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High efficiency adventive embryogenesis on somatic embryos of anther, filament and immature proembryo origin in horse-chestnut (*Aesculus hippocastanum* L.) tissue culture

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Abstract

Adventive embryogenesis was successfully induced in cultures of zygotic and somatic embryos on MS medium supplemented with BA and NAA. A procedure has been proved successful for the *in vitro* multiplication of somatic embryos regenerated at low frequencies from filament and callus cultures. The occurrence and rate of adventive embryogenesis did not depend on the origin of the primary embryos (zygotic and somatic), but did depend on the developmental stage. Primary embryos are capable of embryogenesis in each of the different phases of embryogenesis, though the rate is different. BA concentrations of 22–44 μM increased the rate of adventive embryogenesis and accelerated the development of embryos. The highest proliferation rate (22–25x/5 weeks) was achieved at hormone concentrations of 44 μM BA and 5.4 μM NAA.

Abbreviations: BA – benzyladenine, CH – casein hydrolysate, CM – coconut milk, 2,4-D – dichlorophenoxyacetic acid, MS – Murashige & Skoog medium, WPM – woody plant medium, NAA – 1-naphtaleneacetic acid

Introduction

The horse-chestnut is native only to the Balkan-peninsula, but has been planted throughout Europe and the U.S. as an ornamental and industrial shade tree. It is conventionally propagated by seeds, grafting and budding, but a rapid and low cost vegetative propagation for maintaining the valuable individual genotypes would be of great significance. Plant regeneration via adventive embryogenesis as a tool of micropropagation may offer a solution to the problem (Chalupa 1990).

Embryogenesis of somatic cell cultures of woody plants belonging to the *Fagales* order seems to be very easy to achieve, regarding the results on *Corylus* sp. (Perez et al. 1983; Radojevic et al. 1975), walnut (Tulecke et al. 1988), *Quercus* sp. (Gingas & Lineberger 1989) *Quercus* and *Fagus* (Jörgensen 1988) and *Aesculus* (Radojevic 1988; Jörgensen 1989; Chalupa 1990). Therefore, micropropagation via somatic embryogenesis may be the common method of *in vitro* vegetative propagation of these species.

Somatic embryogenesis of horse-chestnut was reported by Saito (1980), Dameri et al. (1986), Radojevic (1988), Jörgensen (1989) and Chalupa (1990), and regeneration of pollen-derived haploid plantlets was achieved by Radojevic (1978, 1989). Although secondary embryogenesis on

horse-chestnut embryos was mentioned in a few articles (Jörgensen 1989; Radojevic 1988), this phenomenon has not been investigated in detail yet. The aim of our experiments was therefore to explore and characterize adventive embryogenesis of this species.

Materials and methods

Embryo culture

Developing fruits (200 explants/tree) were collected from the central part of the crown of four horse-chestnut tree (*Aesculus hippocastanum* L.) in the Botanical Garden of the Agricultural University (Gödöllö) one month after flowering. The explants were surface sterilized and the proembryos in globular developmental stage (1–2 mm in size) were isolated and placed on nutritive medium. The semisolid MS basal medium was supplemented with 2,4-D (4.4–8.8 μM), NAA (5.4 μM), sucrose (3–11%) and with CM (0–10%).

The cultures were incubated at 26–28°C with a light intensity of 1000 lx and with a 16/8 photoperiod.

Anther and filament culture

To induce somatic callus, green flower buds (2–3 mm in size) were isolated. After sterilization, a portion of the buds (10%) were subjected to cold pretreatment (4°C 1–7 days), whereas from the other part (90%), the anthers and filaments of 1–2 mm were immediately isolated.

The semisolid MS basal medium was supplemented with 2,4-D (4.4–8.8 μM), NAA (5.4 μM), sucrose (3–11%) and CH (0–10%). Cultures were kept in the dark at 28°C.

Embryos developing directly from filament tissue were transferred to semisolid B5 medium (Gamborg et al. 1968) containing CM (5–10%), BA (2.2–8.8 μM), NAA (2.7–5.4 μM) and sucrose (3%).

Calli derived from filaments were transferred to semisolid and liquid B5 medium supplemented with BA (0–8.8 μM), CM (5–10%) and sucrose (3%). They were incubated in light (1000 lx) with 16/8 photoperiod at 26°C.

Adventive embryogenesis

To induce adventive embryogenesis, embryos (2–10 mm in size) were cultured on MS medium containing NAA (0–5.4 μM), BA (2.2–44.0 μM) and sucrose (3%). The developing adventive embryos were transferred to B5 medium, supplemented with BA (2.2–8.8 μM), CM (5%) and sucrose (3%). The medium for plantlet regeneration from adventive embryos was E1 (Gamborg et al. 1983), supplemented with BA (4.4–8.8 μM) and sucrose (2%). The other culture conditions are identical to those used in the embryo and callus culture.

The organogenesis of adventive embryos was analysed by SEM technique.

Plant regeneration

To induce plant regeneration, somatic and adventive embryos were transferred to culture tubes containing hormone free MS and WPM medium with 1/2 amount of macroelements. The other culture conditions are identical to those used in the embryo and callus culture. After germination, plantlets (4–5 cm) were planted to pots and transferred to the greenhouse.

All the experiments were repeated at least twice.

Results

Adventive embryogenesis on zygotic embryos

One group of zygotic embryos (60%) put on nutritive medium completed embryogenesis 3–4 months after isolation. Embryos of different developmental stages [from globular to cotyledonary stages (1–10 mm)] were transferred to MS medium supplemented with various concentrations of BA and NAA. One week after transfer, several adventive embryos started to develop at the radicula of the embryos cultured on medium containing 44 μM BA and 5.4 mM NAA. These reached the globular stage on the tenth day (0.5–1 mm diameter) (Fig. 1a) and the torpedo stage on the fifteenth day (Fig. 1b). On average, there were 20–30 at one primary embryo pole. Adventive embryogenesis also took place on the

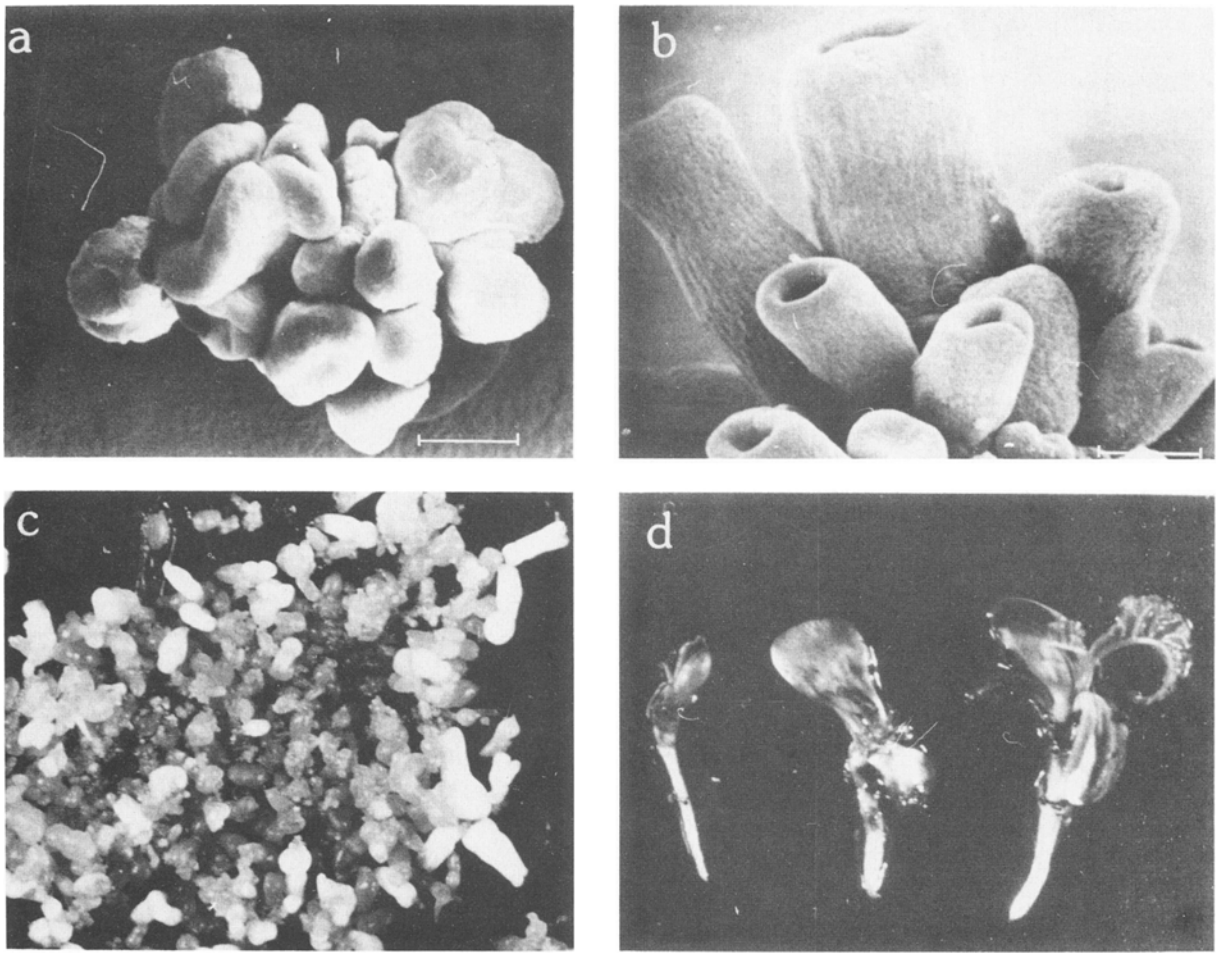

Fig. 1. Adventive embryogenesis and plant regeneration in horse-chestnut tissue culture (*a*) Globular stage proembryos 10 days after isolation of zygotic embryos on MS medium containing BA (10 mg l^{-1}) and NAA (1 mg l^{-1}). Bar represents 1 mm. (*b*) Developing adventive proembryos in the torpedo stage after 15 days in culture. (*c*) High frequency adventive embryogenesis induced on primary somatic embryos cultured on MS medium containing BA (10 mg l^{-1}) and NAA (1 mg l^{-1}), 14 days after isolation. (*d*) Plantlets 20 days after transplantation.

medium supplemented with lower concentrations of BA (2.2–22 μM) but the process required a longer time and the number of embryos was less (Fig. 2). Induction of adventive embryogenesis was also dependent on the size of the isolated embryos. Embryos of 8–10 mm have proved to be the optimum (Fig. 3).

When adventive embryos were separated from the primary embryo, the adventive embryogenesis continued. However, the process was not successful a third time, which can be explained by the fact that, according to our results, the primary embryos have increased to 12–14 mm and in this stage have lost their capability for adventive embryogenesis.

Under similar cultural conditions, adventive embryos removed from the primary embryos also had the potential for induction of adventive embryogenesis. Similar to the precedings, adventive embryos put on the induction media were found to be able to continue repetitive embryogenesis until their size reached 12–14 mm. It is very important to transfer these embryos on the medium with a low hormone concentration (2.2–8.8 μM BA) or containing no hormones at the end of the induction period, as a higher

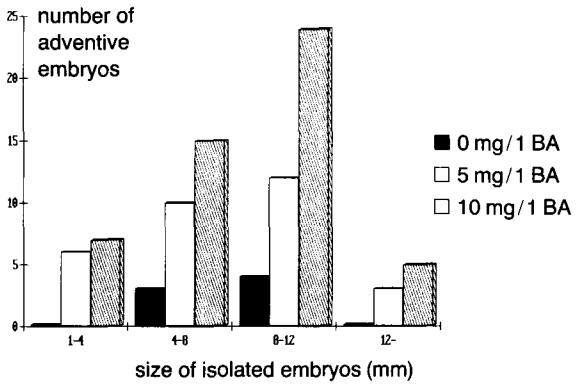

Fig. 2. The effect of the BA concentration and the size of zygotic embryos on the number of adventive embryos 5 weeks after isolation.

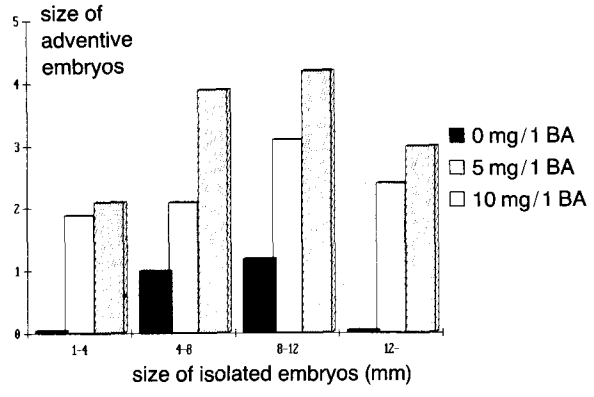

Fig. 3. The effect of the BA concentration and the size of zygotic embryos on the development of adventive embryos 5 weeks after isolation.

hormone concentration 22–44 μM can lead to abnormal embryo development, such as embryos with three or more cotyledons, overgrown embryos, etc. (Radojevic 1988; James et al. 1984; Jörgensen 1989; Merkle & Wiecko 1989).

Induction and multiplication of filament derived embryos

The isolated 1080 anthers became brown and increased twice in size after two weeks in culture. Embryos appeared directly on the surface of filaments 7–8 weeks after isolation. Twenty four embryos were obtained on 24 filaments. The 6% sucrose concentration and the 5.4 μM NAA content proved effective for the induction of horse-chestnut embryogenesis. Cold pretreatment of the anthers gave no response, all the treated anthers died.

After their initial appearance, the embryos were left on the original medium for a week, then were transferred to B5 medium. At this time, the embryos were white and 1–2 mm in size. Four to five weeks after transplantation, they turned green and their size reached 8–9 mm. At this developmental stage, successful adventive embryogenesis was induced on them by the method described above. Within a relatively short period (4–5 weeks), there were about 600 filament derived embryos (Table 1).

On the fifth week of adventive embryogenesis, developing embryos were transferred to different media. The B5 medium supplemented with 3% sucrose, 5% CM, 5.4 μM NAA and 2.2 μM BA gave the most embryos with normal morphology, whereas the higher concentration of sucrose and BA led to abnormal embryo development, as mentioned in the preceding point.

Induction and multiplication of callus derived somatic embryos

Thirteen to fourteen weeks after anther isolation, callus initiation from the somatic tissue of filaments could be observed on MS medium supplemented with 6% sucrose, 5.4 μM NAA, 8.8 μM 2,4-D. On 10% of these, the callus proliferation stopped in the first week and somatic embryos differentiated from them. With the majority of the anthers (90%), callus proliferation continued, but no embryos were directly regenerated.

Table 1. Multiplication of filament and callus derived embryos of horse-chestnut by adventive embryogenesis on MS medium supplemented with BA (10 mg l^{-1}) and NAA (1 mg l^{-1}), 5 weeks after isolation.

Isolated primary embryos			Adventive embryos*	
Origin	Number	Size (mm)	Mean no. and (+SD)	Size (mm)
filament	12	8	26 ± 0.9	1–4
filament	12	9	21 ± 0.7	1–3
callus	13	8	24 ± 0.7	1–4
callus	14	9	25 ± 1.0	1–4
callus	13	10	22 ± 0.8	1–3

* The mean no. and size values of developed adventive embryos are counted to one primary embryo.

Calli induced from filaments reaching 5 mm in diameter were subcultured in hormone free liquid B5 medium. These calli could be maintained as embryogenic in liquid culture for at least 6 months. After several transfers, calli were placed on solid regeneration medium (B5) containing 2.2–8.8 μM BA, 5–10% CM and 3% sucrose.

Callus derived somatic embryos developed on the medium containing 2.2 or 4.4 μM BA and 5% CM. About 4–5 weeks later, these somatic embryos reached 8–10 mm in size. In this developmental stage, they were transferred to appropriate medium applicable for induction adventive embryogenesis (Fig. 1c), and in 4–5 weeks 900–950 adventive embryos were obtained from these embryos (Table 1).

Plant regeneration from somatic and adventive embryos was achieved on hormone free WPM medium with 1/2 concentration of macroelements (Fig. 1d). The low regeneration rate (0.5–1%) indicates, that further detailed study needed to improve this critical step of the system. This problem seems to be a general one among the woody species (Novak et al. 1986).

Discussion

In the case of *Aesculus hippocastanum*, somatic embryos have been produced from filament callus (Jörgensen 1989), from excised immature embryos (Radojevic 1988; Chalupa 1990) and from primary leaves (Dameri et al. 1986). This method can be utilized for cloning horse-chestnut embryos with different origin and for optimizing the process, as regards the highest yield of adventive embryos within the shortest period.

Many articles concerning adventive (or asexual, secondary, etc.) embryogenesis in tissue culture of woody plants have been published recently (James et al. 1984; Gingas & Lineberger 1989; Muralidharan & Mascarenhas 1987; Durzan & Gupta 1987; Sellars et al. 1990). No articles, however, were published on investigation of adventive embryogenesis in horse-chestnut. Adventive embryogenesis in horse-chestnut was mentioned by Jörgensen (1989), Radojevic (1988) and Chalupa (1990) but no further detailed characterization of this phenomenon was carried out. Our findings agree well with the results of Jörgensen (1989) and Chalupa (1990), but we obtained optimum adventive embryogenesis at a higher concentration (44 μM) of BA. The proliferation rate at the first passage of other species, such as *Quercus*, soybean or peanut (Gingas & Lineberger 1989; Sellars et al. 1990) was 1–10 embryos/explant compared to the 22–25 embryos/explant in horse-chestnut tissue culture. Similarly to our observations, adventive embryos were found to appear at the radicula of the primary embryos (Tulecke et al. 1988; Merkle & Wiecko 1989; Radojevic 1988).

Adventive embryos were capable for further repetitive embryogenesis. High concentration (44 μM) of BA was necessary for induction of adventive embryogenesis, but longer (4–5 days) culture on this medium caused abnormal embryo development (policotyledonary, overgrown, etc. embryos). This observation was similar to that of Jörgensen (1989), Radojevic (1988) and Merkle & Wiecko (1989). The efficiency of adventive embryogenesis was strongly dependent on the size of the primary embryos and on the hormone concentration. Contrary to horse-chestnut seeds, *in vitro* embryos first developed the shoot, then later the root, which is the well-known way of germination of this species (Radojevic 1988).

These results indicate, that this method may be very useful in cases, where we need more hundreds or thousands of vegetatively cloned embryos for further experiments, such as vegetative micropropagation or synthetic seed production. Another benefit of this method is the short period needed for the mass multiplication of the *in vitro* embryos (Chalupa 1990). Further investigations are necessary, however, to reduce the number of abnormal embryos, as well as to increase the regenerating potential of the *in vitro* embryos.

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