BIOLOGIA PLANTARUM 49 (3): 431-433, 2005

BRIEF COMMUNICATION

The effect of low temperature on germination of androgenic embryos of *Aesculus hippocastanum* L.

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Abstract

Treating androgenic embryos of *Aesculus hippocastanum* L. with low temperatures (6 °C) improved their germination and regeneration into plantlets. The embryos derived from anther cultures showed better results than those derived from microspore cultures.

Additional key words: anther culture, horse chestnut, microspore culture.

Low temperature treatment improved the germination of somatic embryos of horse chestnut plants (Profumo 1991). However, in the same species androgenic embryos from anther (Radojević 1978, 1991, Ćalić *et al*. 2003) and microspore cultures (Radojević *et al*. 1998, Ćalić *et al*. 2003/4) showed low germination at the cotyledonary stage. The objective of this report was to improve the germination of androgenic embryos by treatment with low temperatures.

Immature flower buds (4 mm long) were collected from a 110-year-old horse chestnut (*Aesculus hippocastanum* L.) tree growing in the Jevremovac Botanical Gardens of the Belgrade University. Flower buds with anthers containing uninucleate microspores were sterilized, the anthers were isolated and cultivated according to the previously elaborated procedure (Radojević 1978, Radojević *et al*. 1998). The basal medium containing Murashige and Skoog (1962; MS) mineral solutions and 2 % sucrose was supplemented with [mg dm^{-3}]: pantothenic acid 10.0, nicotinic acid 5.0, vitamin B_1 2.0, adenine sulphate 2.0, myo-inositol 100, and casein-hydrolysate 200. We isolated approximately seven anthers from each bud. The anthers were cultured in tubes containing MS_1 solid medium supplemented with 2,4-dichlorphenoxyacetic acid (2,4-D) and kinetin (Kin), 1.0 mg dm^{-3} each, while the uninuclear microspores were cultured in MS_2 liquid medium containing the same amount of both hormones (Ćalić *et al*. 2003). One hundred transverse-cut anthers with uninucleate microspores were placed in Erlenmeyer flasks containing 100 cm^3 of MS_2 medium. The liquid cultures were filtered (200 μm) to obtain a suspension. The microspore suspension was subcultured every month refreshed MS_2 medium. After 2 months, the microspore suspension was plated by Bergmann technique (1960) on a MS solid medium with a reduced concentration of 2,4-D (MS_3 = MS + 0.01 mg dm^{-3} 2,4-D + 1.0 mg dm^{-3} Kin). Microspores in both the anther culture and suspension culture were found to have a capacity for androgenesis. Seven days after the plating of the suspension, microspore division occurred and two-, three- and four-celled embryogenic pollen grains appeared. Rapid differentiation and asynchronous development of androgenic embryos were achieved *in vitro* over the following weeks, producing globular, heart-like and torpedo-like embryos, and having different cotyledone numbers. The multiplication of androgenic embryos from the anther and suspension cultures followed as embryo development on MS_3 medium. After the multiplication, the embryos were cultured on a germination medium (MS_4 =MS + glutamine 400 mg dm^{-3}).

Sterilization of all media was performed by autoclaving at 0.9×10^5 kPa and 114 °C for 25 min. Suspension cultures were grown on a horizontal shaker (85 rpm) at 25 ± 1 °C for one month, in the dark, while the other cultures were grown at an identical temperature

Received 24 February 2004, *accepted* 21 April 2005.
Abbreviations: 2,4-D - 2,4-dichlorphenoxyacetic acid; Kin - kinetin; MS - Murashige and Skoog.
Acknowledgement: This work was supported by the Ministry of Science, Technology and Development of Serbia, grant No. 1573.
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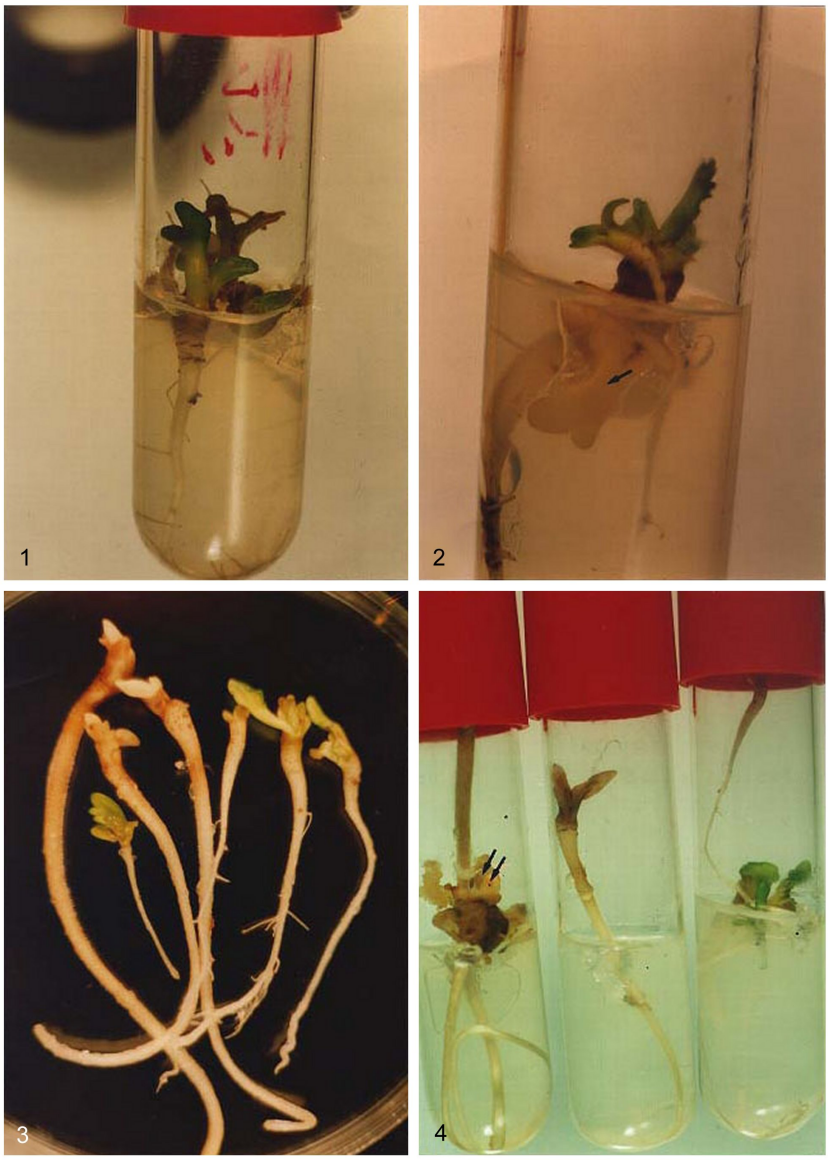
and a 16-h photoperiod. Light was provided by fluorescent lamps supplying a photon flux density of 35 μmol $m^{-2}s^{-1}$. The experiment was repeated 4 times. We analysed 1000 microspore-derived embryos and the same number of embryos derived from anther culture.

Embryos derived from microspore and anther culture with completely formed cotyledons were grown on MS_4 medium and in cold storage at 6 °C, during 4 and 6 months. Adventive embryos were noticed on the roots (Figs. 2, 4).

Embryos that germinated after chilling were transferred to the same medium (MS_4) and grown for one month at 25 ± 1 °C and a 16-h photoperiod.

Low temperature treatment over a period of 6 months had the best effect on the elongation of roots and total growth of plantlets (Table 1). The increase in root and plantlet length was significantly lower in the microspore suspension than in the anther culture (Table 1, Figs. 1 - 4). Our results correspond to the findings of Profumo *et al*. (1991) on the chilling of *A. hippocastanum* somatic embryos. Chilling androgenic embryos for 6 months was more efficient in inducing germination and regeneration into plantlets than when embryos were chilled for only 4 months (Table 1). We noticed that chilling exceeding



Figs. 1 - 2. Germinating primary embryos derived from microspore suspension culture with secondary embryos (*arrow*) growing on the MS_4 medium (×3).
Figs. 3 - 4. Germinating androgenic embryos originating from anther culture and the same type embryos with secondary embryos (*two arrows*) on the MS_4 medium (×3).

6 months was more efficient for the germination of embryos derived from anther culture, while the recovery of embryos into plantlets showed no significant difference between embryos originating from anther culture and those from a suspension of uninuclear microspores. This method of chilling androgenic embryos will be applicable in resolving the problem of dormancy.

Table 1. The influence of chilling for 4 or 6 months on the increase in root and plantlet length and the percentage of germination and regeneration of plantlets originating from anther and microspore cultures of *A. hippocastanum* L. Means ± SE, n = 1000. In each row, values marked with different letters are significantly different at a 0.05 probability level according to the protected LSD test.

Parameter		Anther culture	Suspension culture
Root length [mm]	control	29.1 ± 2.4a	33.9 ± 4.8a
	4 month	137.3 ± 14.6a	94.5 ± 10.2b
	6 month	231.5 ± 24.8a	196.5 ± 20.1b
Shoot length [mm]	control	12.2 ± 1.3a	11.7 ± 1.4a
	4 month	16.4 ± 1.7a	13.6 ± 1.4b
	6 month	38.4 ± 4.1a	26.3 ± 2.9b
Germination [%]	4 month	47.6 ± 5.1b	31.2 ± 3.8a
	6 month	76.6 ± 8.2b	50.8 ± 5.3a
Plantlets [%]	4 month	7.2 ± 0.8a	6.3 ± 0.7a
	6 month	9.6 ± 1.0a	8.1 ± 0.9a

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