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BRIEF COMMUNICATION

Secondary embryogenesis in androgenic embryo cultures of *Aesculus hippocastanum* L.

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Abstract

Secondary somatic embryos appeared on the cotyledons and radiculi of embryos derived from suspension and anther cultures of *Aesculus hippocastanum* L. The highest number of secondary somatic embryos formed on a hormone-free medium.

Additional key words: androgenesis, horse chestnut.

Secondary somatic embryogenesis has been described for several species and is the basis of embryo cloning. It gives somatic embryogenesis a multiplicative potential for clonal mass propagation (Merkle 1995). Secondary somatic embryogenesis on *Aesculus hippocastanum* androgenic embryos has been mentioned in several articles (Radojević 1988, 1995, Jörgensen 1989) and was explained in detail by Kiss *et al*. (1992).

Our experiments aimed to evaluate the induction of secondary somatic embryogenesis and plant regeneration of androgenic embryos of *Aesculus hippocastanum* in order to develop efficient *in vitro* regeneration methods to be used in genetic transformation experiments applicable in the pharmaceutical industry.

Horse chestnut (*Aesculus hippocastanum* L.) androgenic embryos originating from uninuclear microspore and anther cultures were obtained using a previously described protocol for embryo induction (Radojević *et al*. 1999, Ćalić *et al*. 2003/4). The medium for androgenic embryo induction contained 1.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1 mg dm^{-3} kinetin (Kin), while the medium for embryo multiplication was supplemented with 0.01 mg dm^{-3} 2,4-D and 1.0 mg dm^{-3} Kin (Ćalić *et al*. 2003/4). The influence of activated charcoal (AC), abscisic acid (ABA), polyethylene glycol (PEG-4000) and mannitol (Man) on the secondary somatic embryo induction and plant maturation was investigated (data not published). The formed androgenic embryos of horse chestnut were cultured on solid media for embryo maturation supplemented with AC, ABA, PEG and Man (see Table 1). All media for embryo maturation contained 7 g dm^{-3} agar, Murashige and Skoog's (MS) mineral solution, 2 % sucrose and were supplemented with the following substances [mg dm^{-3}]: panthothenic acid 10.0, nicotinic acid 5.0, vitamin B_1 2.0, adenine sulphate 2.0, myo-inositol 100 and casein-hydrolysate 200 and glutamine (Glu) 400. Filter sterilized Glu was added to improve embryo maturation in the later stages of development. The following adjuvents were tested separately: AC (1, 5 or 10 g dm^{-3}); ABA (2.5, 10.0 or 20.0 mg dm^{-3}); PEG-4000 (5, 25 or 50 g dm^{-3}) or mannitol (30 or 60 g dm^{-3}), (media A_1 - D_2, Table 1). Various combinations of the same substances were also tested, namely: ABA (2.5 or 20.0 mg dm^{-3}) and AC (1 g dm^{-3}); ABA (2.5 or 20.0 mg dm^{-3}), AC (1 g dm^{-3}) and PEG (5 or 50 g dm^{-3}); PEG (5 or 50 g dm^{-3}) and AC (1 g dm^{-3}), media E_1 - G_2. (Table 1).

All media were sterilized by autoclaving at 0.9×10^5 kPa and 114 °C for 25 min. Suspension cultures of

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Abbreviations: ABA - abscisic acid; AC - activated charcoal; AE - androgenic embryos; 2,4-D - 2,4-dichlorophenoxyacetic acid; Glu - glutamine; Kin - kinetin; Man - mannitol; MS medium - Murashige and Skoog's (1962) medium; PEG - polyethylene glycol.

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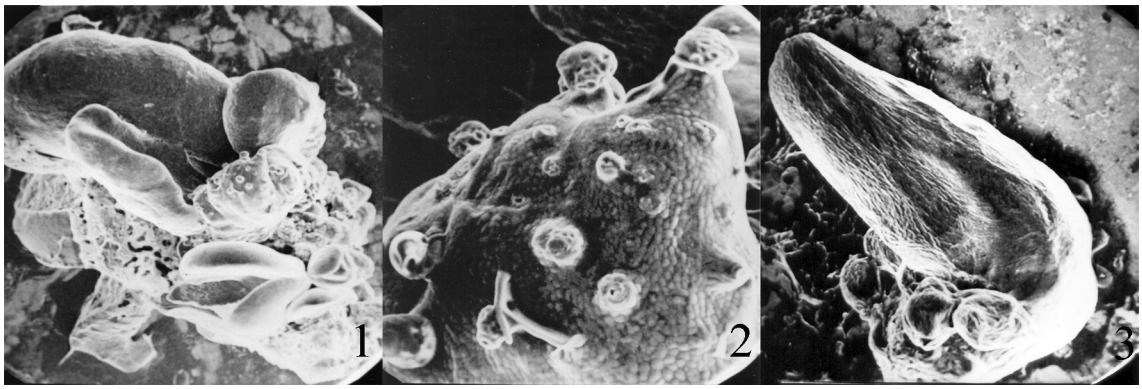
uninuclear microspores were grown on a horizontal shaker (85 rpm) at 25 ± 1 °C temperature for 4 weeks in the dark, while solid cultures were grown under the same temperature, 16 h photoperiod and irradiance of 33 - 45 μmol $m^{-2}s^{-1}$, produced by cool white fluorescent tubes. Each experiment, involving about 20 - 30 androgenic embryos/per medium, derived from suspension or anther culture, was repeated three times. A great number of horse chestnut androgenic embryos were irregular, hypertrophied, with abnormal cotyledons or absent hypocotyl. Similar morphological abnormality has also been frequently observed in the somatic embryos of some other tree species (Pérez *et al*. 1986, Capuana and Debergh 1997). However, ABA supplement to the medium helps overcome this problem. Secondary embryos were formed after 30 and 60 d on the radiculi



Figs. 1 - 3. Scanning electron microscopy micrographs of androgenic embryo at late cotyledonary stage of development forming secondary somatic embryos at cotyledon surface (Fig. 1; ×15), secondary somatic embryos emerging from the primary embryo cotyledon (detail from the previous picture (Fig. 2; ×100), secondary somatic embryos on the radiculi of androgenic embryo of *A. hippocastanum* (Fig. 3; ×20).

Table 1. Effect of different media on the induction of secondary somatic embryos of *A. hippocastanum* derived from suspension and anther cultures following subculture over 30 or 60 d. Percentage of secondary somatic embryos (% ± SE); 70 parental embryos were evaluated.

Medium	Secondary somatic embryos [%]			
	suspension culture		anther culture	
	30 d	60 d	30 d	60 d
A_0 = MS+Glu 400 mg dm^{-3}	100.0 ± 9.1	100.0 ± 8.6	100.0 ± 8.9	100.0 ± 9.2
A_1 = A_0+AC 1 g dm^{-3}	76.8 ± 7.3	83.0 ± 7.8	54.5 ± 5.4	42.8 ± 4.1
A_2 = A_0+AC 5 g dm^{-3}	71.6 ± 7.4	68.0 ± 6.3	22.7 ± 1.9	22.9 ± 2.3
A_3 = A_0+AC 10 g dm^{-3}	53.7 ± 4.8	52.0 ± 4.9	9.1 ± 0.8	14.3 ± 1.3
B_1 = A_0+ABA 2.5 mg dm^{-3}	5.3 ± 0.6	17.0 ± 1.9	13.6 ± 1.5	11.4 ± 1.3
B_2 = A_0+ABA10.0 mg dm^{-3}	3.2 ± 0.2	11.0 ± 1.2	9.1 ± 1.1	8.6 ± 0.9
B_3 = A_0+ABA 20.0 mg dm^{-3}	2.1 ± 0.3	7.0 ± 0.8	4.5 ± 0.5	2.9 ± 0.3
C_1 = A_0+PEG 5 g dm^{-3}	11.6 ± 1.3	15.0 ± 1.7	9.1 ± 1.1	11.4 ± 1.3
C_2 = A_0+PEG 25 g dm^{-3}	27.4 ± 2.9	31.0 ± 3.4	27.3 ± 2.9	20.0 ± 2.2
C_3 = A_0+PEG 50 g dm^{-3}	33.0 ± 3.5	33.0 ± 3.4	59.1 ± 6.1	51.4 ± 6.0
D_1 = A_0+Man 30 g dm^{-3}	1.1 ± 0.1	1.0 ± 0.1	4.5 ± 0.5	2.9 ± 0.3
D_2 = A_0+Man 60 g dm^{-3}	0	0	0	0
E_1 = A_0+ABA 2.5 mg dm^{-3}+AC 1 g dm^{-3}	3.2 ± 0.4	6.0 ± 0.7	13.6 ± 1.4	11.4 ± 1.2
E_2 = A_0+ABA 20.0 mg dm^{-3}+AC 1 g dm^{-3}	1.0 ± 0.1	1.0 ± 0.1	4.5 ± 0.5	2.9 ± 0.3
F_1 = A_0+ABA 2.5 mg dm^{-3}+AC 1 g dm^{-3}+PEG 5 g dm^{-3}	5.3 ± 0.6	5.0 ± 0.6	9.1 ± 1.0	8.6 ± 1.0
F_2 = A_0+ABA 20.0 mg dm^{-3}+AC 1 g dm^{-3}+PEG 5 g dm^{-3}	20.0 ± 2.2	19.0 ± 2.1	9.1 ± 1.1	5.7 ± 0.7
F_3 = A_0+ABA 2.5 mg dm^{-3}+AC 1 g dm^{-3}+PEG 50 g dm^{-3}	9.5 ± 1.0	11.0 ± 1.3	9.1 ± 1.1	8.6 ± 1.0
F_4 = A_0+ABA 20.0 mg dm^{-3}+AC 1 g dm^{-3}+ PEG 50 g dm^{-3}	5.3 ± 0.6	8.0 ± 0.9	4.5 ± 0.5	2.9 ± 0.3
G_1 = A_0+PEG 5 g dm^{-3}+AC 1 g dm^{-3}	1.1 ± 0.1	4.0 ± 0.4	4.5 ± 0.5	5.7 ± 0.6
G_2 = A_0+PEG 50 g dm^{-3}+AC 1 g dm^{-3}	8.4 ± 0.9	9.0 ± 1.0	40.9 ± 4.2	31.4 ± 3.3

and cotyledons of androgenic embryos grown on the hormone-free germination medium (A_0) and all media for embryo maturation (A_1 - G_2). Absence of secondary embryogenesis on the cotyledons of somatic embryos has been reported for several species, such as *Aesculus hippocastanum* (Profumo *et al*. 1986), *Quercus robur* and *Tilia cordata* (Chalupa 1990). In contrast to these authors, Radojević (1995) has noticed that secondary somatic embryos rarely appeared on the cotyledones of somatic embryos derived from the zygotic embryo cultures of *Aesculus hippocastanum*. The somatic embryos in *Picea omorika* culture also originated from superficial cotyledon cells (Budimir 2003/4). In contrast to Profumo *et al*. (1986), we confirmed the presence of secondary somatic embryos on the cotyledons of androgenic embryos of horse chestnut using scanning microscopy (Figs. 1,2,3). Most of the secondary somatic embryos of *Aesculus hippocastanum* were obtained from a medium without hormones (Table 1). With increased concentrations of AC, ABA and Man, there was a slight decrease in their numbers, while increased concentrations of PEG resulted in a slight increase in the number of secondary somatic embryos (Table 1). Media with AC stimulated the production of secondary embryos originating from the suspension, as compared to those originating from anther culture (Table 1).

Under appropriate conditions, the epidermal and sub-epidermal cells of the cotyledons and hypocotyls of androgenic horse chestnut plantlets have been shown to give rise to secondary somatic embryos without passing through a callus phase (Radojević 1995). The largest number of secondary embryos was derived from the solid and suspension anther cultures without supplemented growth regulators (Radojević *et al*. 1999). Somatic embryos of cork oak (*Quercus suber* L.) have also been shown to induce secondary somatic embryos on a medium that lacks plant growth regulators (El Maâtaoui *et al*. 1990, Fernández-Guijarro *et al*. 1995). However, a large number of secondary embryos appeared all around the hypocotyl of the embryo axis but not on the cotyledons (Puigderrajols *et al*. 2000). The formation of secondary embryos of cork oak by meristematic budding began by cell divisions in the outer cell layers of the hypocotyl, which led to the formation of a compact proliferation mass (Puigderrajols *et al*. 2001). Somatic embryo formation has been observed on the hypocotyl surface of immature (Salajová *et al*. 1996) and mature *Abies alba* × *Abies cephalonica* zygotic embryo explants (Salaj and Salaj 2003/4). The species which is the best known producer of secondary embryogenesis is *Juglans regia* (Tulecke and McGranahan 1985). This method may be appropriate in cases where hundreds or thousands of vegetatively cloned embryos are needed for vegetative propagation or synthetic seed production. One of the advantages of this method is the short period required for a mass multiplication of *in vitro* embryos (Chalupa 1990). The origin of embryos is especially relevant to the genetic uniformity of regenerated plants, as multicellular origin may result in the formation of genetically variable chimeric plants. Unicellular origin is therefore a preferred pathway for practical applications of embryo cloning, such as genetic transformation.

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