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BRIEF COMMUNICATION

Somatic embryogenesis from zygotic embryos of *Schisandra chinensis*

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Abstract

We describe the multi-step regeneration system of medicinal plant *Schisandra chinensis* (Turcz.) Baill. The seeds were pre-treated with 0.005 μM thidiazuron. Subsequently the zygotic embryos of the early heart stage were cultured on medium with 50 μM of 2,4-dichlorophenoxyacetic acid (2,4-D) and after three weeks the embryogenic calli were transferred to a medium with 10 μM of 2,4-D and 4 μM of 6-benzyladenine and were sub-cultured at the 4-week intervals. Abscisic acid (30 μM) and polyethyleneglycol (3 %) significantly influenced the synchronization of development of the somatic embryos (SEs) to the globular stage. The following culture on a medium without growth regulators resulted in full developed cotyledonary stage SEs. Indole-3-butyric acid (0.05 μM) contributed to their rapid conversion to plantlets.

Additional key words: abscisic acid, maturation, medicinal plant, micropropagation, somatic embryo conversion.

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Schisandra chinensis (Turcz.) Baillon is a deciduous, woody stem liana from the *Schisandraceae* family, which belongs to primitive dicotyledon plants with undifferentiated embryos. Seed germination requires cyclic stratification, and the rate of germination is low (Saunders 2000). *Schisandra chinensis* contains dibenzo-[a,c]cyclooctadiene lignans that are unique natural substances. This compounds display significant antioxidant action and some of them were identified as potent anti-human immunodeficiency virus agent (Perez 2003). The medical effects of *Schisandra* fruits together with its low toxicity and well-established traditional use have therefore potential clinical and therapeutic applications in Western medicine (Weinberg *et al*. 1999).

Although *Schisandra* is an important medicinal plant, no reports of its morphogenic capabilities *in vitro* have been published yet. The aim of our experiments was to induce somatic embryogenesis and establish an *in vitro* culture that could overcame problems with low germination rate and need of seed stratification and could be used for biochemical, physiological, molecular and transformation studies and/or breeding and biotechnology.

Immature berries of two cross pollinated plants of *Schisandra chinensis* cultivated in the Centre of Medicinal Plants of the Faculty of Medicine of Masaryk University in Brno, Czech Republic, were collected at the end of June in 2000. Seeds were removed from berries and surface-sterilised by a 30-s rinse in 70 % (v/v) ethanol, followed by 25 min in 20 % (v/v) *Savo* (*Bochemie*, Brno, Czech Republic) and then rinsed three times in sterile distilled water. The seed coat was removed and the longitudinally cut seed was placed on the pre-treatment Murashige and Skoog (1962; MS) medium with 5.0 nM thidiazuron (TDZ). After one-week ZEs were excised and used as explants.

For embryogenic tissue initiation medium WV5 (*Duchefa*, Haarlem, The Netherlands) with 50 μM 2,4-D, 2 % sucrose, 2 mM glutamine and 0.15 % of *Gellan Gum* was used. Embryogenic calli were sub-cultured on a monthly basis on the same medium with 4 μM BA and

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Abbreviations: ABA - abscisic acid; AC - activated charcoal; BA - 6-benzyladenine; 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indole-3-butyric acid; MS medium - Murashige and Skoog medium; PEG - polyethyleneglycol 4000; SEs - somatic embryos; TDZ - thidiazuron; WV5 - Westvaco medium; ZEs - zygotic embryos.

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10 μM 2,4-D for about one year. Ten actively growing embryogenic calli (each about 100 mg) were transferred to the pre-maturation medium WV5 with 3 % of polyethylene glycol 4000 (PEG), 3 % sucrose and 0 - 50 μM (±)-abscisic acid (ABA) (*Sigma*, St. Louis, MI, USA).

After 49 d somatic embryos (SEs) from the best treatment (30 μM ABA) were dispersed on activated charcoal (AC)-impregnated filter paper discs (*Whatman No. 3*) placed on the maturation medium WV5 without growth regulators and cultured for 14 d until the torpedo and early cotyledonary stages developed. Then they were individually isolated and cultured on the conversion medium WV5 with 1.5 % sucrose, 0.1 % AC and 0.05 μM indole-3-butyric acid (IBA). After three weeks the plantlets were transferred to the acclimation medium (MS/2) with the same additives except IBA.

All cultures at the pre-treatment, initiation, maintenance and maturation phases were grown in glass Petri dishes in the dark at temperature of 23 ± 2 °C. Conversion was achieved in Magenta boxes (*Sigma*) under diffused light (irradiance of 15 μmol m^{-2} s^{-1}) and 16-h photoperiod. All media were autoclaved at 121 °C for 15 min and the growth regulators and amino acid were filter-sterilised and added to the molten medium. The pH was adjusted to 5.8 prior to autoclaving and filtration. Each treatment consisted of three replications and the experiments were repeated twice. The average frequency of developed globular SEs was calculated from three Petri dishes, and each sample consisted of ten clusters of embryonic calli (approx. 100 mg). The average frequency of converted SEs of each line was calculated from three separate Petri dishes, and each sample consisted of 30 SEs.

The plants were hardened in multiplates filled with the *Seedling substrate* (*Klasmann-Deilmann*, Geste-Gross Hesepe, Germany) and cultivated in the growth chamber (*Climacell*, *BMT*, Brno, Czech Republic) under a 16-h photoperiod, irradiance of 75 μmol m^{-2} s^{-1}, temperature of 25 °C, and gradually decreasing humidity (90 - 60 %). The hardened plants were kept in a greenhouse (Fig. 1*F*) and planted out in a garden.

Even if the zygotic embryos of *S. chinensis* in fresh seeds were undifferentiated and very small, the possibility to establish embryogenic culture from them was studied. Pre-treatment with a very low concentration of TDZ (0.005 μM) was used to enlarge the zygotic embryo according to Thomas and Katterman (1986). In woody plants, higher concentration of TDZ than 1 μM may result in the formation of callus and regeneration of adventitious shoots and somatic embryos (Huetteman and Preece 1993). In our experiments ZEs enlarged and developed to early heart stage (Fig. 1*A*) after one week on medium with TDZ without callus formation on the other parts of the seed. Then they could be removed without damage and transferred to induction medium.

After about following three weeks twenty percent of *Schisandra* ZEs developed different callus types, which were cultured separately on the maintenance medium. Creamy, potentially embryogenic calli (Fig. 1*B*) developed on the basal parts of seven zygotic embryos (11.6 %). Frequency of embryogenic callus development was relatively low in comparison with herbaceous plants, *e.g.*, barley with 64 to 100 % (Halámková *et al*. 2004) and cotton with 92 to 100 % (Aydin *et al*. 2004) but it was similar to quince with 15 to 50 % (D'Onofrio and Morini 2003/4) and to hybrid fir with 27 % (Salaj and Salaj 2003/4).

Three most viable embryonic lines were successfully established and maintained for more than two years with about the same embryogenic capacity. To obtain mature SEs, the embryogenic calli were transferred to the pre-maturation medium supplemented with an osmotic regulator PEG 4000 and various concentrations of ABA. The highest frequency of globular SEs (Fig. 1*C*) was achieved on medium with 30 μM ABA (Fig. 2). It corresponded with the results of Tian and Brown (2000), who observed the beneficial effect of ABA treatment on SE growth and normal development and Leung and Giraudat (1998) who reported that ABA treatment partially synchronized development of SEs and prevented precocious conversion of SEs in *Arabidopsis*. Our experiments showed differences in sensitivity to ABA treatment among three lines of *S. chinensis*. The lowest sensitivity to 50 μM of ABA was found in line 1, while lines 2 and 3 did not form any SEs and died on this ABA concentration (Fig. 2).

The subculture of *S. chinensis* SEs at the globular and heart stages on activated charcoal-impregnated filter discs which was used *e.g.* by Smith and Krikorian (1991) and Kumria *et al*. (2003) seemed to be a prerequisite for obtaining torpedo and early cotyledonary SEs. The torpedo and early cotyledonary stage of SEs developed on activated charcoal-impregnated filter paper discs cultured on maturation medium within two weeks (Fig. 1*D*).

When we compared three embryogenic lines of mature SEs we found that in the most embryogenic line 1, 72.3 % SEs converted into plantlets (38.9 % showed normal and 33.4 % abnormal morphology) and 27.7 % SEs did not convert at all. The percentage of conversion into normal plants of line 2 and 3 was lower (19 and 8 %, respectively) and a high percentage of malformed SEs with multiple cotyledons (71 and 62 %, respectively) was observed. The positive correlation between morphological responses to ABA treatment and the highest percentage of conversion of line 1 was in agreement with the data of Lelu *et al*. (1994) who reported that ABA was important for the accumulation of storage reserves in the cotyledons of hybrid larch and directly impacted conversion of SEs. Developed plants of *S. chinensis* were transferred to *ex vitro* conditions. Most of malformed plants stopped growing and died. Two growth types were observed among the 40 % of survived plants: normal and dwarf (Fig. 1*F*).

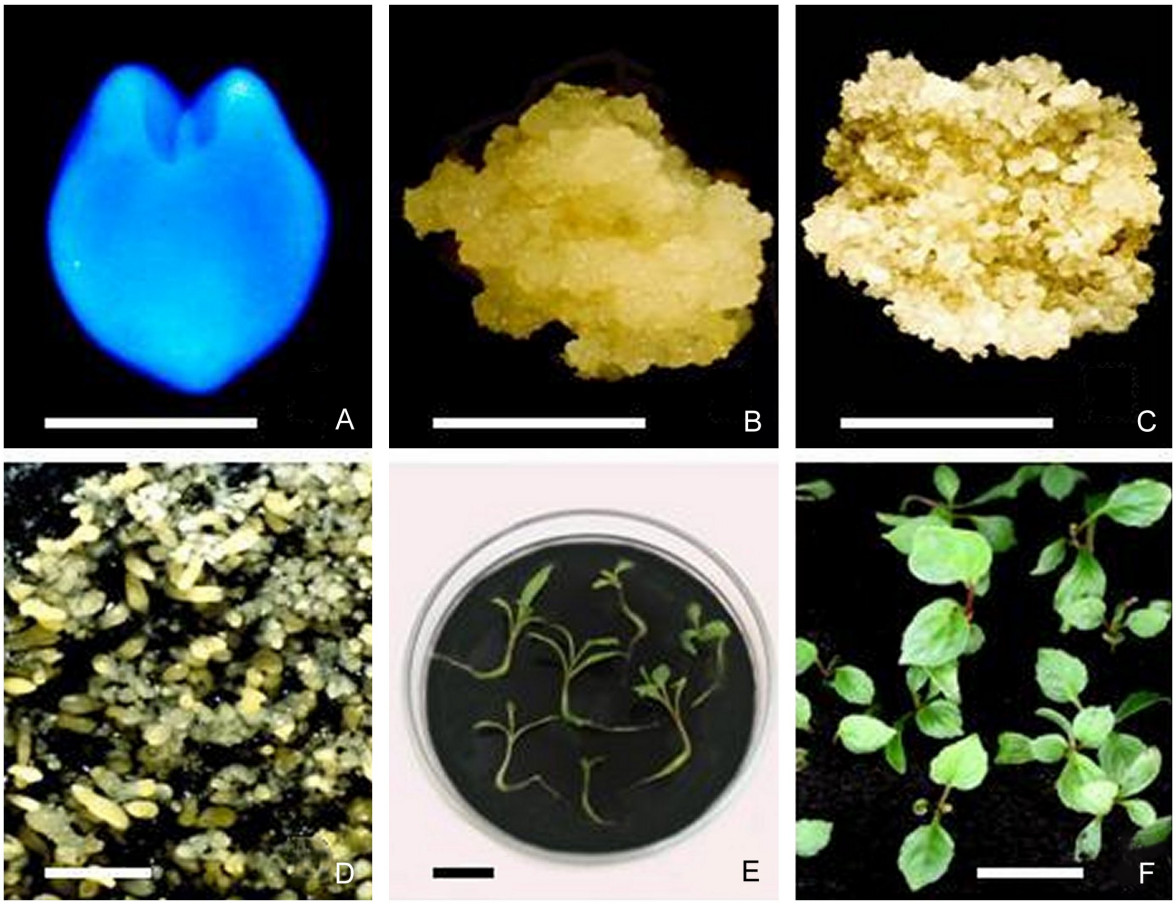

Fig. 1. *A* - *Schisandra chinensis* immature zygotic embryo after one-week pre-treatment with 0.005 µM of TDZ (*bar* = 0.1 cm), image was artificially coloured for better visualization of its apex. *B* - Actively growing embryogenic callus on the maintenance medium (*bar* = 1 cm). *C* - SEs in the globular and heart stages developed after 7-week-culture on the pre-maturation medium with 30 µM ABA (*bar* = 1 cm). *D* - SEs in the torpedo and early cotyledonary stages developed after two weeks on activated charcoal-impregnated filter paper discs placed on the maturation medium without growth regulators (*bar* = 1 cm). *E* - Converted plantlets after three week culture on the conversion medium (*bar* = 1.5 cm). *F* - Acclimated plantlets in peat substrate (*bar* = 5 cm).

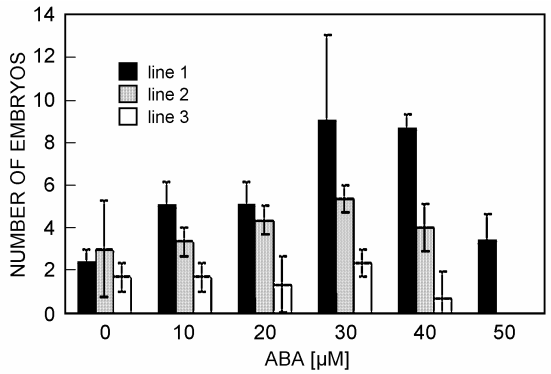

Fig. 2. Effect of ABA treatment on the number of somatic embryos of three lines of *S. chinensis* after 7-week-culture. The average number SEs in globular stage per 100 mg calli is shown (confidence intervals are indicated by *vertical bars*, *P* = 0.05).

This is the first report describing somatic embryogenesis in *Schisandra chinensis.* The results with TDZ could be generally applied for development of somatic embryos on minute zygotic embryos. To achieve sufficient quantity and quality of the SEs a multi-step regeneration system which included ABA and PEG treatment was developed. The primitive dicotyledon plants *Schisandra* seems to have similar physiological requirements as conifers, where ABA stop the cleavage, stimulate storage reserve accumulation and prevent precocious germination (*c.f.* Salaj and Salaj 2003/4).
The analysis of main lignans in developed culture of *Schisandra* are in progress because the system could serve as a tool for further selection of improved genotypes and/or biotechnological production of lignans.

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