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Somatic embryogenesis and plant regeneration from suspension cultures of *Acanthopanax koreanum* Nakai

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Abstract High-frequency somatic embryogenesis was achieved from an embryogenic cell suspension culture of *Acanthopanax koreanum* Nakai. Stem segments were cultured on Murashige and Skoog (MS) medium containing auxins and cytokinins. Opaque and friable embryogenic callus formed on MS medium with 4.5 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 2.0 μM kinetin or zeatin, but was highest on medium containing 4.5 μM 2,4-D alone. Embryogenic calli were transferred to MS liquid medium containing 4.5 μM 2,4-D and maintained by subculture at 2-week intervals. Initiation of somatic embryogenesis and development up to the globular stage from embryogenic cell clumps occurred in medium containing 0.45 μM 2,4-D, whereas maturation and germination of somatic embryos occurred in MS medium lacking 2,4-D. Cytokinin treatment suppressed the normal growth of embryos, but stimulated secondary somatic embryogenesis from the surfaces of primary embryos. Plants from somatic embryos were acclimatized in a greenhouse.

Key words *Acnathopanax koreanum* · Cell suspension culture · Somatic embryos

Abbreviations *BA* Benzyladenine · *2,4-D* 2,4-Dichlorophenoxy acetic acid · *MS* Murashige and Skoog · *NAA* α-naphthalene acetic acid

Introduction

Most species of *Acanthopanax* (Araliaceae) are woody. The cortical tissues of their roots are used for medicinal purposes. These species are endangered due to overharvesting. Zygotic embryos at harvest time are in an immature globular stage; therefore, stratification and low-temperature treatment for several months are required for germination or, alternatively, they can germinate naturally after about 2 years (Isoda and Shoji 1994). It is difficult to propagate these species by stem cuttings. Thus, plant propagation by conventional methods is quite limited.

Plant production through tissue culture in *Acanthopanax* could allow mass propagation. In one species, *A. senticosus* Herms (Gui et al. 1991; Choi and Soh 1993), direct somatic embryogenesis from zygotic embryos was reported. However, somatic embryogenesis from callus or suspended cell clumps has not been achieved due to poor or no callus formation from the explants (Gui et al. 1991; Choi and Soh 1993). Both reports mentioned that the radicle portion of the direct somatic embryos was fused to the parent explants, hampering normal plant regeneration.

The development of somatic embryos from embryogenic callus or cell suspension culture might be an efficient method for mass propagation of the *Acanthopanax* species. The present work was carried out to establish plant regeneration via somatic embryogenesis from embryogenic cell suspension cultures of *A. koreanum* Nakai.

Materials and methods

Plant material and callus initiation

Plants of *A. koreanum* Nakai were grown at the experimental garden of the Chonbuk National University. Young stems of 3-year-old plants were used as explants. The stems were surface disinfected in 70% ethanol for 1 min, followed by 1% sodium hypochlorite for 10 min, and then rinsed three times with sterile distilled water. The apical regions (2 cm from the shoot apex) were discarded and the subjacent 6-cm sections were used. Internodes were cut transversely into 2-mm-long segments and placed onto 20 ml Murashige and Skoog (MS) (1962) solid medium in 50-ml Erlenmeyer flasks. Three stem segments were inoculated in each flask and ten flasks were prepared for each treatment. The experiment was repeated three times. The medium for callus induction was composed of MS basal medium containing 3% sucrose, 0.7% agar and varied concentrations and combinations of auxins [0.45–4.5 μM 2,4-dichlorophenoxyacetic ac-

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id (2,4-D) or α-naphthaleneacetic acid (NAA)] and cytokinins [2.0 μM benzyladenine (BA), kinetin or zeatin]. All media were adjusted to pH 5.8 before autoclaving at 121°C for 15 min. The culture room was maintained at 25±1°C with a 16-h photoperiod under 24 μmol m^{-2} s^{-1} cool white fluorescent light. After 10 weeks, embryogenic callus formation and frequency of somatic embryo formation were assessed.

Initiation of somatic embryos

Opaque and friable embryogenic calli formed on MS medium containing 4.5 μM 2,4-D were selected and transferred to medium containing the same concentration of 2,4-D in 100-ml Erlenmeyer flasks containing 30 ml MS liquid medium. The embryogenic cell suspensions were maintained by adding 5 ml of the old suspension to 25 ml of fresh medium at 2-week intervals. Cultures were agitated at 100 rpm on a gyrating shaker under 12 μmol m^{-2} s^{-1} cool white fluorescent light with a 16-h photoperiod. After 2 weeks of culture, embryogenic cell clumps were filtered using a 200-μm stainless steel sieve to remove the large clumps and settled by centrifugation for 5 min at 1000*g*. Filtered cells (200 μl) were transferred to liquid medium supplemented with various concentrations of 2,4-D ranging from 0 to 4.5 μM in 100-ml Erlenmeyer flasks containing 30 ml MS medium. After 2 weeks, the number of globular somatic embryos per flask was counted.

Maturation of somatic embryos

Embryogenic suspensions consisting of globular embryos were passed through a 350-μm stainless steel sieve to eliminate the embryos larger than the globular stage, and were then allowed to settle for 10 min. The supernatant was decanted carefully and 0.2 ml of packed globular embryos was transferred to medium containing 2.0 μM kinetin, BA, zeatin, or no cytokinin in 100-ml Erlenmeyer flasks. Five replicates were used per treatment. After 2 weeks, maturation of somatic embryos was observed. The experiment was repeated three times. The culture environment was as described above for embryo initiation.

Germination and plant regeneration

Cotyledonary-stage somatic embryos grown in a liquid medium without growth regulators were removed by forceps, and transferred to the same solidified MS medium in 50-ml Erlenmeyer flasks containing 30 ml liquid medium. Five embryos were placed in each flask. Five replicates were used for each treatment and the experiment repeated three times. After 2 weeks, germination (root elongation) of somatic embryos was observed. Germinating embryos about 10–20 mm in length were transferred to a semi-solid MS basal medium (0.5% agar) in 200-ml glass culture bottles containing 50 ml medium, for continued growth. One seedling was cultured in each bottle. After 2 months, morphologically normal plants with both shoot and root were counted. The culture environment for germination and subsequent growth was the same as that for callus induction. Plants with both shoots and roots, about 7–10 cm in height, were transferred to pots with a mixture of soil, sand and peat (4:4:3 vol/vol). Plants were covered with a glass beaker for 2 weeks, and then acclimatized to greenhouse conditions.

Statistical analysis of data

Results were subjected to analysis of variance, and significant differences between means calculated by Duncan's multiple-range tests (P=0.05).

Results and discussion

Embryogenic callus formation

Stem segments were cultured on medium containing auxins and cytokinins as shown in Table 1. Calli were observed from the cut ends of the stem segments after 2 weeks. The optimum amounts of callus were formed on media with auxin-cytokinin combinations, especially 4.5 μM 2,4-D with either 2.0 μM BA or kinetin (Table 1). The calli formed on all media were mucilaginous and translucent until 5 weeks after initiation (Fig. 1A). By prolonging culture to over 8 weeks, opaque, white, and friable embryogenic callus formed on medium with 4.5 μM 2,4-D and 2.0 μM kinetin or zeatin, and was highest on a medium containing 4.5 μM 2,4-D alone (Fig. 1B). Various stages of somatic embryos developed from embryogenic callus when left on the same medium for 10 weeks (Fig. 1C). Cotyledonary-stage somatic embryos developed into shoot clumps after transfer to a solid medium lacking 2,4-D (Fig. 1D). While callus formed on medium containing NAA alone or NAA in combination with cytokinins, only adventitious roots were formed. These results indicate that the auxin type was found to be critical for inducing embryogenic callus. Media containing 2,4-D alone were more efficient at induc-

Table 1 Effects of various concentrations and combinations of auxins and cytokinins on callus and somatic embryo formation from cultured stem segments of *Acanthopanax koreanum* after 10 weeks of culture. The total fresh weight of the three explants per flask at culture initiation was 50 mg. Weight data represent the mean values ±SE from three independent experiments. Within columns, values followed by the same lowercase letter are not significantly different using Duncan's multiple-range tests (P=0.05)

Growth regulators (μM)	Fresh weight of callus (mg)	Explants producing embryogenic callus and/or somatic embryos (%)	Explants producing roots (%)
2,4-D 0.45	230 ± 15	0 c	0 c
2,4-D 4.5	320 ± 20	12 a	0 c
2,4-D 22.5	265 ± 14	0 c	0 c
2,4-D 4.5 + BA 2.0	396 ± 25	0 c	0 c
2,4-D 4.5 + kinetin 2.0	405 ± 30	6 b	0 c
2,4-D 4.5 + zeatin 2.0	342 ± 23	4 b	0 c
NAA 0.45	74 ± 7	0 c	4 b
NAA 4.5	189 ± 11	0 c	14 a
NAA 22.5	204 ± 16	0 c	6 b
NAA 4.5 + BA 2.0	268 ± 18	0 c	0 c
NAA 4.5 + kinetin 2.0	307 ± 24	0 c	2 b, c
NAA 4.5 + zeatin 2.0	293 ± 21	0 c	0 c

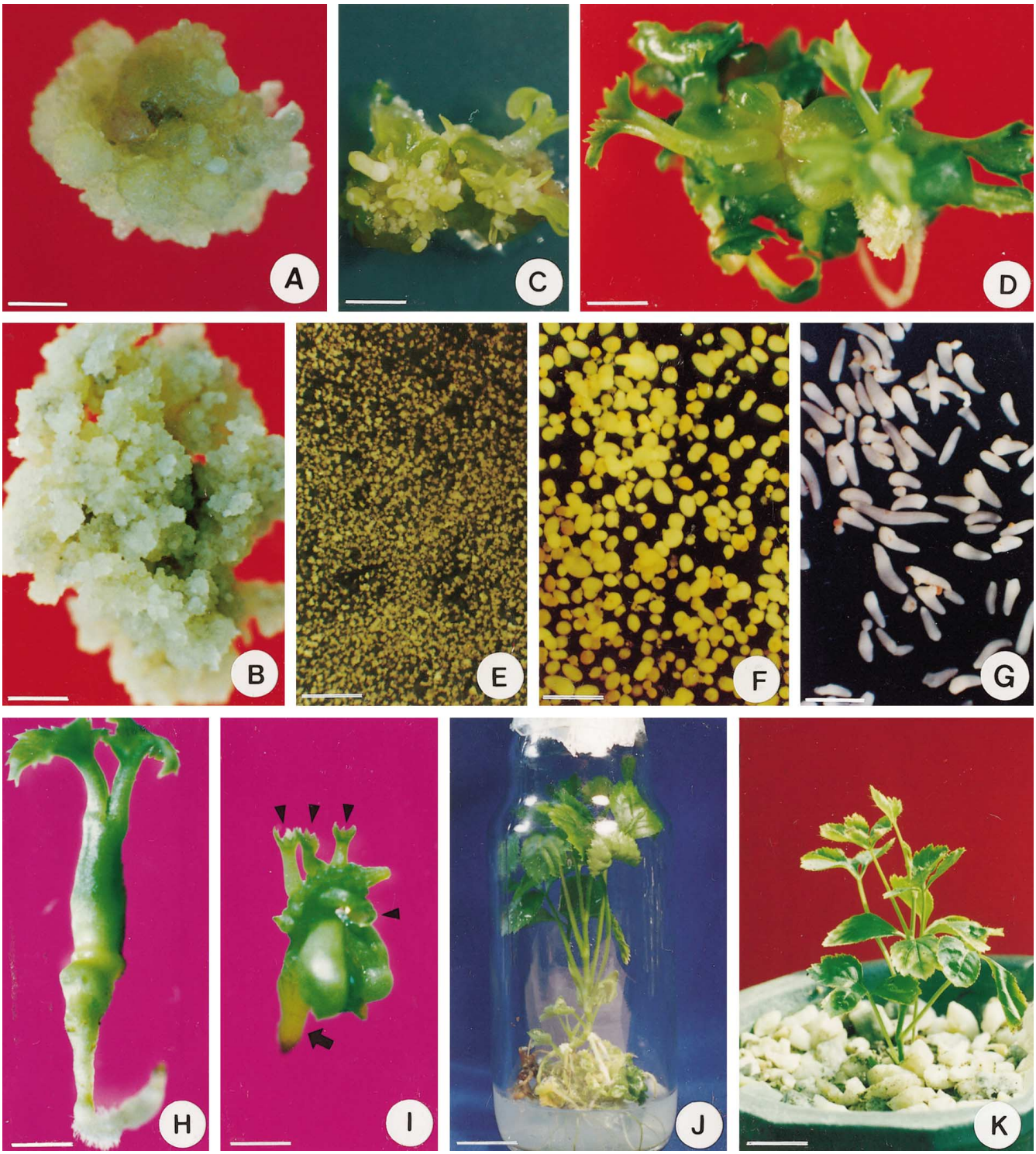

Fig. 1A–K Somatic embryogenesis from callus or cell suspension cultures derived from stem segments. **A** Translucent and mucilaginous callus formed from a stem segment on MS medium containing 4.5 μM 2,4-D after 5 weeks (*bar* 250 μm). **B** Embryogenic callus formed on medium containing 4.5 μM 2,4-D after 8 weeks (*bar* 200 μm). **C** Somatic embryos at various stages after 10 weeks (*bar* 300 μm). **D** Shoot clumps from somatic embryos after transfer to solid MS medium lacking growth regulators (*bar* 500 μm). **E** Embryogenic cell clumps before transfer to liquid MS medium containing 0.45 μM 2,4-D (*bar* 150 μm). **F** After 2 weeks, globular-stage embryos formed from the embryogenic cell clumps in liquid MS medium containing 0.45 μM 2,4-D (*bar* 150 μm). **G** Torpedo-stage embryos after transferring the globular embryos to a MS liquid medium lacking growth regulators (*bar* 350 μm). **H** Germinated somatic embryo on a solid medium lacking growth regulators (*bar* 350 μm). **I** Secondary somatic embryos (*arrowheads*) formed on the hypocotyl surface of primary somatic embryo (*arrow*) on a medium containing 2.0 μM BA (*bar* 350 μm). **J** Plantlet grown in a semisolid medium lacking growth regulators after 2 months (*bar* 0.8 cm). **K** Plantlet growing in the greenhouse 3 months after planting in soil (*bar* 2.5 cm)

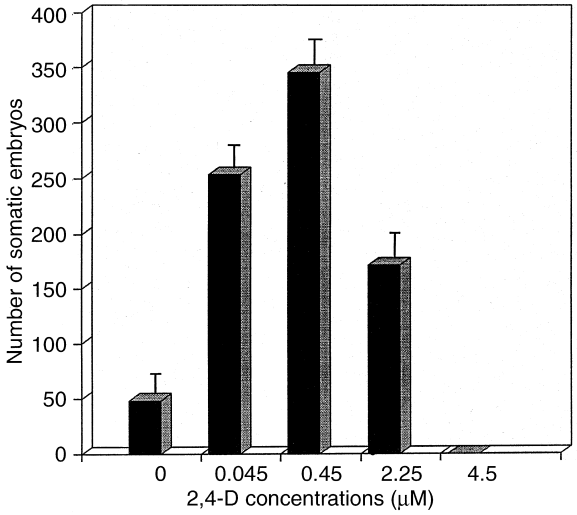

Fig. 2 Number of globular somatic embryos per flask from embryogenic cell suspension culture in liquid MS media containing various concentrations of 2,4-D. Packed cells (200 μl) were transferred to a 100-ml Erlenmeyer flask containing 30 ml liquid medium with five replications. Data were collected from three independent experiments (mean +SE)

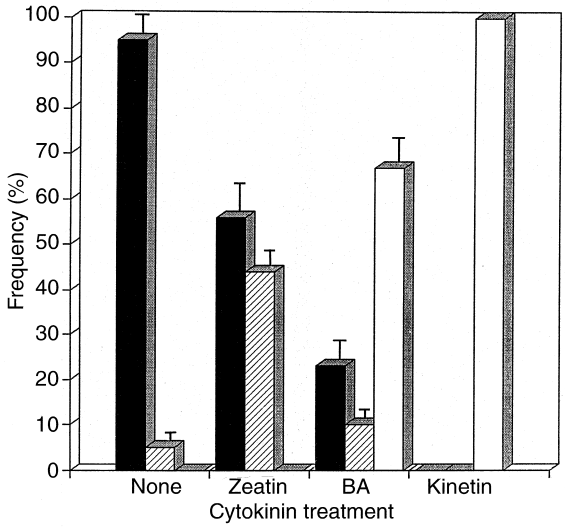

Fig. 3 Effects of cytokinins (2.0 μM) on the maturation of globular somatic embryos after 2 weeks. [■ normal cotyledonary embryos; ▨ embryo with secondary embryos; □ embryos without growth (browned)]. Packed globular embryos (200 μl) were cultured in a 100-ml Erlenmeyer flask containing 30 ml liquid medium. Results are expressed as the mean +SE

ing embryogenic callus than those combining 2,4-D with cytokinins, although the cytokinin combination stimulated the increase in the fresh weight of the callus (Table 1). A similar result was observed in *A. senticosus* (Gui et al. 1991), which direct somatic embryogenesis occurred on medium containing only 2,4-D but not on medium containing 2,4-D and BA.

Somatic embryogenesis from suspended cell clumps

Opaque, white, friable embryogenic calli were transferred to liquid MS medium containing 4.5 μM 2,4-D and maintained by subculture at 2-week intervals. The frequency of embryogenic callus formation from stem segments of *A. koreanum* was low. However, once the embryogenic callus was established in liquid medium, proliferation of embryogenic cell clumps actively occurred without loss of embryogenic competency through consecutive subcultures. To observe the effect of 2,4-D on somatic embryo development, the embryogenic cell clumps were transferred to media containing 0–4.5 μM 2,4-D. Somatic embryo development from embryogenic cell clumps occurred in medium containing low concentrations of 2,4-D (Fig. 1F). The number of globular somatic embryos was 350 per flask in the medium containing 0.45 μM 2,4-D (Fig. 2). With 2.25 μM 2,4-D, somatic embryos developed normally up to the globular stage, but subsequent development was suppressed and they eventually became callused. In 2,4-D-free medium, globular somatic embryos formed at a very low rate (Fig. 2). In carrot, growth regulators must be omitted for somatic embryo initiation from the embryogenic cell clumps (Halperin and Weherell 1964), but in *A. koreanum*, somatic embryo formation from embryogenic cell clumps required 2,4-D. This auxin requirement was similar to that of monocotyledonous plants, such as *Sorghum bicolor* L. (Wernicke and Brettell 1980), *Pennisetum americanum* L. (Vasil and Vasil 1982) and *Allium fistulosum* L. (Kim and Soh 1996).

Globular embryos were transferred to medium without growth regulators or with varied cytokinins (kinetin, zeatin, or BA). Continued culture of globular embryos in media containing more than 0.45 μM 2,4-D hampered maturation. In growth-regulator-free medium, 95% of the embryos matured (Figs. 1G, 3). However, in media containing kinetin or BA, embryos rapidly deteriorated and became brown. In medium containing zeatin, secondary embryos developed on the surface of the somatic embryos (Fig. 1I, arrowheads). In many plant species, cytokinins have been used to support the growth of somatic embryos (Ammirato 1983), but in *A. koreanum*, cytokinin treatment suppressed both maturation and germination of somatic embryos, while stimulating secondary embryogenesis.

Plant regeneration

When the cotyledonary-stage somatic embryos were transferred to MS agar medium lacking growth regulators or containing cytokinins (2.0 μM BA, kinetin, or zeatin), 91% germinated normally within 1–2 weeks (Fig. 1H). However, cytokinin treatment suppressed the germination of embryos, and stimulated secondary embryogenesis, similar to the results of cytokinin treatment of globular embryos (data not presented).

In some instances, for somatic embryos from plants such as *Eschscholzia californica* Cham *(*Kavathekar et al. 1977), *Vitis vinifera*×*V. rupestris* (Takeno et al. 1983) and

Panax ginseng C. A. Meyer (Lee et al. 1991), low temperature (or gibberellic acid) treatment is necessary for their germination because they are dormant, like the zygotic embryos of seeds. In seeds of *Acanthopanax* species, which belong to the same family as *P. ginseng*, stratification and dormancy breaking are prerequisites for normal germination of the zygotic embryos of seeds (Isoda and Shoji 1994). However, somatic embryos of *A. koreanum* were not dormant in contrast to those of *P. ginseng*.

In many of the germinated seedlings, shoot growth was not active, compared to root growth. To support the shoot growth of seedlings, they were cultured on a semisolid (0.5% agar) MS basal medium for 1 month (Fig. 1J). Of the plants, 85% developed normally with both shoots and roots, but in the others, shoots did not grow or secondary embryos formed. Normal plants with both shoots and roots were transferred to greenhouse soil and were successfully acclimatized (Fig. 1K).

In this report we have established high-frequency somatic embryogenesis through cell suspension cultures of *A. koreanum*. The procedure could be applied to generate mass propagation of this endangered medicinal plant.

References

Ammirato PV (1983) Embryogenesis. In: Evans DA, Sharp WR, Ammirato PV, Yamada Y (eds) Techniques for propagation and breeding. MacMillan New York, pp 82–123

Choi YE, Soh WY (1993) Structural aspects of somatic embryos derived from cultured zygotic embryos in *Acanthopanax senticosus* L. Korean J Plant Tissue Cult 20:261–266

Gui Y, Guo Z, Ke S, Skirvin RH (1991) Somatic embryogenesis and plant regeneration in *Acanthopanax senticosus*. Plant Cell Rep 9:514–516

Halperin W, Wetherell DF (1964) Adventive embryogeny in tissue cultures of the wild carrot, *Daucus carota*. Am J Bot 51:274–283

Isoda S, Shoji T (1994) Studies on the cultivation of *Eleutherococus senticosus* Maxim. II. On the germination and raising of seedling. Nat Med 48:75–81

Kavathekar AK, Ganapathy PS, Johri BM (1977) Chilling induces development of embryoids into plantlets in *Eschscholzia*. Z Pflanzenphysiol 81:358–363

Kim JW, Soh WY (1996) Plant regeneration through somatic embryogenesis from suspension cultures of *Allium fistulosum* L. Plant Sci 114:215–220

Lee HS, Leu JR, Yang SG, Lee YH, Lee KW (1990) In vitro flowering of plantlets regenerated from zygotic embryo-derived somatic embryos of ginseng. Hort Sci 25:1652–1654

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Takeno K, Koshioka M, Pharis RP, Rajasekran K, Mullins MG (1983) Endogenous gibberellin-like substances in somatic embryos of grape (*Vitis vinifera′Vitis rupestris*) in relation to embryogenesis and the chilling requirement for subsequent development of mature embryos. Plant Physiol 73:803–808

Vasil V, Vasil IK (1982) Characterization of an embryogenic cell suspension culture derived from cultured inflorescences of *Pennisetum americanum* (pearl millet, gramineae). Amer J Bot 69: 77–81

Wernicke W, Brettell R (1980) Somatic embryogenesis from *Sorghum bicolor* leaves. Nature 287:138–139