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Cryopreservation of encapsulated gentian axillary buds following 2 step-preculture with sucrose and desiccation

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Abstract

Alginate beads containing axillary buds of *in vitro*-grown gentian (*Gentiana scabra* Bunge var. *buergeri* Maxim.), were successfully cryopreserved following 2 step-preculture with sucrose and desiccation. The optimal preculture conditions were as follows: axillary buds were excised from *in vitro*-grown gentian plants and precultured on semi-solid Murashige and Skoog (MS) medium containing 0.1 M sucrose for 10 days (25 °C, 16-h photoperiod) (first step). This was followed by incubation on semi-solid MS media containing 0.4 M (1 day) and then 0.7 M sucrose (1 day) (second step). After preculture, the buds were encapsulated in alginate beads and desiccated aseptically on silica gel for 9 h to a water content of 10% (fresh weight basis), followed by immersion in liquid nitrogen (LN). With this protocol, 87% of the gentian buds survived exposure to LN and showed normal development of shoots and roots *in vitro* and *in vivo*. Depletion of NH_4NO_3 in the regeneration medium did not improve survival following desiccation and exposure to LN. The results show that 2 step-preculture with sucrose is effectively applicable in encapsulation–desiccation based cryopreservation of gentian axillary buds. This preculture can replace the conventionally used lengthy cold-hardening treatment and is useful for routine cryopreservation of gentian germplasm.

Abbreviations: LN – liquid nitrogen; MS medium – Murashige and Skoog (1962) medium

Introduction

Gentian (*Gentiana scabra* Bunge var. *buergeri* Maxim.) is a perennial herb that has gardening, ornamental and medicinal values and is commercially propagated using seeds due to difficult multiplication by stem cuttings. Therefore, to maintain genotypes, propagation of clones *in vitro* is necessary and cryopreservation of such clones would allow long-term conservation of gentian genetic resources (Grout, 1995).

Previously, we developed an efficient cryopreservation protocol for gentian axillary buds of *in vitro*-grown plants using a desiccation method (without encapsulation). There, a 2 step-preculture with sucrose was the key step in the protocol. This allowed the gentian buds to tolerate extreme desiccation to a water content of 10% (fresh weight basis) and to survive cryopreservation (Suzuki et al., 1998).

Encapsulation–desiccation methods were first used in cryopreservation by Dereuddre's group (Dereuddre et al., 1990). Encapsulation of explants in alginate beads for cryopreservation has some merits over non-encapsulated ones: enhanced protection of dried material from mechanical and oxidative stress during storage and easier handling of small sample during the pre- and post-cryopreservation procedures (Niino and Sakai, 1992). Therefore, it has been applied for cryopreservation

of many plant species such as grape (Plessis et al., 1991), *Citrus* (Gonzalez-Arnao, 2003), kiwifruit (Suzuki et al., 1994), apple, pear, mulberry (Niino and Sakai, 1992), rape (Uragami et al., 1993), and sweet potato (Bhatti et al., 1997).

The objective of this study was to verify if the 2 step-preculture with sucrose could be applied for inducing desiccation tolerance in encapsulated gentian axillary buds and to optimize an encapsulation–desiccation protocol for gentians. The effect of removing NH_4NO_3 from the regeneration medium on the recovery of cryopreserved explants were also determined.

Materials and methods

Plant materials

In vitro nodal stem segments of gentian containing axillary buds were propagated on half strength semi-solid MS medium (25 °C; 16-h photoperiod) as previously described (Suzuki et al., 1998).

Preculture and encapsulation

Two step-preculture with sucrose was performed following the method of Suzuki et al. (1998). In the first step, nodal stem segments (approximately 2 mm) with axillary buds (excised from 60-day-old *in vitro*-grown plantlets), were precultured for various periods (1–20 days) on full strength semi-solid MS medium containing 0.1 M sucrose and 8 g l^{-1} agar (pH 5.8) at 25 °C and a 16-h photoperiod (50 μmol s^{-1} m^{-2}). In the subsequent step, these segments were consecutively cultured on full strength semi-solid MS media supplemented with 0.4 M sucrose for 1 day, followed by 0.7 M sucrose for 1 day (25 °C; under the same light conditions). Precultured nodal stem segments with axillary buds (hereafter referred to just as axillary buds or buds) were subjected to encapsulation, desiccation and cryopreservation (Figure 1).

Precultured axillary buds were suspended in liquid MS medium (without $CaCl_2$) containing 3% (w/v) sodium alginate and 0.5 M sucrose. An aliquot of this medium containing one single bud, was dropped into the solidifying solution (0.1 M $CaCl_2$, 0.5 M sucrose) using an appropriate pipette and left there for 30 min at 25 °C. This allowed the formation of an alginate bead (diameter: 4 mm) containing one single axillary bud. Before desiccation, the beads were incubated in liquid MS medium containing 1 M sucrose for 16 h at 5 °C.

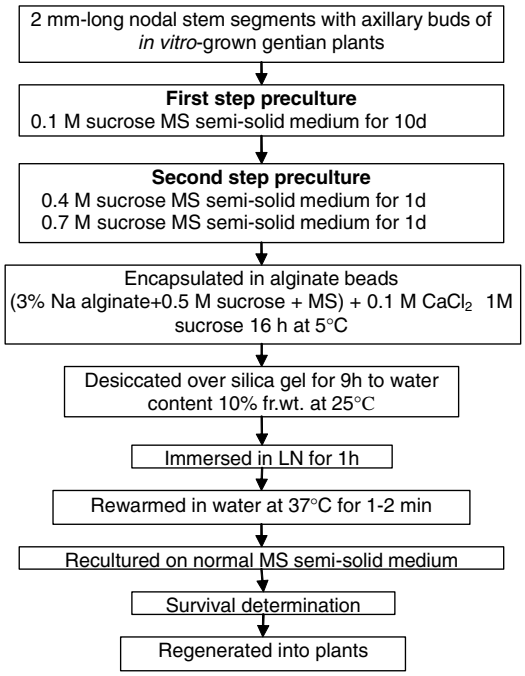

Figure 1. A schematic representation of the experimental procedures including a 2 step-preculture method used in this study.

Desiccation and LN immersion

After removing the excess of medium, the alginate beads were transferred to a drying apparatus: filter paper (9 cm diameter) was layered on 50 g of silica gel in a Petri dish. The drying apparatus was previsouly pre-sterilized by heating at 110 °C for 16 h. The Petri dish (with lid) was hermetically sealed and left in a laminar flow hood for various times (0–24 h; 25 °C). Following desiccation, approximately 10 dried beads were transferred to a 10-ml conical glass centrifuge tube (Maruemu NS-10) and submerged directly into LN and stored for at least 1 h, before being warmed by shaking the centrifuge tube in a water bath at 37 °C for 1–2 min. Glass tubes were used for clearly viewing samples inside and did not significantly affect the survival rates after cryopreservation compared with plastic cryotubes. The water content of dried

beads containing buds was determined after oven-drying at 70 °C for 48 h.

Determination of the survival rate of encapsulated axillary buds

The encapsulated buds subjected to desiccation alone or to desiccation and cryopreservation, were placed on full strength semi-solid MS medium containing 0.088 M sucrose and were recultured under a 16-h photoperiod at 25 °C for 1 week. In experiments for determining the effect of the NH_4NO_3 concentration in MS medium on survival and regeneration (as described in the literature: Kuriyama et al., 1990; Kikuchi and Yashiro, 1995), some sets of cryopreserved beads were also cultured on NH_4NO_3-free, full strength semi-solid MS medium to compare survival rates. After 1 week of reculture following cryopreservation, the encapsulated buds were transferred to fresh ½ MS medium (with 0.044 M sucrose) and incubated under a 16-h photoperiod at 25 °C for 60 days to verify if they grew into normal plants. The survival rate of encapsulated axillary buds was defined as the percentage of buds producing normal shoots and roots after 20 days of reculturing.

Each experiment was replicated at least three times (each using 10–11 explants for each data point) and expressed as the mean ± SE.

After 60 days of reculturing, some regenerated plantlets *in vitro* (300 or more) were transferred to soil following an appropriate process for acclimating the plants to lower humidity conditions and grown for 2 years to check abnormalities during further growth and flowering.

Results

Changes in the water content of the alginate beads during drying

A typical example of the changes in the water content during drying over silica gel of alginate beads encapsulating 2 step-precultured axillary buds is shown (Figure 2a). The initial water content of beads (containing one single axillary bud) was approximately 62% (fresh weight basis), but rapidly decreased to 12% within the first 6 h and then gradually decreased to approximately 10% after 9 h and 6% after 24 h.

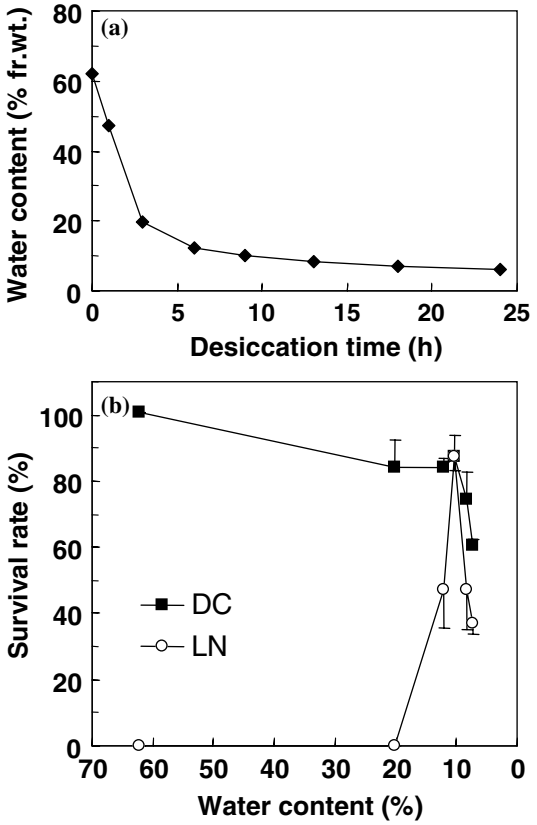

Figure 2. (*a*) A typical desiccation curve of alginate beads containing axillary buds (1 bud/bead). Nodal stem segments with axillary buds were precultured (0.1 M sucrose for 10 days (Step1), then 0.4 M sucrose and 0.7 M sucrose for 1 day each at 25 °C (Step 2)), encapsulated in alginate beads and desiccated for up to 24 h at 25 °C over silica gel in a Petri dish. Ten to eleven axillary buds were sampled for each data point (most SE were smaller than the simbols and not shown). (*b*) Survival rates of encapsulated axillary buds, desiccated to various water contents and subsequently frozen in LN. The results for beads undergoing desiccation alone (DC; not frozen) or desiccation and LN exposure (LN), are presented. The data are the mean ± SE of three replicates (10–11 buds for each replicate).

Water content of the beads encapsulating axillary buds vs. survival before and after exposure to LN

The survival before and after exposure to LN of encapsulated axillary buds (with various water contents) is shown in Figure 2b. The survival rate of the non-frozen explants, was 100–85% in a water content range of 62–10% and then abruptly decreased with further desiccation (water content: 8–7%). Survival after exposure to LN was only attained with beads having a water content of 12%

and less. The maximum survival rate (87%) was obtained with buds having a water content of 10% (dried for 9 h). Beads with a water content of 8–7% had reduced survival rates both before and after cryopreservation.

Effect of the preculturing conditions on the survival after drying and exposure to LN

The effect of the first preculture step (0.1 M sucrose) on the survival of encapsulated axillary buds after drying and cryopreservation was investigated (Figure 3). The axillary buds precultured with 0.1 M sucrose for 3–20 days before transferring them to media containing 0.4 and 0.7 M sucrose showed high survival rates (74–94%) following encapsulation and desiccation for 9 h to water content of 10%. High survival rates following LN exposure were achieved when explants were precultured for more than 7 days on 0.1 M sucrose. The highest survival rate (87%) was attained with the first preculture period of 10 days. Longer periods for the first preculture (12–20 days) resulted in gradually reduced survival rates (78–60%) after cryopreservation.

Effect of NH_4NO_3 contained in the regeneration medium on survival

The beads encapsulating axillary buds were desiccated to a water content of 10% and immersed in LN (the optimal conditions for survival). Then the beads were recultured on medium with or without NH_4NO_3 to see the effect of NH_4NO_3 removal on the regeneration. The results showed that removal of NH_4NO_3 did not affect survival of desiccated buds but slightly reduced survival of cryopreserved buds (Table 1).

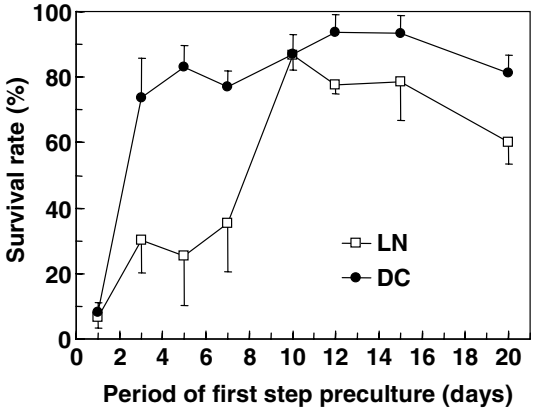

Figure 3. Effect of the first preculture step (0.1 M sucrose) on the survival of encapsulated gentian axillary buds after desiccation and immediately before (DC) and after exposure to LN (LN). After the first preculture step (various periods; 2–20 days), the buds were precultured in 0.4 M and 0.7 M sucrose (1 day each), and then encapsulated before being dried to a water content of 10% (9 h) and submerged in LN. The data are the mean ± SE of three replicates (10–11 buds for each replicate).

Shoot development from the cryopreserved buds

Successfully cryopreserved explants could be visually detected after 3 days, resumed growth after 7 days reculturing (Figure 4a), developed normal shoots and roots within 14 days without callus formation and grew into plants *in vitro* (Figure 4b). No signs of abnormalities were observed in the regerated plants *in vitro* (Figure 4b) and in the plans transferred to *in vivo* conditions (Figure 4c, d).

Discussion

In cryopreservation protocols using desiccation, survival is often increased by encapsulation of the sample; this was reported for rapeseed microspore embryos and apple shoot tips (Niino and Sakai, 1992; Uragami et al., 1993). In the case of gentian, the optimal water content (10%) and the maximum survival rates (87–90%) after cryopreservation with or without encapsulation, were similar (Suzuki et al., 1998 and the present study). But the optimum window of the water content for successful cryopreservation was more narrow for encapsulated buds than buds without encapsulation and a precise control of the water content is

Table 1. Effect of ammonium nitrate in regeneration medium on survival of alginate coated axillary buds of gentian after preculture, desiccation and immersion in LN

Regenaration medium	Survival rates (% ± SE)	
	DC	LN
Normal MS medium	83.7 ± 3.0	85.7 ± 8.2
NH_4NO_3-free MS medium	80.0 ± 8.8	67.4 ± 10.3

Following 2 step-preculturing, encapsulated gentian buds were desiccated to water content 10% (DC) and exposed to LN (LN) as shown in Figure 1 and 2. The beads thus treated were recultured on either full strength MS medium or NH_4NO_3-free MS medium containing 0.088 M sucrose and 8 g l^{-1} under a 16-h photoperiod at 25 °C. The data are mean ± SE of three replicates.

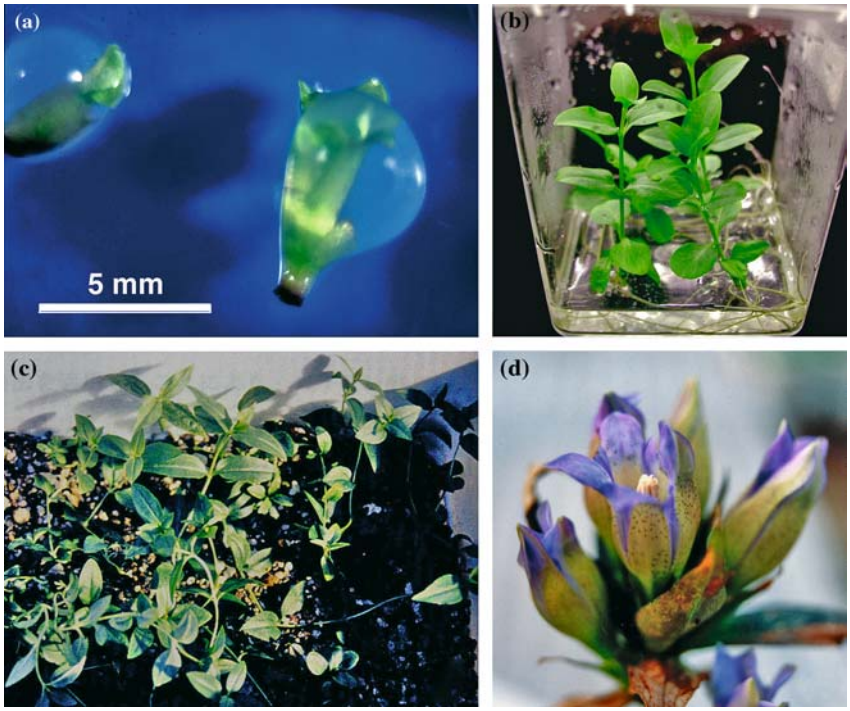

Figure 4. Regenerating explants encapsulated in alginate beads that were cryopreserved following 2 step-preculture, encapsulation and desiccation (9 h), subsequently recultured for 7 days (*a*), transferred to 1/2 MS medium and grown *in vitro* for 60 days (*b*). Some of the regenerated plants were transferred to soil and formed normal plants in 90 days (*c*) and flowers after 2 years (*d*).

required, which is similar for apple shoot tips (Niino and Sakai, 1992). The optimal effect of the first preculture step was observed after a longer period in encapsulated gentian buds (10–15 days) compared to naked buds (8–13 days). The reasons for these differences in the optimal conditions remain unclear. When handling of desiccated buds is taken into account, alginate bead-encapsulated buds are superior as naked buds often became fragile. Alginate bead coating would also be beneficial for protecting the explants from mechanical and oxidative stresses during storage in a similar manner that seed coats protect the embryos in seeds. For both of the methods (with or without encapsulation), the 2 step-preculture with sucrose was effective to induce desiccation tolerance and useful for cryopreserving gentian axillary buds.

Kikuchi and Yashiro (1995) used medium without NH_4NO_3 as regeneration medium for cryopreserved, encapsulated gentian buds. Our results showed that removing NH_4NO_3 from the regeneration medium did not improve survival (Table 1) and regeneration (data not shown) of cryopreserved, encapsulated buds. It has been reported that NH_4NO_3 in regeneration medium tends to retard or inhibit recovery or regeneration of cryopreserved cells or tissues (Kuriyama et al., 1990). Such a deteriorating effect of NH_4NO_3 was not observed in the regeneration of cryopreserved, encapsulated gentian buds. Therefore, to simplify the protocol, we used normal MS medium (with NH_4NO_3) for regeneration.

Cold-hardening has been reported to be important for successful cryopreservation of most *in vitro*-grown, cold-hardy plants such as apple, pear, *Rubus*, *Prunus*, kiwifruit, birch and mulberry (Reed and Ragerstedt, 1987; Dereuddre et al., 1990; Niino and Sakai, 1992; Suzuki et al., 1994; Brison et al., 1995; Ryynanen, 1998; Wu et al., 1999; Chang and Reed, 2000). Gentians fall into this category of plants. Cryopreservation of gentian axillary buds using an encapsulation–desiccation procedure has already been performed with cold-hardened buds (10 days at 15 °C and 24 days at 5 °C) after preculture for 2 days in 0.7 M sucrose (Kikuchi and Yashiro, 1995). More recently, Tanaka et al. (2004) have cryopreserved gentians

by using a vitrification protocols after cold-hardening for 20–30 days at 5 °C and subsequent preculturing for 1–2 days in 0.3 M sucrose.

The successful cryopreservation of gentian axillary buds we described using encapsulation–desiccation, shows that the first preculture step (0.1 M sucrose for 10 days at 25 °C) can replace cold-hardening. This greatly reduces the total preculture period and reduces the cost, energy and facilities required. The optimal survival rate obtained (87%) with our protocol was higher than the result obtained (80%) using cold-hardening by Kikuchi and Yashiro (1995). Moreover, the 2 step-preculture was applicable to cryopreservation of gentian buds by vitrification (data not shown). We are currently applying this preculture technique to cryopreserving some perennials that require lengthy cold-hardening treatments such as kiwifruits and *Paulownia*. Physiological changes in gentian buds during the 2 step-preculture that confer tolerance to desiccation and cryopreservation are also being investigated (Suzuki et al., 2005).

In conclusion, we developed a protocol using 2 step-preculture with sucrose that successfully induces desiccation tolerance and allows subsequent cryopreservation of gentian axillary buds. This protocol is based on encapsulation–desiccation and will be an asset to routine cryopreservation of gentian germplasm together with the desiccation (Suzuki et al., 1998), vitrification and encapsulation-vitrification protocols (Tanaka et al., 2004). Successfully cryopreserved gentian buds in beads regenerated shoots and roots without callus formation and showed normal growth and development *in vitro* and *in vivo*.

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