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SUSTAINABLE USE OF FOREST ECOSYSTEMS
The Challenge of the 21st Century

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SECONDARY REGENERATION FROM SOMATIC EMBRYOS OF *AESCULUS CARNEA* HAYNE

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Abstract: *Somatic embryos of red horse chestnut were subjected to an induction of secondary regeneration. The embryos were divided in 4 classes on the basis of their size (I-1, II-5, III-10 and IV-30 mm), and sub-cultivated on MS media containing 0, 1, 5 or 10 μM kinetin (Kin) or benzyladenine (BA). The pathway of secondary regeneration (somatic embryogenesis or caulogenesis) depended solely on the primary somatic embryo (PSE) stage of development. The PSE of the I and II class produced solely secondary somatic embryos (SSE), the III class PSE formed SSE on media containing Kin, and both SSE and adventitious buds on media containing BA, whereas the IV class PSE developed almost solely adventitious buds. BA promoted bud induction at much higher rate than Kin and slightly higher embryogenic response. The histological study confirmed these findings.*

Key words: adventitious buds, red horse chestnut, secondary somatic embryos

1. INTRODUCTION

Somatic embryogenesis is the process of an embryo formation from a somatic cell(s). Somatic embryos (SE) induced from primary somatic embryos (PSE) are called secondary somatic embryos (SSE) or adventive embryos. The process is usually repetitive and thus also called repetitive or recurrent somatic embryogenesis. This character enables embryogenic capacity maintenance by repeated cycles of secondary embryogenesis over a long period of time (Raemarkes *et al*., 1995). Secondary somatic embryogenesis has been reported in a number of woody species, including related horse chestnut (Dameri *et al*., 1986; Radojević, 1988; Kiss *et al*., 1992; Jörgensen, 1989). The influence of plant growth regulators (PGR) on horse chestnut secondary somatic embryogenesis was studied in details using scanning electron microscopy by Kiss *et al*. (1992) and Ćalić *et al*. (2005). Kiss *et al*. (1992) found a distinct correlation between the embryo-forming capacity and the PSE size. They found that the PSE of 8-10 mm were optimal for SSE induction, whereas those reaching 12-14 mm lost the SSE forming capacity. The process was independent of the origin of the PSE (zygotic or somatic). The presence of adventitious buds was not reported. Another morphogenetic pathway, caulogenesis, is the process of adventitious bud formation. As it demands a root-inducing step, this kind of regeneration is assumed to be inferior to somatic embryogenesis. However, somatic embryogenesis and caulogenesis are frequently simultaneous within the same explant (Budimir, 2003/4).

In *Aesculus carnea* androgenesis through anther culture and subsequent haploid plant regeneration were achieved (Radojević *et al*., 1989). The aim of the current study was to examine

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a correlation between the stage of PSE development and the process of secondary regeneration (somatic embryogenesis and caulogenesis).

2. MATERIALS AND METHODS

SE of *A. carnea* were induced from androgenic embryos obtained according to previously described procedures for *A. carnea* anther culture (Radojević *et al*., 1989) and *A. hippocastanum* microspore suspension culture (Ćalić *et al*., 2003/4). The SE cultures were maintained through repetitive somatic embryogenesis on solid MS medium containing 0.01 mg/L 2,4-D + 1 mg/L Kin.

The basal medium contained the following: MS mineral solution (Murashige and Skoog, 1962), 2 % sucrose, 0.7 % agar, 100 mg/L myo-inositol, 200 mg/L casein hydrolysate, 2 mg/L thiamine, 2 mg/L adenine, 5 mg/L nicotinic acid, 10 mg/L panthotenic acid. The cultures were maintained under cool white fluorescent tubes with fluency rate of 33-45 μmol m^{-2} s^{-2} and a 16 *h* photoperiod, at 25 ± 2 °C. The media were sterilized by autoclaving at 114 °C for 25 min.

The PSEs of different size at different stage of development (globular, cotyledonary, germinating embryos) were used for the current experiment. They were divided in 4 classes based on their size (I-1, II-5, III-10 and IV-30 mm), and sub-cultivated on MS media containing 0, 1, 5 or 10 μM Kin or BA. At least thirty PSE were used for each treatment, in six replicates with five sub-samples. Experimental design was completely randomized block design. The numbers of SSE and adventitious buds were recorded after 4 weeks by dissecting the PSE and counting the number of secondary regenerants (SSE or adventitious buds) under stereomicroscope. The results are expressed as the percentages of PSE forming secondary regenerants and the mean number of secondary regenerants per PSE.

3. RESULTS AND DISSCUSION

SE of *A. carnea* were induced from androgenic embryos. The SE cultures have been maintained through repetitive adventive embryogenesis on solid MS medium containing 0.01 mg/L 2,4-D + 1 mg/L Kin for a few years. Adventitious buds were never noticed. When germinating SE were placed on media containing BA, the occurrence of adventitious buds was apparent. We tested the effects of media containing Kin or BA at 0, 1, 5 and 10 μM and found no differences in the type of regeneration present. However, when we utilized embryos of different size, the differences in the morphogenic pathway became apparent. The smallest PSE (I and II class, 1 and 5 mm) produced solely SSE, the III class PSE (10 mm) formed SSE on media containing Kin, and both SSE and adventitious buds on media containing BA, whereas the IV class PSE (30 mm) developed almost solely adventitious buds. This is in agreement with Kiss *et al*. (1992) who reported on the loss of the embryo-forming capacity of PSE bigger than 12-14 mm in horse chestnut. When the I and II class SE were placed on the induction media, only a slight proliferations of epidermal layer could be seen during the first two weeks. The highest activity was in the radicle, especially at the base and the tip of radicle (Figs. 1 and 2). These proliferations usually peeled off, and SSE emerging from them could be seen all around the PSE within the next week. The same region (radicle) was the most active in the IV class PSE, although in this case adventitious buds were formed (Fig. 3). Within the IV class PSE, activity of the superficial layer became apparent after a few days. Epidermis burst, but no visible callus was formed. Green regenerants were observed after ten days (Fig. 4), mainly on the radicle, and only seldom on the cotyledons (Fig. 3). As the PSE grew, the radicle elongated and formed the root. Secondary roots formed abundantly at 1 μM Kin or BA, whereas at 5 and 10 μM Kin or BA the roots began to thicken. From root's epidermal and sub epidermal layers voluminous white-snowish callus formed, without any form

of regeneration. After removal of this callus, small nodules emerging from the inner tissues with tightly bound green regenerants were observed starting from the third week.

Spontaneous formation of SSE on PGR-free medium was observed within all classes, being maximal within the II and III dass (40 % both, means 9.4 and 11.7),but in the IV dass SSE occurred only on 8 % of PSE (mean 1.0 SSE) (Table 1). However, adventitious bud formation occurred only in the IV class PSE at 36 % (mean 3.3). Our results are in accordance with the results of Ćalić *et al.* (2005) who showed spontaneous SSE formation in horse chestnut androgenic embryos on PGR-free medium. The highest embryogenic response of PSE (70 %) was reached at 5 μM Kin within the II PSE class, and maximal SSE number per PSE (32.7) was recorded at 10 μM BA, within the III class (Table 1). The maximal organogenic response (100 % and 96.30 %) of PSE and maximal bud number per PSE (48.8 and 68.8) were achieved at 5 and 10 μM BA respectively, within the IV PSE group (Table 1). Higher cytokinin concentrations were not tested due to high hiperhydricity that was already present at 10 μM.

The histological study was undertaken to confirm these findings. Paraffin sections through the IV class PSE revealed the meristematic zones embedded in calli , with obvious connection with the maternal tissue (Fig. 5). These structures developed into more advanced bud forms (Fig. 6). SSE were seen only occasionally on these sections.

Figs. 1 - 6. Secondary regeneration from somatic embryos of Aesculus carnea. 1. and 2. – Secondary somatic embryo (SSE) formation at the root pole of the II class primary somatic embryo (PSE). 3. – Adventitious bud regeneration from the radicle of the IV class PSE. 4. – Adventitious buds emerging from the radicle of the IV class PSE. 5. – Meristematic zones embeded in callus from the radicle of the IV class PSE. 6. – An adventitious bud regenerated from the IV class PSE radicle.

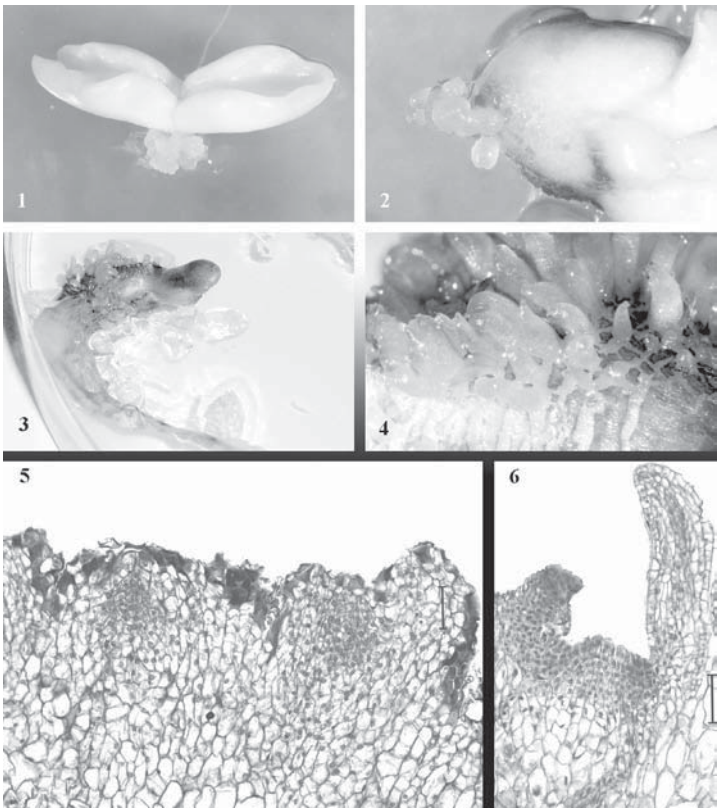

Table 1. Secondary somatic embryo (SSE) and adventitious bud formation from the primary somatic embryos (PSE) of Aesculus carnea at different stages of development.

PSE class	Kin (μM)	BA (μM)	% PSE with SSE	Mean SSE	% PSE with buds	Mean buds
I 1 mm	-	-	9.1	17.3de	0.0	0.0a
	1	-	30.0	7.3c	0.0	0.0a
	5	-	60.0	10.0d	0.0	0.0a
	10	-	20.0	9.5cd	0.0	0.0a
	-	1	50.0	7.2c	0.0	0.0a
	-	5	60.0	20.2e	0.0	0.0a
	-	10	20.0	3.0bc	0.0	0.0a
II 5 mm	-	-	40.0	9.4cd	0.0	0.0a
	1	-	30.0	29.0f	0.0	0.0a
	5	-	70.0	12.0d	0.0	0.0a
	10	-	50.0	19.2e	0.0	0.0a
	-	1	20.0	22.5ef	0.0	0.0a
	-	5	30.0	18.3e	0.0	0.0a
	-	10	40.0	8.7c	0.0	0.0a
III 10 mm	-	-	40.0	11.7d	0.0	0.0a
	1	-	0.0	0.0a	0.0	0.0a
	5	-	10.0	11.0d	0.0	0.0a
	10	-	40.0	8.7c	0.0	0.0a
	-	1	0.0	0.0a	10.0	2.0b
	-	5	20.0	17.0de	40.0	23.5c
	-	10	30.0	32.7f	60.0	19.2c
IV 30 mm	-	-	8.0	1.0b	36.0	3.3b
	1	-	5.3	8.0c	21.0	2.2b
	5	-	0.0	0.0a	26.3	1.4b
	10	-	0.0	0.0a	64.3	5.0b
	-	1	10.5	1.0b	78.9	7.5b
	-	5	0.0	0.0a	100.0	48.8d
	-	10	3.7	14.0d	96.3	68.8e

Data within a column followed by a different letter are significantly different according to LSD test at $P \leq 0.05$, n=30.

We assume that the differences in response at different stages of PSE development could be attributed to differences in the physiological status of the competent cells. The response was simmilar regardless of the presence of a PGR in a medium, the cytokinine type and concentration applied. The embryogenic potential of an explant is affected by numerous factors. The most important are: the level of endogenous hormones within the explant, the interaction between applied PGR and endogenous hormones, sensitivity of the explants to PGRs, PGR uptake levels etc. (reviewed by Jiménez, 2005). Although there was no clear correlation between endogenous hormone levels and SE induction among different species and general conclusions could not be drawn (Jiménez, 2005), many authors found levels of endogenous hormones to be one of the most important factors determining embryogenic potential of an explant (Fehér *et al.*, 2003; Gaj, 2004). Centeno *et al.* (1997) demonstrated that the embryogenic capacity of hazelnut cotyledonary explants declined with the degree of the zygotic embryo maturity. They found a correlation between embryogenic capacity and endogenous PGR content: 10-20 times higher iP-type/Z-type cytokinin ratio, and 5-10 times lower Z-type cytokinins/IAA ratio in embryogenic than in non-embryogenic explants. The loss of embryogenic potential of the germinating PSE could be caused

by the reduction of endogenous ABA level. Prewein *et al.* (2004) found a reduction in ABA level during germination of somatic embryos. Higher levels of endogenous ABA were reported to favor somatic embryogenesis in many species (reviewed by Jiménez, 2005). Disturbing a native explant's physiological status, by applying polar auxin transport inhibitors, Charrière and Hahne (1998) demonstrated change in morphogenic pathway in *Helianthus annuus* from embryogenic to organogenic. There are numerous contributions stating that developmental stage of the zygotic embryo is critically important for SE initiation (Gaj, 2004; Park *et al.* 2006).

To conclude, we reported on the differences in response of the primary somatic embryos of *A. carnea* depending on the stage of development. The PSE of up to 10 mm formed SSE, whereas bud formation prevailed from the germinating PSE. However, there were some differences between the effects of the two cytokinins. BA promoted bud induction at much higher rate than Kin and slightly higher embryogenic response.

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