in embryogenic potential of anthers positioned along the inflorescence's axis was observed and it declined acropetally. Anthers isolated from basal third of inflorescence (A-anthers) exhibited the highest frequency of embryo formation (36.6%), comparing to anthers from the middle third of inflorescence (B-anthers) and particularly the top third anthers (C-anthers). The embryo average number per anther did not differ significantly between A- and B-anthers, whereas it was significantly lower in C-anthers. The same tendency was observed in microspore suspension cultures. Microspore suspension culture was more efficient method for embryo induction, as it yielded twice more embryos then anther culture. Androgenic embryos obtained by both techniques were maintained and multiplied by repetitive somatic embryogenesis on solid RIM medium.*

Key words: androgenesis, anther culture, microspore suspension, yellow buckeye

1. INTRODUCTION

The members of the genus Aesculus are deciduous ornamental trees or shrubs, commonly planted for shade and ornamental purposes in parks, arboreta and home landscapes. The genus Aesculus (Hippocastanaceae) comprises 13 species distributed throughout the temperate parts of the northern hemisphere. Seven species are native to North America, one species to Europe and five species to Asia. In addition, Aesculus species crossed in nature and were cultivated and bred by man, consequently leading to a number of varieties, cultivars and interspecific hybrids (Chanon, 2005). A. hippocastanum is the only species native to the Balkan Peninsula, but some other members of the genus (A. carnea, A. flava and A. parviflora) are grown as ornamentals in parks of Serbia.

Most temperate trees are characterized by a long reproductive cycle with several years of a juvenile phase. They are highly heterozygous, outbreeding species, propagated asexually or by seeds. For these reasons, their genetic improvement by conventional methods is time-consuming (Höfer and Lespinasse, 1996). Classical breeding may be enhanced and accelerated by exploiting in vitro approaches. Androgenesis is a morphogenic pathway that can be used for this purpose. The process of androgenesis represents a shift from the gametophytic to the sporophytic pathway of microspore development, eventually leading to the formation of a new embryo without fertilization. As microspores undergo recombinations during meiosis, genetic variability of the acquired embryo lines is tremendously high. Haploids obtained this way enables recessive traits to be expressed. As haploid plants are sterile, obtaining of dihaploids, either spontaneously or

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colchicine-induced, is essential. By using this techniques, homozygous plants may be obtained in a few months.

Androgenesis can be achieved by anther culture or isolated microspore culture. Although anther culture is less laborious method than microspore suspension culture, the latter has a number of advantages. The main disadvantage of anther culture is the coincidence of callus and embryo formation from somatic anther tissue, leading to the formation of a mixed population of diploids and haploids. Furthermore, microspore suspension allows more accurate and highly controlled experimentation for studying both fundamental and applied aspects of androgenesis.

Up to date, androgenesis was induced from anther culture of horse chestnut (Radojević, 1978), red horse chestnut (Radojević et al., 1989) and yellow buckeye (Ćalić et al., 2005a). This study aimed at the establishment of A. flava microspore suspension culture and its comparison to anther culture in terms of the efficiency of androgenic embryo production.

2. MATERIALS AND METHODS

Plant material

Inflorescences were collected from A. flava tree growing in the Botanical Garden "Jevremovac", University of Belgrade. The inflorescences were washed with running water and a few drops of detergent and sterilized by immersing in 96% and 70% ethanol for 3 min and 5 min, respectively and rinsed with plenty of sterile deionisated water. Sterilized inflorescences were divided in thirds: the basal third designated A-anthers, the middle B-anthers, and the top C-anthers. Anthers were isolated under aseptic conditions from closed flower buds of 4 5 mm and immediately placed on solid callus induction medium (CIM), containing MS (Murashige and Skoog, 1962) mineral salts, 1 mg/l 2,4 dichlorophenoxyacetic acid (2,4 D) and 1 mg/l 6 furfurylaminopurine (Kin).

Basal medium

The media contained MS mineral solution and 20 g/l sucrose, 100 mg/l myo-inositol, 200 mg/l casein hydrolysate, 2 mg/l thiamine, 5 mg/l nicotinic acid and 2 mg/l adenine, 10 mg/l panthotenic acid. The media were gelled with 0.7% (w/v) agar (unless is differently specified), and pH was adjusted to 5.8 before sterilization. Media were sterilized by autoclaving at 114ºC for 25 min.

Anther culture and microspore suspension establishment

Anthers were kept on CIM in darkness for eight weeks and then transferred to solid regeneration induction medium (RIM) containing MS mineral salts and 0.01 mg/l 2,4 D + 1 mg/l Kin.

The anthers that were used for microspore suspension initiation were incubated on solid CIM for 3-5 days, and then noncontaminated anthers were picked out, macerated with a sterile blade, suspended in 50 ml of liquid CIM and sieved through a 50 □m mesh. The cultures were shaken on a platform shaker (85 rpm) in darkness for eight weeks. Cell suspensions were then mixed with equal volumes of cooled RIM medium (30 °C) with 0.8% agar and dispensed in Petri dishes (5 plates per a suspension culture). The cultures were maintained under cool white fluorescent tubes with a photon flux density of 55 □mol m-2 s-1 and a 16 h day length at 25 ± 2ºC.

Recordings and statistical analysis

All cultures were placed in a completely randomized design. For anther culture, the experiment was performed in three replicates with 3 samples (Petri-dishes) and 20 subsamples

(anthers) for each inflorescence segment (n = 180). For microspore suspension culture, twenty anthers were used per a suspension, and three suspensions were prepared per each segment. The experiment was performed in triplicate (n = 9).

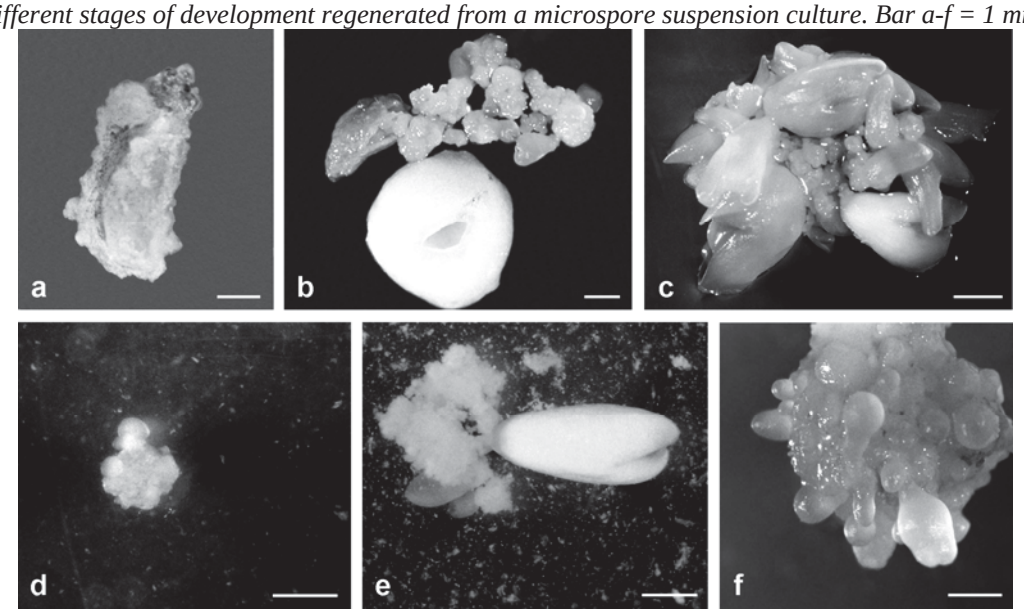
The callus formation rate was recorded after eight weeks of anther culture and the embryo appearance had been monitored over a 2-month period upon the transfer of anthers to RIM, without subcultivation. The frequency of anthers differentiating embryos and the number of embryos per anther were recorded in 2-week intervals, with the aid of a stereomicroscope. For suspension cultures, the number of embryogenic clusters and the number of embryos per cluster have been monitored in 2-week intervals after suspensions' planting over a 2-month period.

All percentage data were subjected to angular transformation before analysis. The data were subjected to standard analysis of variance (ANOVA) and the means were separated using Duncan's test at $P \leq 0.05$. For the presentation, the percentage data were inverse-transformed.

3. RESULTS

Anthers cultivated on CIM turned dark yellow and swelled (Fig. 1a). The frequency of callus formation ranged from 45% to 63% (Table 1) and the differences in response to CIM treatment among A-, B and C anthers were not significantly different (p= 0.5542). Upon transfer to RIM, the anthers burst to release androgenic embryos and embryogenic callus (Fig. 1b).

Figure 1 (a-f). *Sequential phases of androgenic embryo differentiation from A. flava anther culture and microspore suspension culture. (a) Anther forming callus after 8 weeks on callus induction medium (CIM). (b) Androgenic embryos released from burst anther after 4 weeks on regeneration medium (RIM). (c) Androgenic embryos regenerated from anther at the cotyledonary stage of development. (d) Androgenic embryos regenerating from microcallus that was formed from a microspore suspension after 8 weeks on CIM and two weeks on RIM. (e) An androgenic embryo at cotyledonary stage of development after four weeks on RIM. (f) Androgenic embryos at different stages of development regenerated from a microspore suspension culture. Bar a-f = 1 mm.*



First embryos were observed two weeks following the transfer to RIM. They completed development on this medium (Fig. 1c) and even germinated. The highest embryogenic response (36.6%) was observed in A anthers, and decreased in B and particularly in C anthers. However, the mean embryo number per anther was not significantly different between A- and B-anthers, while in C anthers it was significantly lower. The overall embryo number obtained from A anthers was three times higher then from B anthers (Table 1).

In microspore semisolid culture, new cell aggregates were observed ten days after suspension planting, and five days later first androgenic embryos emerged from microcalli (Fig. 1d). One to two weeks later the embryos at the cotyledonary stage of development were observed (Fig. 1e) and they multiplied further on (Fig. 1f). As the microspores were suspended, the embryogenic clusters were numerous, reaching 111.67 clusters per suspension derived from A anthers (Table 2). However, the mean number of embryos per cluster was rather low (1.57-5.96 on average). As was observed in anther culture, the embryogenic response of microspore declined from A- to C anthers. In addition, the mean embryo number was not significantly different between A and B microspores, but it was also significantly higher than in C microspores (Table 2).

Table 1. *Callus formation frequency of anthers isolated from A, B and C inflorences' segments of A. flava after 8 weeks of cultivation on solid MS medium supplemented with 1 mg/l 2,4 D + 1 mg/l Kin and androgenic embryo differentiation from A. flava anthers during 8-week culture on a medium for the induction of regeneration (0.01 mg/l 2,4 D + 1 mg/l Kin).*

Segment	Callusing anthers (%) Mean ± SE	Time (weeks)	Embryogenic anthers (%) *Mean ± SE*	Embryos per anther Mean ± SE	Overall embryo number
A	50.1 ± 1.0a	2	10.6 ± 0.1 **bc**	18.7 ± 4.3 **ab**	56
		4	17.3 ± 0.4 **abc**	20.2 ± 5.9 **ab**	155
		6	30.2 ± 0.2 **ab**	25.8 ± 4.7 **ab**	182
		8	36.6 ± 0.6 **a**	30.3 ± 6.1 **ab**	333
B	44.9 ± 0.2 a	2	3.2 ± 3.2 **c**	12.0 ± 0 **ab**	12
		4	3.2 ± 3.2 **c**	25.0 ± 0 **ab**	45
		6	11.2 ± 0.4 **abc**	33.0 ±13.4 **ab**	66
		8	16.8 ± 1.0 **abc**	31.3±13.2 **ab**	94
C	62.9 ± 0.3 a	2	0	0 **c**	0
		4	0	0 **c**	0
		6	0	0 **c**	0
		8	4.4 ± 4.4 **c**	2.0 ± 0 **c**	2

Data in the Table present means ± standard error (SE). The sample size was n = 180 for each inflorescence segment. Treatments denoted by the same letter in a column are not significantly different ($P \leq 0.05$) according to Duncan's test.

Table 2. *Androgenic embryo differentiation from A. flava microspore suspension culture, during eight-week period cultivation on solid MS medium containing 0.01 mg/l 2,4 D and 1 mg/l Kin.*

Segment	Time (weeks)	Embryogenic cluster per suspension Mean ± SE	Embryos per cluster Mean ± SE	Overall embryo number
A	2	1.67 ± 0.44 **b**	2.00 ± 0.32 **ab**	11
	4	22.00 ± 5.79 **b**	3.13 ± 0.70 **ab**	89
	6	26.33 ± 5.99 **b**	3.32 ± 0.48 **ab**	111
	8	111.67 ± 30.75 **a**	5.96 ± 0.97 **a**	510
B	2	3.50 ± 0.67 **b**	1.57 ± 0.30 **ab**	15
	4	10.00 ± 2.24 **b**	2.80 ± 0.54 **ab**	35
	6	12.00 ± 2.68 **b**	3.12 ± 0.57 **ab**	41
	8	23.00 ± 5.81 **b**	5.37 ± 0.95 **ab**	173
C	2	0 **c**	0	0
	4	0 **c**	0	0
	6	0 **c**	0	0
	8	1.0 ± 0.22 **c**	1.0 ± 0 **b**	1

Data in the Table present means ± standard error (SE). The sample size was n = 9 for each inflorescence segment. Treatments denoted by the same letter in a column are not significantly different ($P \leq 0.05$) according to Duncan's test.

As the initial sample size was the same for both the techniques used, we compared the embryo yield from anther culture and suspension culture. Although ANOVA showed no significant difference ($p = 0.947$) between the two ways of embryo acquisition, the overall embryo number was twice higher from the microspore suspension culture as compared to anther culture (Tables 1, 2).

Androgenic embryos originating from both anther and microspore suspension culture were maintained and multiplied by secondary (also called repetitive or recurrent) somatic embryogenesis. The somatic embryo cultures are still available, four years after androgenic embryo initiation. Flow cytometry analysis revealed that majority of tested embryos were diploid (not shown).

4. DISCUSSION

We reported here on efficient and reproducible embryo regeneration from both anther culture and microspore suspension culture of A. flava. In a previous study we reported on successful embryo regeneration from anther culture of A. flava (Ćalić et al., 2005a). In this experiments the regeneration system was improved further by employing microspore suspension technique.

One of decisive factors influencing the efficiency of androgenesis is the stage of microspore development. However, for its routine identification it is essential to determine an associated morphological marker. For efficient androgenesis in A. flava the uninuclear stage of microspore development was optimal, and the associated morphological marker was the flower bud length of 4-5 mm. It was demonstrated that the majority of A. flava microspores isolated from flower buds of this size were at the uninuclear stage of development (Ćalić-Dragosavac et al., 2009). These findings are in agreement with results obtained in A. hippocastanum (Radojević, 1978) and A. carnea (Radojević et al., 1989), as well as for many woody species (Höfer and Lespinasse, 1996).

Embryogenic response of A. flava anthers was rather high (up to 36.6 %) and comparable to other members of the genus. In A. carnea Radojević et al. (1989) obtained up to 38% and in A. hippocastanum 52.7 % (Radojević, 1991) of embryogenic anthers. By contrast, Höfer and Lespinasse (1996) reported on rather low the frequency of embryogenic anthers (up to 6.4 %) in apple.

In this study we obtained a gradient of embryogenic capacity along the inflorescence axis. The embryogenic capacity declined from A to C anthers. Our findings are in agreement with the results of Radojević et al. (2000), who reported on the same phenomenon in A. hippocastanum anther culture. Flowers of A , B and C segments of A. hippocastanum, although being all bisexual, differ physiologically (Radojević et al., 2000), so that C flowers never set seeds (Heywood, 1978).

Microspore suspension culture was more efficient method for embryo induction in A. flava, as it yielded twice more embryos then anther culture. The same result was obtained in apple by Höfer et al. (1999), and in horse chestnut it was even more pronounced (Ćalić et al., 2003).

Androgenic embryos obtained in this study by both the techniques were healthy and able for further development, although some malformations were also noted. They readily developed until late cotyledonary stage of development and even germinated on RIM medium. Embryos multiplied by secondary somatic embryogenesis, enabling the maintenance of acquired embryogenic lines for a long period of time. This feature is well known for A. hippocastanum (Ćalić et al., 2005b) and A. carnea (Zdravković-Korać et al., 2008). Formation of diploid embryos, as was shown by flow cytometry, may point to spontaneous diploidization during

culturing, at least for those formed from microspore suspension, as no somatic cells could be present after filtration through a 50 □m mash. Spontaneous polyploidization was demonstrated in androgenic embryos of horse chestnut (Ćalić-Dragosavac et al., 2006), even on plant growth regulator free medium.

To conclude, an efficient and reproducible method for androgenic embryo induction was developed in A. flava. The efficiency of this process was doubled by employing microspore suspension culture. Obtained embryos were healthy and able to develop further. Further studies of molecular markers suitable for homozygote/heterozygote discrimination are needed and are in progress in our lab.

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