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Polyethylene glycol and abscisic acid improve maturation and regeneration of *Panax ginseng* somatic embryos

Abstract Embryogenic culture was initiated from mature zygotic embryos of *Panax ginseng*. Multiple somatic embryos formed and proliferated on Murashige and Skoog medium supplemented with 2,4-dichlorophenoxyacetic acid (2.26 μM) and kinetin (0.046 μM). Mature as well as immature somatic embryos grew into plantlets lacking roots on the same media. Histomorphological analysis of somatic embryos treated with abscisic acid (ABA) and polyethylene glycol (PEG 4000) showed a slight improvement in the root meristem organization of torpedo-stage embryos (embryos were more compact and their cells exhibited a lower degree of vacuolation). Shoot regeneration of non-treated somatic embryos was 31% while that for somatic embryos treated with PEG 4000 and ABA was 70%. Moreover, 75% of plants regenerated from PEG- and ABA-treated embryos formed roots while plants from non-treated embryos did not form roots.

Keywords ABA · *Panax ginseng* · Somatic embryogenesis

Abbreviations *ABA:* (±)-Abscisic acid ·
BAP: N^6-Benzyladenine ·
2,4-D: 2,4-Dichlorophenoxyacetic acid ·
GA$_3$: Gibberellic acid · *Kin:* Kinetin · *MS:* Murashige and Skoog medium · *PEG 4000:* Polyethylene glycol 4000 · *PGR:* Plant growth regulators

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Introduction

Panax ginseng C. A. Meyer is an herbaceous plant belonging to the family *Araliaceae*. Ginseng has had a strong reputation in oriental medicine since ancient times as being tonic, regenerating, and rejuvenating, even though its pharmacological activity is difficult to pin down. It grows wild, but has been overexploited, in mountain areas, from Nepal to Manchuria, and from eastern Siberia to Korea (Bruneton 1995). Nowadays, wild ginseng is rarely available and the current supply of ginseng on the markets mainly depends on field cultivation, which is an extremely long-lasting and labor-intensive process. Native ginseng plants need 5–7 years prior to harvest and the content of ginsenosides is low (Shoyama et al. 1995). One of the most critical steps in field cultivation is seed germination. Seeds have to be stratified in order to accelerate completion of embryo development, though this process takes 7–9 months (Hong 1982). In vitro mass production in large-scale systems seems to be a potentially more efficient alternative for production of bioactive ginseng components, as well as being capable of shortening the production cycle of outstanding clones with established, efficient in vitro plant regeneration protocols (Choi et al. 1998; Tang 2000).

Somatic embryogenesis (the development of embryos from somatic cells) is a powerful tool for large-scale vegetative propagation of many plant species. Several reports have shown initiation of somatic embryos from zygotic embryos of *P. ginseng* (e.g. Lee et al. 1990; Arya et al. 1993; Choi and Soh 1996; Choi et al. 1997, 1998; Tang 2000). Although substantial progress has been made towards the development of systems for somatic embryogenesis in ginseng, routine production cannot be used in practice due to persisting problems with the efficiency of embryo maturation and embryo conversion. Similarly to other plant tissue cultures, the rooting phase is frequently a very problematic step in the ginseng regeneration process (Tirajoh et al. 1998). Many reports on ginseng somatic embryogenesis are focused mainly on improve-

ment of the embryo induction stage. Further development to regeneration and rooting phases was usually done very shortly after initiation, when the culture preserves its regeneration ability (e.g. Arya et al. 1993; Choi et al. 1998; Tang 2000). Nevertheless, in these cases, somatic embryos are frequently reported to be regenerated into multiple shoots that either miss a root system (Butenko et al. 1968; Chang and Hsing 1980; Choi 1988; Čeliárová et al. 1992) or have an inadequate or poor root formation (e.g. Arya et al. 1993; Shoyama et al. 1995; Kevers et al. 2002). Choi et al. (1998) suggest that the inability of regenerated plants to form roots might be related to embryonic structural abnormalities since morphologically abnormal somatic embryos (e.g. multicotyledonary and multiple embryos) have also been frequently observed (Butenko et al. 1968; Chang and Hsing 1980; Lee et al. 1990; Arya et al. 1993).

After long-term maintenance of embryogenic culture, difficulties with culture stability and regeneration may increase. Tang (2000) reported morphogenic callus to maintain its embryogenic potential over 12 months only and Shoyama et al. (1995) reported a gradual decrease in embryo propagation ability and regeneration efficiency after 2 years of subculture. Also culture hyperhydricity, usually related to long-term cultivation, causes regeneration of plants with abnormal morphological appearance and physiological functions (Debergh et al. 1992). Such propagules usually do not survive the transfer to ex vitro conditions (Kongbangkerd and Wawrosch 2003).

The literature data show that at least in some species the addition of abscisic acid (ABA) into the medium improves the efficiency of the process of somatic embryogenesis (e.g. von Arnold and Hakman 1988; Linossier et al. 1997). It promotes the transition of the cultures from proliferation (multiplication) to maturation stage and prevents precocious germination. Polyethylene glycol 4000 (PEG 4000) was reported to improve germination frequencies (root and shoot emergence) without limiting embryo histodifferentiation in soybean somatic embryos (e.g. Walker and Parrott 2001). Likewise in spruce, it was reported that PEG might improve the quality of somatic embryos by promotingnormal differentiation of the embryonic shoot and root (e.g. Stasolla et al. 2003). Non-penetrating osmotica cannot penetrate into the plant cells, but restrict water uptake and provide a simulated drought stress during embryo development. A combination of ABA and a non-penetrating osmoticum as a supplement in the culture medium can help prevent precocious germination (Attree et al. 1991) and allow embryo development to proceed (Linossier et al. 1997). As far as we know the effect of PEG and ABA has been described in a number of conifers (Stasolla et al. 2002), but for non-conifer plant we found only a few reports, on *Hevea brasiliensis* by Linossier et al. (1997) and on *Corydalis yanhusuo* by Sagare et al. (2000).

The aim of the present paper is to study the effect of the non-penetrating osmoticum (PEG 4000) and ABA on the structure of developing somatic embryos and their further regeneration capacity.

Materials and methods

Somatic embryogenesis induction and plant regeneration

Korean ginseng (*P. ginseng* C. A. Meyer) seeds were obtained from Kyoto botanical garden in Japan. The stratified seeds were surface sterilized with 70% ethanol for 1 min and 30% commercial bleach (v/v) (approx. 1.58% NaOCl) for 20 min and than washed three times in sterile distilled water. After aseptic removal of the hard mesocarp, the seeds were placed on Murashige and Skoog medium (1962) (MS) that was free of plant growth regulators (PGR) in plastic Petri dishes. The medium was adjusted to pH 5.7 and 0.75% (w/v) agar (Lachema, Czech Republic) was added to the medium before autoclaving at 125°C and 120 MPa for 25 min.

After 1 month, zygotic embryos were carefully excised from sterile seeds and placed on MS medium supplemented with 3.0% (w/v) sucrose, 0.01% (w/v) myo-inositol, 4.5 μM 2,4-dichloro-phenoxyacetic acid (2,4-D) and 0.046 μM kinetin (Kin) (Sigma, USA). Zygotic embryos were cultivated in the dark. Somatic embryos started to form on cotyledons, and germination of somatic embryos was observed on the same medium. For higher efficiency of proliferation (multiplication), embryogenic mass was transferred to Magenta vessels (Osmotek, Israel) with floating rafts. The 2,4-D content of the liquid MS medium was decreased to 2.26 μM, with the same content of Kin (0.046 μM). Static tissue cultures were cultivated in the dark at 24°C. Cultures were subcultured at 5-week intervals.

To study the effects of ABA and the high molecular-mass osmoticum PEG, the cultures were treated with 3.75% or 7.50% PEG 4000 (Fluka, Switzerland) and 20 μM ABA (Sigma, USA). PEG and ABA were added to solid MS medium, and cultivation was done in dark conditions. This 4-week maturation stage was inserted between the proliferation stage (maintenance of the culture) and the regeneration stage. In the non-treated (control) variant, this stage was omitted. In each treatment, 5–6 cultivation flasks were inoculated with 20–30 embryos (embryogenic mass with embryos of different developmental stage).

Somatic embryos regenerated into plantlets on solid half-strength MS medium supplemented with 28.87 μM gibberellic acid (GA$_3$) (Sigma) (Fig. 1A). In each treatment 5–6 cultivation flasks were inoculated with 20–30 embryos (embryogenic mass with embryos of different developmental stage) after PEG and ABA treatment or directly from multiplication medium as a control. The frequency of plant regeneration was determined after 4 weeks of culture by counting the number of regenerating plantlets from somatic embryos. Regenerating plantlets further rooted on solid PGR-free MS medium with third-strength salts, 1.0% (w/v) sucrose and full MS vitamin concentration. Into each cultivation flask we transferred 10 plantlets and 5–6 flasks were inoculated from each treatment. The frequency of rooting was determined after 4 weeks of culture by counting rooted plantlets on rooting medium after another 4 weeks of culture. The cultures during regeneration and plantlet rooting stages were cultivated under a 16-h photoperiod at a temperature of 24°C. Cultures were subcultured at 4-week intervals.

Anatomical study

For light microscopy, samplings were made after PEG and ABA treatment by the end of the subcultivation period (4 weeks), taking 3 samples per vessel and 3 vessels per variant. The paraffin sections of embryogenic tissues were prepared essentially according to Johansen (1940). Briefly, samples were fixed with 50% formaldehyde/acetic acid/ethanol/water (1:1:9:9, v/v/v/v) for at least 48 h. After washing with 50% ethanol, the samples were dehydrated gradually in an ethanol-butanol series, and infiltrated with paraffin. Serial longitudinal sections of 12 μm were cut on a Leitz microtome. Sections were stained by a two-step staining procedure using alcian blue and nuclear fast red (Poláčková and Beneš 1975). Observation was made using a binocular microscope (Olympus Provis, Japan).

Fig. 1A–F Histomorphological analysis of *Panax ginseng* somatic embryos and their regeneration capacity. **A** Plantlet forming "multiple shoots" regenerated from somatic embryo on regeneration medium (half-strength Murashige and Skoog medium supplemented with 28.87 *μM* gibberellic acid). **B–F** Paraffin longitudinal sections, stained with alcian blue and nuclear fast red (meristematic areas are stained red), under bright field microscopy. **B** Somatic embryo formed on the callus surface, in multiplication (maintenance) medium [without abscisic acid (ABA) and with 3.75% polyethylene glycol (PEG)]. *Bar* represents 100 μm. **C** Somatic embryo formation on the callus surface, in maturation medium supplemented with ABA and 3.75% PEG. *Bar* represents 200 μm. **D** Somatic embryo formed on maturation medium supplemented with ABA and 3.75% PEG. *Arrows* show apical meristem (*a*) and cotyledons (*c*). *Bar* represents 100 μm. **E** Somatic embryos formed on the callus surface, in maturation medium supplemented with ABA and 7.5% PEG. *Bar* represents 200 μm. **F** Friable callus without embryogenic potential illustrating degradation of cultures after prolonged cultivation on multiplication (maintenance) medium. *Bar* represents 100 μm
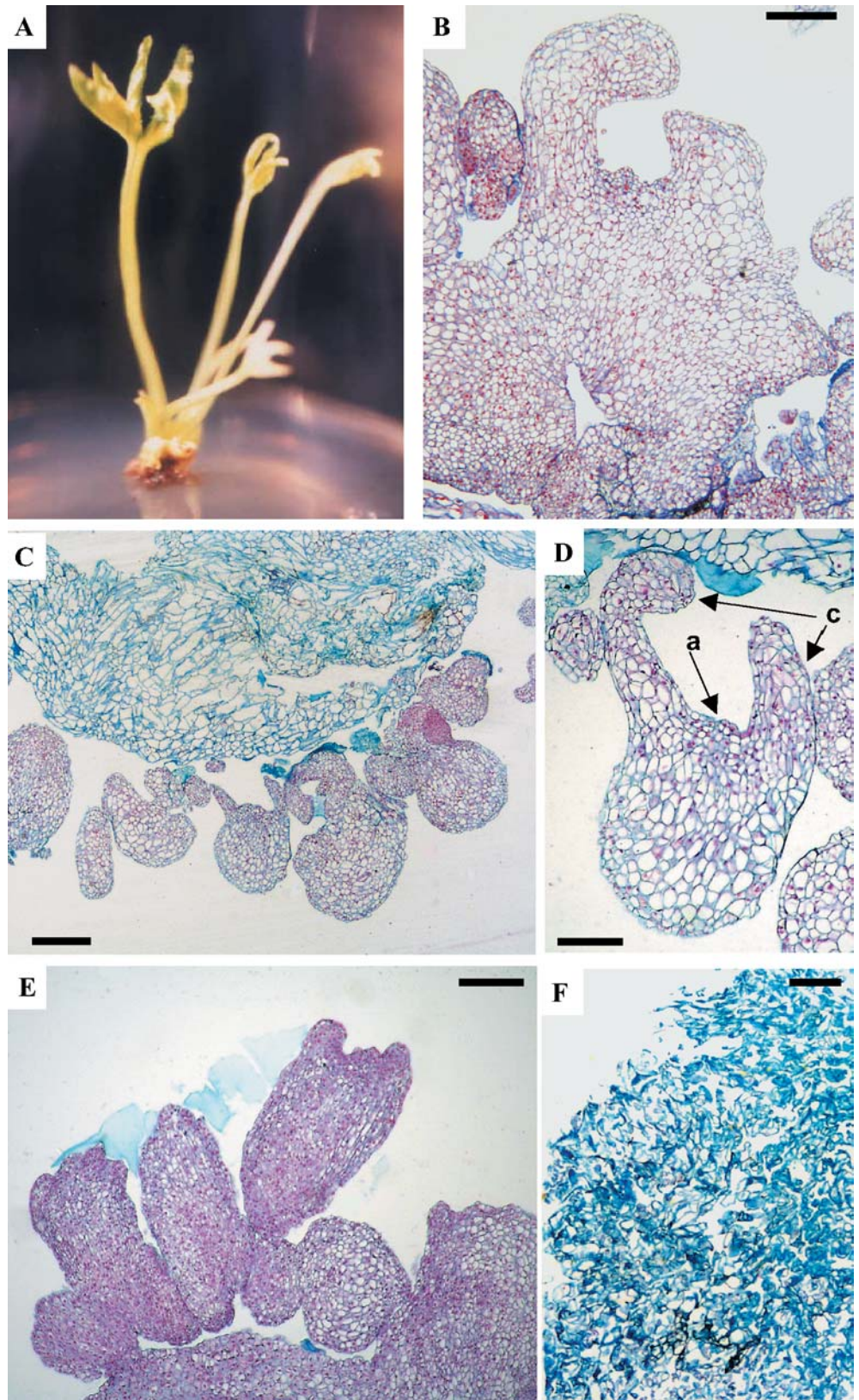
Table 1 Process of *Panax ginseng* micropropagation through somatic embryogenesis including the insertion of a maturation stage. Regeneration and rooting efficiency were determined on 100–180 embryos/50–60 plants

Somatic embryo initiation	Multiplication (maintenance) stage (5-weeks subculture)	Embryo maturation stage (4 weeks)	Plant regeneration stage (4 weeks)		Rooting stage (4 weeks)	
			PGR (μM) in half-strength MS	Regeneration efficiency (%)	Medium	Rooting efficiency (%)
MS + 4.52 μM 2,4-D + 0.05 μM Kin	MS + 2.26 μM 2,4-D + 0.05 μM Kin	Skipped	2.89 μM GA$_3$ + 4.44 μM BAP	24	Third-strength MS + 1.0% sucrose	0
		Skipped	2.89 μM GA$_3$ + 44.40 μM BAP	31		0
		Skipped	28.87 μM GA$_3$	69		0
		MS + 20 μM ABA + 3.75% PEG	28.87 μM GA$_3$	70		75
		MS + 20 μM ABA + 7.50% PEG		68		74

Abbreviations: *ABA* abscisic acid; *BAP* N [6]-benzyladenine; *2,4-D* 2,4-dichlorophenoxyacetic acid; *GA$_3$* gibberellic acid; *Kin* kinetin; *MS* medium according to Murashige and Skoog; *PEG* polyethylene glycol; *PGR* plant growth regulators

Results and discussion

Somatic embryo morphogenesis and plantlet regeneration

Embryogenic culture was initiated from mature zygotic embryos of *P. ginseng* on MS supplemented with 2,4-D (4.5 μM) and Kin (0.046 μM) (initiation stage). Multiple somatic embryos formed on the same medium and the culture proliferated on medium supplemented with 2,4-D (2.26 μM) and Kin (0.046 μM) (multiplication medium for further culture maintenance). In the embryogenic mass at this stage, a continuous and unsynchronized formation of secondary embryos was observed and the culture was therefore composed of embryos in different developmental stages appearing simultaneously. Mature torpedo-stage embryos as well as immature somatic embryos after long-term maintenance developed into plantlets lacking roots on the same media (Table 1) and their transfer to ex vitro conditions was not successful. Similar results were observed for example by Arya et al. (1993), Shoyama et al. (1995), Kevers et al. (2002) and Choi et al. (1998).

The anatomical study performed on the cultures confirmed their heterogeneous character where somatic embryos in different developmental stages appeared simultaneously. Most of the torpedo-stage embryos were lacking root meristem or the meristem was loosely organized (Fig. 1B). In some torpedo-stage embryos, the meristematic character of cells was even questionable due to the high degree of embryo cell vacuolation. We regard this abnormality as one of the possible reasons for regeneration of plantlets lacking roots. Moreover, in embryogenic culture we observed the formation of somatic embryos with numerous morphological abnormalities. We observed many anomalous structures, especially in the torpedo-stage of development. Among the most frequent abnormalities we have found, for example, are fasciation of two embryos, asymmetrical cotyledon development or multicotyledonarity. Similar results were reported by Čeliárová et al. (1992). They observed secondary embryogenesis especially on the surface of cotyledons and leaf-like structures, and many anomalous structures, especially in the torpedo stage. Various malformed embryoids were observed also by other authors without detailed histological studies (e.g. Chang and Hsing 1980; Choi 1988).

On some of the embryos we observed overgrowth of leaf primordia over the apical meristem, which stops further development. These embryos were determined as being precociously germinated, regenerated in complete plantlets without roots, or plantlets of abnormal morphogenesis. Some of these embryos contained more than two cotyledons (multicotyledonarity) and regenerated in a bunch of leaves.

To solve the problem of plantlets lacking roots we tested different rooting media using different auxin concentrations or PGR-free medium, with no response (data not shown). Moreover, the spontaneous somatic embryo germination rate on multiplication medium (MS supplemented with 2.26 μM 2,4-D and 0.046 μM Kin) tended to decrease. Therefore our investigation was aimed at finding a suitable regeneration medium by testing different N [6]-benzyladenine (BAP) and GA$_3$ combination (see Table 1). The best regeneration was reached on half-strength MS medium supplemented with 28.87 μM GA$_3$ (Fig. 1A). Nevertheless, the regeneration efficiency was only 31% and plantlets regenerated from somatic embryos did not form roots (Table 1). As mentioned above, we suggest that poor regeneration of plants lacking roots is caused by unsatisfactory embryo development where the characteristic root meristem is not present in the torpedo stage (Fig. 1B).

The effect of PEG and ABA on somatic embryogenesis and plantlet regeneration

The attempt to increase the quality of somatic embryos by using the high molecular mass osmoticum, PEG 4000,

and ABA was accomplished by insertion of a maturation phase of culture between multiplication (maintenance) and regeneration phases. The combined application of ABA and PEG has become a routine method for stimulation of somatic embryo maturation in some genera of *Coniferales* (Bozhkov and von Arnold 1998) and selected tree species such as *H. braziliensis* (e.g. Linossier et al. 1997). We tested the effect of ABA (at a concentration of 20 *µM*) in combination with PEG 4000 [at a concentration of 3.75% or 7.5% (w/v)]. Compared to non-treated embryos [cultures where the maturation phase was omitted (see Table 1)], cultures treated with PEG 4000 and ABA exhibited better development (see below). Similar results were obtained for both PEG concentrations tested.

After PEG and ABA treatment, embryos were transferred to regeneration medium where they differentiated into plantlets and, subsequently, responded satisfactorily to rooting medium [PGR-free, third-strength MS medium with 1% (w/v) sucrose] (Table 1). Therefore, the insertion of a cultivation period including PEG and ABA (maturation medium) between multiplication and regeneration stages significantly promotes regeneration and rooting efficiency of plantlets.

An anatomical study performed on embryogenic cultures after 4 weeks of cultivation on maturation medium containing ABA and PEG 4000 revealed that the cultures were not more synchronized compared to non-treated cultures at the multiplication stage and contained somatic embryos in different development stages appearing simultaneously. The somatic embryos started their development at the stage of meristematic centers through globular and heart-shape to torpedo-shape embryos. In spite of the fact that typical fine distinct inner histological differentiation of torpedo-shape embryos was not present, the PEG- and ABA-treated embryos were structurally more developed than untreated embryos (compare Fig. 1B and C). Treated somatic embryos were formed mainly of compact cells of meristematic character, of a small shape with dense cytoplasm, and an obviously dense nucleus. The apical meristem was well developed as well as cotyledons and distinct root pole (Fig. 1D). The majority of embryos formed a typical single-cell layer protoderm on their surface very soon. Nevertheless, many cells in embryo root pole showed a more or less vacuolated character. Desirable root meristem development was not observed even in the furthest-developed embryos (Fig. 1D). In addition to the typical embryos, we observed many anomalous structures, similar to those of cultures not treated with PEG or ABA (fasciation of two embryos, asymmetrical cotyledon development, and multicotyledonarity).

Somatic embryo cells cultivated on higher PEG concentration (7.5%) were less vacuolated (Fig. 1E) comparing to those cultivated on lower PEG concentration.

Morphogenic non-stability of ginseng cultures during long-term maintenance

After long-term cultivation, part of the culture preserved meristematic character with formation of different developmental stages (somatic embryos, buds, and regenerated plantlets) but a certain level of hyperhydricity was observed, too. Part of the culture dedifferentiated to friable callus, without any further morphogenic development (Fig. 1F). In this callus we observed the presence of disintegrating meristematic centers, shown by very weak dye reactions of nuclei, and their disorganization. These observations are in tune with other reports showing the instability of *Panax* cultures and support the opinion that we need efficient methods for long-term preservation of cultures, e.g. cryopreservation (Seitz and Reinhard 1987; Joshi and Teng 2000).

Improvement of ginseng somatic embryogenesis was important also for production of secondary metabolites. Separated roots of the rooted plants produced by the PEG and ABA treatment were cultivated in liquid medium and a culture of multiple adventitious roots was established. In our corresponding study we were comparing saponin production in different ginseng tissue cultures such as callus, suspension culture and adventitious roots (data not shown). We found adventitious roots able to produce the full range of ginsenosides distributed analogously to the roots of native plants (Langhansová et al. 2002) in contrast to undifferentiated cultures.

In conclusion, we described the improvement of *P. ginseng* somatic embryo regeneration capacity from cultures that matured on the medium with ABA and PEG. In contrast to the somatic embryos formed without this treatment, ABA- and PEG-treated embryos exhibited improved histological differentiation, improved meristematic development of embryoids, and finally better regeneration and rooting of plantlets. Therefore, the improved method of somatic embryo production described in present paper might be important for micropropagation as well as for obtaining cultures producing medicinally important substances.

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