Induction of In Vitro Cultured Masses of Shoot Primordia of Hybrid Statice and Its Cryopreservation by Vitrification

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Abstract. Bulbous structures consisting of meristematic clumps (designated “shoot primordia”) were induced from a meristematic culture of a hybrid statice (*Limonium altaica* Mill. x *L. caspium* Mill., cv. Blue Symphonet). The shoot tips were cultured in 25 mL of liquid 1/2 Murashige & Skoog (MS) medium supplemented with 0.44 μM BA and 0.054 μM NAA and 3% sucrose at pH 5.8 by vertically shaking at 2 rpm on rotating stages (1 m in diameter) at 25 °C. One month after inoculation of shoot tips, numerous small globular structures were formed and propagated vegetatively at a high rate following subculture. Segments of shoot primordia had developed into plantlets 2 weeks after transfer to solidified 1/2 MS medium supplemented with 0.44 μM BA and 1% sucrose. Plantlets successfully acclimated and grew into normal plants in a greenhouse. Cold-hardened, precultured small segments of shoot primordia were successfully cryopreserved in liquid N by vitrification. Vitrified and warmed segments plated on solidified 1/2 MS medium produced shoots about 21 d after plating. Cultured masses of shoot primordia appear promising for large-scale production and cryopreservation of annual and biennial statice. Chemical names used: 6-benzyladenine (BA); 1-naphthaleneacetic acid (NAA).

Statice (*Limonium* Mill.) belongs to the family *Plumbaginaceae*. The chief genera are *Limonium* (≈ 300 species), which are cosmopolitan and are periodically found in dry or saline habitats, such as sea coasts and salt steppes in temperate and tropical zones (Heywood, 1978). Many species of *Limonium* are grown in borders and beds as cut flowers that can be dried and used toward providing “everlasting” decorations. Recently, many varieties of hybrid statice have been developed as ornamental plants, such as ‘Misty Blue’, ‘Blue Fantasia’, and ‘Blue Star’. However, these hybrid statice demonstrate a low efficiency in propagation, since the seedlings cannot be used for nursery multiplication. More recently, mass propagation of hybrid statice was developed with shoot tips (Harazy et al., 1985; Martin and Perez, 1992) and protoplast culture techniques (Kunitake and Mii, 1990).

Recently, we succeeded in inducing in vitro-cultured masses of shoot primordia using a hybrid statice ‘Blue Symphonet’. This culture may prove more efficient for mass propagation of hybrid statice than meristem cultures. “Shoot primordia” culture was first reported by Tanaka and Ikeda (1983) with *Haplopappus gracilis* (Nutt.) Gray. The mass of shoot primordia propagates vegetatively at a very high rate and readily regenerates plantlets by organogenesis.

Cryopreservation is a commonly used method for long-term storage of germplasm or experimental materials with unique attributes. Small segments of hybrid statice shoot primordia were successfully cryopreserved by vitrification. Here we report the induction for masses of hybrid statice shoot primordia and its cryopreservation.

We used in vitro-grown plantlets of hybrid statice (‘Blue Symphonet’). Original explants of in vitro growth for statice plantlets were maintained on modified MS medium (Murashige and Skoog, 1962) (half strength of NH_4NO_3 and KNO_3 , 1/2 MS), containing 0.44 μM BA plus 0.054 μM NAA, 3% (w/v) sucrose and 0.8% (w/v) agar powder for plant media (Wako Pure Chemical Industries, Osaka, Japan) at pH 5.8. The stock cultures were subcultured every 30 d. Tissue-cultured plantlets were grown on 75-mL medium in a 200-mL mericlone flask under cool-white fluorescent light (52 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ with a 16-h photoperiod at 25 °C). Shoot tips of ≈ 1 mm in diameter were dissected from 30-d-old plantlets. They were cultured in vials (30 $\times$ 200 mm, 30 mL) containing 1/2 MS medium, supplemented with a various concentrations of BA and NAA to make 25 separate media (Table 1). The shoot tips were incubated at 25 °C on rotating stages (1 m in diameter) at two cycles/min under continuous fluorescent illumination (80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). One month after inoculation of shoot tips, numerous dark-green shoot primordia formed and individual masses were ≈ 2 to 3 mm in diameter. A large globular mass of shoot primordia was divided into small segments (2 to 3 mm in diameter) and subcultured every 2 weeks. In preparation for histological observation of shoot primordia segments, specimens were dehydrated using a graduated ethyl alcohol series to absolute alcohol, and embedded in methacrylate. Sections (10 μm) were cut and stained with toluidine blue for observation.

Shoot primordia segments (≈ 2 mm in diameter) were cold-hardened in the same medium at 4 °C for 10 d by shaking at 2 rpm on rotating stages and then precultured on solidified 1/2 MS medium supplemented with 0.3 M sucrose at 25 °C for 16 h. For cryoprotection, 10 hardened and precultured segments were placed in a 1.8-mL cryotube and then loaded with a mixture of 2 M glycerol plus 0.4 M sucrose and held 20 min at 25 °C. Segments were sufficiently dehydrated by exposure to a highly concentrated vitrification solution (PVS2; Sakai et al., 1990) at 0 °C for various lengths of time. PVS2 contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol (EG), and 15% (w/v) dimethyl sulfoxide (DMSO) in a 0.4 M sucrose solution (pH 5.8). Finally, shoot primordia were resuspended in 1 mL of fresh PVS2, directly plunged into liquid N (LN),

Table 1. Structure produced by shoot tip culture of statice in 1/2 MS medium modified with several combinations of BA and NAA.

NAA (μM)	Structure produced			
	BA (μM)			
	0	0.044	0.44	22.2
0	B(10/10) ^a	BC(10/10)	SP(10/10)	C(10/10)
0.054	B(8/10)	BC(10/10)	SP(10/10)	C(10/10)
0.54	C(10/10)	BC(10/10)	SP(8/10)	C(10/10)
5.4	C(10/10)	C(4/10)	C(1/10)	---
27.0	---	---	---	---

^aNumber of shoot tips developed/number of shoot tips plated. B = branches; SP = shoot primordia; BC = bud clusters; C = callus; --- = No growth or death.

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and held for more than 1 h. After rapid warming in a water bath at 40 °C for 1 min, PVS2 was drained, replaced with 1.8 mL of a 1.2 M sucrose solution, and held for 20 min. Segments were then transferred onto sterilized paper disks in a petri dish containing the same medium. Shoot formation from vitrified and warmed segments was recorded as the percentage of the total number of segments forming normal shoots 21 d after plating.

Ten segments of shoot primordia were tested in triplicate for each experiment.

Results and Discussion

Ten masses of hybrid static shoot primordia were induced only in 1/2 MS medium that contained a combination of 0.44 μM BA with 0 to 0.54 μM NAA (Table 1). All other combinations produced precocious branches, bud clusters, or callus. The higher concentrations of BA and NAA produced callus and lower concentrations of BA produced bud clusters or branches. During 1 month of subculture, the mass of shoot primordia increased as much as ≈ 4 times (data not shown). Histological observation on segments of shoot primordia revealed many small rises shaped like a dome (Fig. 1A). These differentiated structurally into an outer single meristematic cell layer where the inner cell mass was composed of many large parenchyma cells (Fig. 1B). However, distinct bud formation was not evident on the cross sections, as described by others for multiple bud clusters (Banerjee et al., 1991; Kohmura et al., 1992). A mixture of 1% (w/v) sucrose and 0.44 μM BA produced the most shoots (Table 2). Plants successfully regenerated from a mass of shoot primordia segments within 21 d after transfer to solidified 1/2 MS medium supplemented with 1% sucrose and 0.44 μM BA (Fig. 2). Most of the plants rooted after 30 d of reculturing on solidified hormone-free 1/2 MS medium, acclimated, and successfully grew into normal plants in a greenhouse. The ability to regenerate whole plants is a vital objective of *in vitro* tissue-culture techniques and is necessary for the application of molecular and somatic genetics for plant improvement and for efficient germplasm conservation.

Exposure to PVS2 to 0 °C for various lengths of time resulted in a variable rate of shoot formation following vitrification (Fig. 3). The highest rate of shoot formation was

Table 2. Number of static shoots regenerated from each mass (5 mm in diameter) of shoot primordia segments 14 d after transfer to solidified 1/2 MS medium supplemented with several combinations of BA and sucrose.

BA (μM)	Shoots regenerated (no.)	
	Sucrose (%) (w/v)	
	1	3
0	7.6 a ^c	4.0 e
0.44	18.0 b	7.8 a
4.44	5.9 c	2.8 d
22.2	2.9 d	3.3 f

^cMean separation at $P \leq 0.05$ by Duncan's multiple range test. Values represent ≈ 10 segments each, tested in triplicate.

Fig. 1. (A) Clump of dark-green bulbous (globular) structures consisting of numerous meristematic clusters designated "shoot primordia." (B) Thin cross section of shoot primordia segment. Bar = A: 5 mm, B: 50 μm .

Fig. 2. Developmental progress of static through to shoot primordia. (A) Mass of shoot primordia. (B, C) Developmental stage of shoot primordia. (D) Whole plantlet. Bar = A–C: 2 mm, D: 20 mm.

Fig. 3. Effect of exposure to a vitrification solution (PVS2) at 0 °C on shoot formation for shoot primordia segments cooled to -196 °C by vitrification. Precultured and loaded segments were treated with PVS2 for various lengths of time before immersed into liquid N. About 10 segments were tested in triplicate for each value.

Fig. 4. Shoot formed from shoot primordia cryopreserved by vitrification, 21 d after reculture. Bar = 3 mm.

obtained when the segments of shoot primordia treated with PVS2 for 60 min at 0 °C. Successfully vitrified and warmed segments of shoot primordia turned brown, but recovered within 7 d and eventually developed shoots 20 to 25 d after plating (Fig. 4). The vitrification procedure for cryopreservation eliminates the need for controlled slow freezing and permits cells and meristems to be cryopreserved by direct transfer to LN (Sakai et al., 1991). Vitrification is a potentially valuable cryogenic protocol for cryopreserving cells (Sakai et al., 1990, 1991; Uragami et al., 1989) and meristems (Langis et al., 1990; Matsumoto et al., 1994, 1995; Niino et al., 1992; Reed, 1992; Towill, 1990). With vitrification, exposure of cells and meristems to a highly concentrated vitrification solution leads to harmful effects

resulting from osmotic stress or chemical toxicity. In the present study, these harmful effects were overcome by preconditioning segments using a cold-hardening, preculturing and cryoprotection (collectively known as a loading treatment).

Cultured masses of shoot primordia can be divided into small segments to provide adequate material for cryopreservation. Thus, shoot primordia appear promising for mass propagation and cryopreservation of 'Blue Symphonet' statice. Further study is necessary to induce and cryopreserve the cultured shoot primordia in other hybrid statice.

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