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Somatic embryogenesis from ovaries, developing ovules and immature zygotic embryos, and improved embryo development of *Castanea sativa*

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Abstract

Somatic embryogenesis of European chestnut (*Castanea sativa* Mill.) was obtained using juvenile tissue cultured on P24 medium with 5 μM 2,4-dichlorophenoxyacetic acid plus 0.5 μM 6-benzylaminopurine (BA) for three weeks and then cultured on 0.89 μM BA. Induction frequency with ovaries ranged from 2.0 to 19.1 % and was observed in tissue collected 2 to 8 weeks postanthesis, ovules used as a starting tissue gained 0.8 to 7.8 %, 3 to 9 weeks postanthesis. Zygotic embryos collected 5 to 10 weeks postanthesis formed 10.5 to 57.1 % somatic embryos, respectively. The culture lines were maintained *via* secondary embryogenesis on P24 medium with 0.89 μM BA. Development and maturation were stimulated on P24 medium with increased agar concentration (1.1 %). Five plantlets were transferred to substrate and acclimatized successfully in greenhouse.

Additional key words: 6-benzylaminopurine, 2,4-dichlorophenoxyacetic acid, European chestnut, *Fagaceae*.

Introduction

From the Black Sea to the Iberian Peninsula, European chestnut (*Castanea sativa* Mill.) is an important tree species for both timber and nut production. However, this tree species is threatened by pollution, social and economic changes, and two major fungal diseases; ink disease (*Phytophthora* ssp.) and chestnut blight [*Cryphonectria parasitica* (Murr.) Barr.].

In the area of tree biotechnology, propagation *via* somatic embryogenesis (SE) is regarded as a system of choice for mass propagation of superior tree genotypes (Cervelli *et al*. 1995). The advantages of somatic embryos (SE) include high multiplication rates and the potential for scale-up in bioreactors (Eeva *et al.* 2003) and for direct delivery to the greenhouse or field as artificial seeds. Production of artificial seeds has been reported in many species, in conifers as well as in angiosperms (Gupta *et al*. 1993).

For the genus *Castanea*, SE has been reported for *C. mollissima* Blume × *C. dentata* (Marsh.) Borkh. hybrids (Skirvin 1981), *C. dentata* (Merkle 1991, Xing *et al*. 1999) and *C. sativa* × *C. crenata* Sieb. & Zucc. hybrids (Vieitez *et al.* 1990). Regeneration of plants *via* somatic embryogenesis in *C. sativa* × *C. crenata* hybrids was achieved by Vieitez *et al*. (1994). Xing *et al*. (1999) recovered plants from developing ovules through somatic embryogenesis in American chestnut (*C. dentata*). Carraway *et al*. (1994) transferred neomycin phospho-transferase and β-glucuronidase genes into American chestnut embryogenic cultures via particle bombardment. However, the application of somatic embryogenesis for the improvement of chestnut is limited as a result of problems with low initiation frequencies, maintenance of embryogenic cell lines and low conversion rates. In the present study, we investigated the frequencies for initiation of SE from different juvenile explants of European chestnut: ovaries, ovules and immature zygotic embryos. To stimulate secondary somatic embryogenesis, two basal media, GD (Gresshoff and Doy 1972) and P24 (Teasdale 1992) were tested. The effect of glutamine was also tested. In order to improve development and maturation of SE, an experiment with increased agar concentration was performed.

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Abbreviations: ABA - abscisic acid; BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; GD - Gresshof and Doy; IBA - indole-3-butyric acid; LSD - least significant difference; PGR - plant growth regulator; SE - somatic embryos, somatic embryogenesis; WPM - woody plant medium.

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Materials and methods

Plants: Burs were collected weekly starting from the end of July until the end of August (2 to 10 weeks postanthesis) during 1998 and 1999, from three open pollinated European chestnut (*Castanea sativa* Mill.) trees growing in the park of Castle Schönbrunn in Vienna, Austria. Spikes were removed and burs were surface sterilized (2 × 10 min) with a commercial bleach containing 2.5 % NaOCl in an ultrasonic bath (Wilhelm 1997), with an intermittent washing step with 70 % ethanol for 15 s. Burs were rinsed twice in a sterile solution of 300 mM Na_2HPO_4 + 42 mM citric acid + 100 mM KI + soluble starch (5 g dm^{-3}), then transferred to a sterile solution of starch (5 g dm^{-3}) and rinsed twice in sterile distilled water (Dirks *et al.* 1991). The cupule was removed aseptically and nuts were excised. Longitudinally halved nuts (ovaries), clusters of ovules, single ovules and developing embryos were tested as explants. Sizes of burs, ovules and zygotic embryos were measured by determining length (l) and width (w) in mm and a size index was calculated as l × w for morphological description. Seventy seven zygotic embryos with a size index exceeding 40 were separated into axes and cotyledons. Developing ovules with a size index exceeding six were defined as immature zygotic embryos, as the remaining ovules stopped growing beyond this size.

Induction of somatic embryogenesis: To induce SE, explants were placed in Petri dishes containing 25 cm^3 P24 medium (Teasdale 1992) with 5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) plus 0.5 µM 6-benzylaminopurine (BA), and 2 cm^3 dm^{-3} *Preservative for Plant Tissue Culture Media* (*PPM*™, *Plant Cell Technology, Inc.*, Washington, USA), supplemented with 3 % sucrose and gelled with 0.8 % agar (*Daishin, Brunschwig Chemie*, Amsterdam, The Netherlands). The pH of the medium was adjusted to 5.6 - 5.7 prior to autoclaving at 121 °C for 20 min. To avoid the inhibitory influence of polyphenols, the explants were transferred to fresh induction medium within the first week. After 3 weeks explants were transferred to P24 medium with 0.89 µM BA. The latter medium was used for maintenance of embryogenic cell lines. Subculture interval was 4 weeks. Cultures were incubated in the growth chamber at temperature of 24 ± 2 °C, irradiance of 50 µmol m^{-2} s^{-1} (white fluorescent light) and a 16-h photoperiod. After 6 weeks, cultures were scored for induction of embryo like structures (white and yellow globular objects).

Development of embryogenic lines: To stimulate secondary SE formation, two basal media, GD (Gresshoff and Doy 1972) and P24 (Teasdale 1992) were tested. Experiments with 300 and 1 000 mg dm^{-3} L-glutamine were also performed. L-glutamine was autoclaved with the medium. Three embryogenic lines, B8/117, B7/65 and B7/89 were cultured on P24 or GD with 0.89 µM BA. For the experiment with glutamine, P24 medium with 0.89 µM BA was used with the cell lines C6/57, B7/65 and B7/89. Six plates, 0.5 - 0.7 g of tissue per plate, were used for each line and treatment and the entire experiment was repeated twice. Percentages of cultures with proliferating embryogenic tissue were evaluated after 4 weeks.

Maturation and germination: For maturation, the effect of two agar concentrations (0.8 and 1.1 %) was tested. Clusters of SE of cell line C6/57, 0.5 - 0.7 g per Petri dish, were placed on P24 medium with 0.89 µM BA, solidified with 0.8 or 1.1 % agar. After 5 weeks the number of well formed embryos (size < 3 mm, Fig. 2*c*), was scored and calculated as numbers of SE produced per g tissue. Data were compared with a one-sided *t*-test. For germination, harvested SE of two cell lines (C6/57 and B7/89) were cultured on P24 medium with 0.1 µM indole-3-butyric acid (IBA) and 0.89 µM BA or on P24 medium without plant growth regulators (PGRs). After 5 weeks the germination response was classified as either conversion (both root and shoot development) or as shoot or root formation only.

Seedlings were transferred to *Magenta™ GA-7* vessels containing PGR-free P24 medium supplemented with 1 % (m/v) activated charcoal.

Data analysis: Statistical analysis was performed with *SPSS for Windows 6.0.1* (χ^2 contingency table) and *Microsoft Excel 97* (*t*-test). Data from proliferation and germination experiments were compared with χ^2 contingency table.

Results and discussion

SE induction: Data were pooled for all three source trees per sampling date, as source tree did not effect embryogenic response (χ^2 = 2.4, P = 0.3). In total, 142 embryogenic lines out of 3163 explants were initiated during 1998 and 1999, which corresponded to an overall induction frequency of 4.5 %. Twenty-five embryogenic cell lines were formed from 491 ovaries (5.1 %), 76 lines were derived from 2520 ovules (3.0 %) and 41 lines were induced from 152 zygotic embryos (27.0 %). According to χ^2 contingency table embryogenic response of ovules and ovaries did not differ significantly, whereas induction frequencies of immature

zygotic embryos were significantly higher than of ovules ($P < 0.01$) and ovaries ($P < 0.05$).

Embryogenic tissue from halved nuts (ovaries) was creamy-white, nodular and friable and developed after 3 to 4 weeks. In contrast, yellow and green compact callus showed no further organized development. SE were initiated from bisected ovaries 4 to 8 weeks postanthesis in 1998, and 4 to 6 weeks postanthesis in 1999 (Fig. 1).

In 1998, clusters of ovules turned brown and necrotic; therefore single ovules were explanted in 1999. After 2 to 3 weeks in culture, direct SE was observed from several isolated ovules, where SE formed without any intervening tissue. Size index of isolated ovules ranged between 1.5 and 5. One ovary contained 10 to 18 ovules. Up to six ovules out of them showed an embryogenic response (Fig. 2*a*,*b*), indicating that either at least six ovules per ovary were fertilized, or that non zygotic tissue was responsive as well.

In 1998, SE frequencies from zygotic embryos ranged between 41 and 47 % from immature zygotic embryos collected 9 and 10 weeks after anthesis (Fig. 1), whereas the highest induction rate (57.1 %) was obtained from material collected only 6 weeks postanthesis in 1999 (Fig. 1). Twenty one % of isolated axes from immature zygotic embryos gave rise to direct SE, while only 1 % of cotyledon tissue responded.

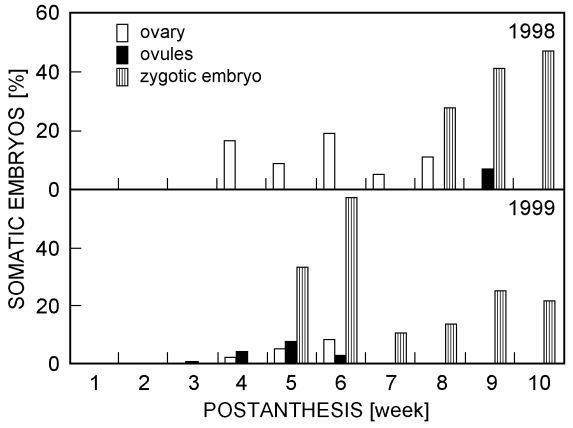

Fig. 1. Effect of collection date on formation of somatic embryos [%] in ovaries, ovules and immature zygotic embryos from *Castanea sativa* in 1998 and 1999.

In the present study we were able to induce high frequencies of SE in *C. sativa* using immature zygotic embryos. Xing *et al.* (1999), using developing ovules, achieved initiation rates of 1.6 %. In *C. sativa* × *C. crenata* hybrids, formation of SE was restricted to 15 - 20 mm immature nuts, collected 10 to 12 weeks post fertilisation, with a frequency of 2 % (Vieitez 1995). This variation may be explained by different protocols, such as culture regimes, media and PGRs as well as by species. Merkle *et al.* (1991) and Xing *et al.* (1999) induced SE in the dark, on woody plant medium (WPM) with high auxin content (18.1 µM 2,4-D). Carraway and Merkle (1997) tested the embryogenic response of different genotypes and explant types in *C. dentata* and reported an induction frequency of 2.8 %. Initiation of SE was only possible from *C. dentata* zygotic embryos less than 5 mm.

The frequency of SE initiation was related to the developmental stage of the zygotic embryo in European chestnut. This phenomenon has also been observed in several broad-leaved tree species such as *Quercus* ssp. (Endemann and Wilhelm 1999, Wilhelm 2000) and *Liriodendron tulipifera* (Sotak *et al.* 1991). The optimal stage for formation of SE in *C. sativa* was between 5 and 10 weeks post anthesis and a size index ranging from 6 to 350 (Fig. 3). Sizes of seeds differed from year to year and even within one harvesting date. The growth of the cupule and the zygotic embryo started at the same time during both years, but was retarded in 1998. Therefore seasonal influences have to be considered when choosing the optimal induction time.

Development of embryogenic lines: The ability for proliferation *via* repetitive SE differed among the cell lines. Stable embryogenic cell lines were initiated from all types of explants 6 and 7 weeks postanthesis in 1998, and 4, 5 and 6 weeks postanthesis in 1999. Twenty-one SE lines were selected for their proliferation capacity and subcultured on P24 with 0.89 µM BA (Fig. 2*d*). High ratios of vitamins and amino acids, especially glutamine and serine, stimulated androgenesis in *Quercus petraea* and *Fagus sylvatica* (Jörgensen 1988). In chestnut hybrids, glutamine was necessary for repetitive embryogenesis (Vieitez 1995). However, in our experiments, glutamine had no significant beneficial effect on secondary embryogenesis for any of the tested cell lines (Table 1). Embryogenic cell lines were maintained without glutamine for 2 years and did not loose embryogenic competence. Carraway and Merkle (1997) considered addition of an auxin necessary to maintain SE in *C. dentata*, whereas formation of *C. sativa* SE was halted by 1µM 2,4D.

The tested basal media, GD and P24, produced no

Table 1. Effect of addition of glutamine to P24 medium with 0.89 µM BA on proliferation of embryogenic cell lines of *Castanea sativa.* Values are percentages of samples with embryogenic proliferation. According to χ^2 contingency table, cell lines ($\chi^2 = 1.2$, $P = 0.5$), and treatments ($\chi^2 = 2.1$, $P = 0.3$) do not differ significantly.

Cell line	Glutamine [mg dm^{-3}]		
	0	300	1000
C6/57	67	75	58
B7/65	58	58	50
B7/89	75	17	75

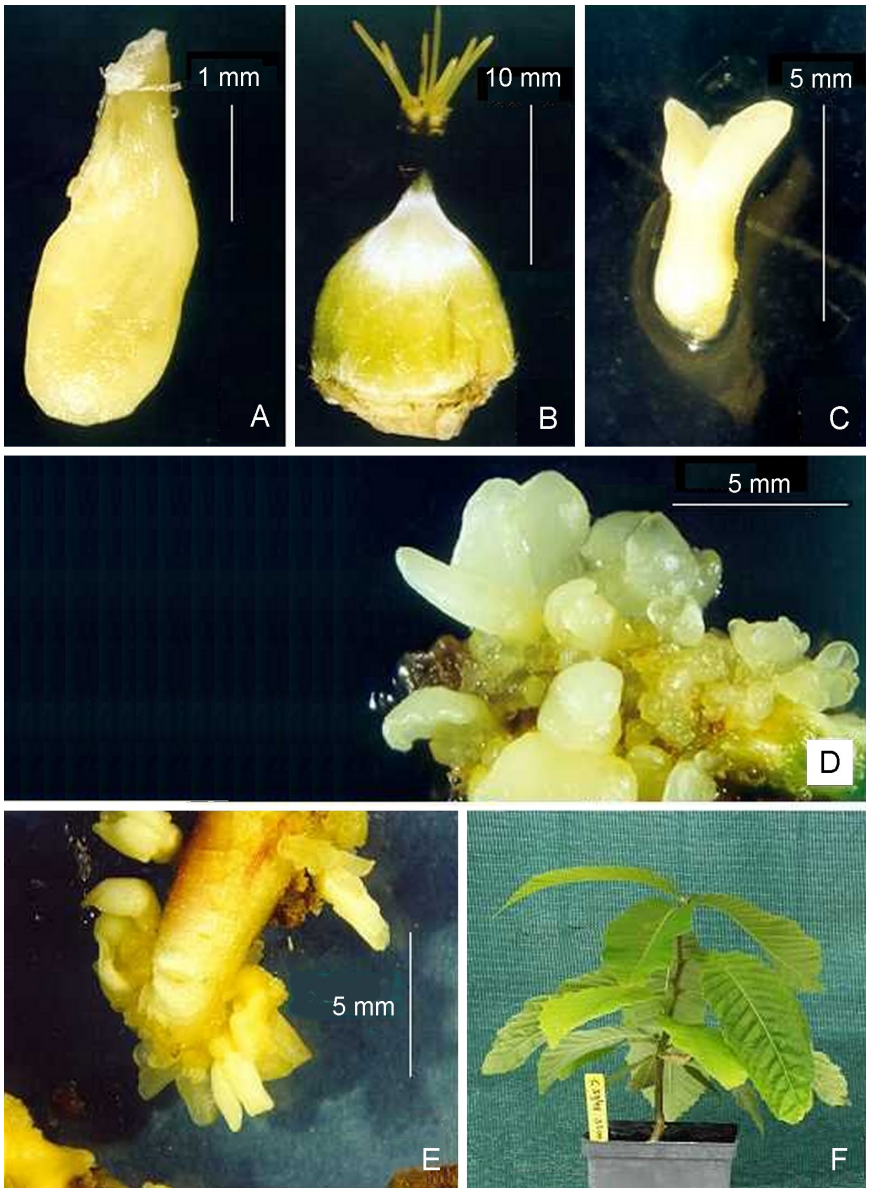

Fig. 2. Somatic embryogenesis in *Castanea sativa*. *A* - single ovule as explant, *B* - ovary as explant, *C* - isolated cotyledonary somatic embryo on maturation medium with 1.1 % agar, *D* - secondary embryos at various stages of development on proliferation medium, *E* - secondary embryos developed from hypocotyl of a germinated somatic embryo, *F* - plant regenerated from somatic embryo.

differences in proliferation capacity of the tested cell lines, and differences among cell lines were also not significant (Table 2).

In many broadleaved tree species such as oak (Wilhelm 2000), pecan, black locust and bigleaf magnolia (Merkle 1995, Merkle *et al*. 1995) the embryogenic lines are normally maintained *via* repetitive secondary embryogenesis. However the proliferaton of SE was found to be difficult in European chestnut as a result of development of many different tissue types. As described by Carraway *et al*. (1994) explants produce a mixture of callus types and continuous selection of embryogenic cells is necessary. Direct formation of secondary embryos was frequently observed at the hypocotyl region of a germinating SE (Fig. 2*e*). Unicellular or multicellular pathways are possible origins of secondary embryogenesis. Zegzouti and Favre (1999) revealed that both pathways were possible in *Quercus robur*, depending on the applied PGRs. The application of BA promoted indirect secondary embryogenesis from cortical parenchyma cells, indicating multiple cell origin in oak, which may explain the polycotyledonary embryos and fused embryoids. In *C. sativa* we also frequently observed anomalous formations of SE, which may have been evoked by the use of BA.

Table 2. Effect of basal media P24 and GD on proliferation of SE [%] of *C. sativa*. Values are percentages of samples with embryogenic proliferation. GD *versus* P24 $\chi^2 = 2.0$, $P = 0.2$; B8/117 *versus* B7/89 $\chi^2 = 3.0$, $P = 0.1$; B8/117 *versus* B7/65 $\chi^2 = 2.3$, $P = 0.1$; B7/89 *versus* B7/65 $\chi^2 = 0.3$, $P = 0.6$.

Cell line	Medium	
	GD	P24
B8/117	33	33
B7/65	42	58
B7/89	42	75

Maturation and germination: Maturation and germination are considered to be major bottlenecks in SE of many broadleaved species. Spontaneous germination of *C. sativa* SE was rarely observed on proliferation medium after one year in culture. With a higher agar concentration (1.1 %), the average number of single cotyledonary SE produced per gram increased significantly (Table 3).

Within the *Fagaceae*, a wide range of treatments and culture conditions lead to regeneration of plants *via* SE. Most often, radicles elongate first and epicotyls form with delay or fail to elongate. Increasing sucrose concentration from 20 to 60 g dm^{-3} enhanced embryo maturation and conversion in American chestnut (Xing *et al*. 1999). Vieitez (1999) employed a cold treatment (4 °C) to improve germination of *Castanea sativa* × *C. crenata* hybrids. The combination of increased agar concentration (1.1 %) and partial desiccation treatment improved conversion rates in *Quercus robur* (Wilhelm *et al*. 1999) Additionally Sunderlikova and Wilhelm (2002) showed that high expression of storage protein genes during maturation of oak somatic embryos was associated with increased conversion frequency. Although we did not determine the transcript accumulation of storage protein genes in chestnut somatic embryos, we also found significantly more embryos converted in both cell lines when harvested from maturation media with the higher agar concentration, followed by culture on P24 with 0.1 M IBA and 0.89 µM BA or P24 without PGRs. This result suggests that the regulation of storage proteins is under developmental control and can be influenced by manipulation of the culture conditons also in chestnut somatic embryos. No differences in shoot formation were observed between the different treatments (Table 4).

Table 3. Effect of 0.8 and 1.1 % agar on maturation of SE of *C. sativa*. Means ± SE; values are significantly different as determined by *t*-test (one-tail): $P = 0.01$, $n = 24$.

Cell line	Agar [%]	Number of SE [g^{-1}]
C6/57	0.8	4.5 ± 0.5
C6/57	1.1	6.8 ± 0.8

SE derived plantlets were transferred to PGR-free medium supplemented with 1 % m/v activated charcoal. Five plants with well developed shoots and roots were transplanted into pots containing a 1:1 peat moss:perlite mixture and acclimatized in a growth chamber under artificial light with a 14-h photoperiod at 20 - 22 °C and

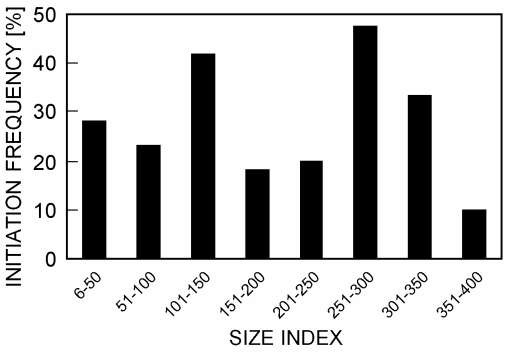

Fig. 3. Initiation frequencies [%] of zygotic embryos of *C. sativa*, explants grouped into nine classes by size index (l × w).

Table 4. Effect of different concentrations of agar in maturation media (1: P24 + 0.89 µM BA + 0.8 % agar; 2: P24 + 0.89 µM BA + 1.1 % agar) followed by culture on two different germination media (I: P24 without PGRs; II: P24 + 0.89 µM BA + 0.1 µM IBA), tested with cell lines C6/57 and B7/89. C6/57 *versus* B7/89 $\chi^2 = 3.0$, $P < 0.1$; cell line C6/57 1 *versus* 2 $\chi^2 = 10.3$, $P < 0.01$; I *versus* II $\chi^2 = 3.5$, ns; cell line B7/89 1 *versus* 2 $\chi^2 = 7.3$, $P < 0.01$; I *versus* II $\chi^2 = 0.005$, ns.

Cell line	Agar [%]	Germination medium	Number of embryos tested	Number of plantlets	roots only	shoots only
C6/57	0.8	–PGR	86	2	12	2
C6/57	0.8	+PGR	57	3	6	3
C6/57	1.1	–PGR	76	8	17	5
C6/57	1.1	+PGR	89	15	4	3
B7/89	0.8	–PGR	36	2	7	3
B7/89	0.8	+PGR	32	2	8	3
B7/89	1.1	–PGR	32	8	7	3
B7/89	1.1	+PGR	52	10	10	4

a relative humidity of 97 %. After 4 weeks the plants were kept under daylight conditions at 20 to 25 °C in the greenhouse (Fig. 2*f*).

Conclusion: We were able to induce somatic embryogenesis from ovules and immature zygotic embryos at high frequencies. The conversion rate of chestnut somatic embryos was improved by using a maturation medium containing 1.1 % agar. However, the number of resultant plantlets was low. Further work is needed to elucidate the biochemical and molecular regulation of chestnut somatic embryo development with the aim of adjusting maturation and germination treatments for the production of high quality plants.

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