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Cryopreservation of carnation (*Dianthus caryophyllus* L.) shoot tips by encapsulation-vitrification

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Abstract

Shoot tips excised from in vitro cultured plants of *Dianthus caryophyllus* L. (cv. Pallas, cv. Pink Candy and cv. Wanessa) were successfully cryopreserved using an encapsulation-vitrification method. Shoot tips (2–3 mm in length) were encapsulated in sodium alginate, precultured on liquid Murashige and Skoog (1962) medium supplemented with various sucrose concentrations (0.25, 0.5, 0.75, 1.0 M) for 24 h or 48 h and dehydrated with the vitrification solution PVS2 (up to 4 h) at 24 °C or 0 °C prior to direct immersion in liquid nitrogen (−196 °C). A maximum of shoot regeneration from cryopreserved shoot tips was obtained with the following combinations: preculture in 0.5 M sucrose and 180 min dehydration treatment at 0 °C for cv. Pallas (60% shoot formation), or preculture in 0.75 M and 200 min dehydration at the same temperature for cv. Pink Candy (66.6% shoot formation) and cv. Wanessa (73% shoot formation).

Keywords: Conservation; *Dianthus*; Encapsulation; Shoot tip; Liquid nitrogen; Vitrification

1. Introduction

Over 300 *Dianthus* species have been identified (Galbally and Galbally, 1997). Various species of the genus *Dianthus* (*Caryophyllaceae* family) (Tutin et al., 1993) are known in the horticultural literature carnations and pinks.

In recent years, considerable work has been done on various aspects of *Dianthus* tissue culture, genetic engineering, disease resistance and storage of plant material. Among the traits that have received attention are: (a) induction of direct somatic embryogenesis and plant regeneration from leaf explants (Yantcheva et al., 1999); (b) in vitro fertilization (Hoshino et al., 2000); (c) Agrobacterium-mediated transformation (Lim et al., 2005); (d) disease resistance of plants (Tanaka et al., 2005) or (e) floral scent (Jürgens et al., 2003).

Cryopreservation has an enormous potential for the long-term storage of plant genetic resources (Engelmann, 1997). Cryogenic procedures such as vitrification, encapsulation-dehydration and encapsulation-vitrification have been developed and the number of species or cultivars that have been cryopreserved has sharply increased (Sakai, 1997). The encapsulation-vitrification procedure derived from the encapsulation-dehydration technique which was developed for cryopreservation of pear shoot-tips (Dereuddre et al., 1990). During this procedure the shoot tips are encapsulated in alginate beads, cultured for different times on medium with sucrose, dried (in silica gel or under sterile air flow) and directly transferred to liquid nitrogen. In the encapsulation-vitrification technique the alginate coated shoot tips are treated with a vitrification solution (Tannoury et al., 1991; Dereuddre and Tannoury, 1995; Hirai et al., 1998; Wang et al., 2005).

Cryopreservation of carnation was first carried out by Seibert (1976). In this procedure prior freezing shoot tips were precultured in the dark at 26 °C; the regrowth level after storage in liquid nitrogen was only 2%. A combination between hardening of donor plants at 4 °C (Seibert and Wetherbee, 1977) and slow cooling (Uemura and Sakai, 1980) improved considerably survival after cryopreservation and 70–80% shoot formation was obtained. A further improvement (90% shoot regrowth) was achieved using a two-step cooling (Dereuddre et al., 1987) or a slow-cooling procedure (Fukai, 1989).

The objective of this study was to investigate the effects of preculture (sucrose concentration and treatment duration), the

Abbreviations: BA, N^6-benzyladenine; IAA, indole-3-acetic acid; MS, Murashige and Skoog (1962) medium; LN, liquid nitrogen; PVS2, plant vitrification solution 2

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exposure to the vitrification solution (duration and temperature of treatment) and their interaction on shoot regrowth from encapsulated shoot tips of three *Dianthus caryophyllus* L. cultivars (cv. Pallas, cv. Pink Candy and cv. Wanessa) following cryopreservation using the encapsulation-vitrification method.

2. Materials and methods

2.1. Plant material

In vitro grown *Dianthus caryophyllus* L. (cv. Pallas, cv. Pink Candy and cv. Wanessa) plants were selected for cryopreservation in this study. Stock cultures of the three cultivars were cultured on Murashige and Skoog (1962) (MS) basal medium supplemented with 1.5 mg/l N^6-benzyladenine (BA), 0.5 mg/l indole-3-acetic acid (IAA), 20 g/l sucrose and 7 g/l agar (Sigma) (noted C-medium). The pH was adjusted to 5.7 before autoclaving. The plants were grown at 24 °C during a 16 h light photoperiod with a light intensity of 40 μmol/m^2 s photosynthetic active radiation (PAR) provided by cool white fluorescent tubes. For micropropagation nodal segments consisting of a piece of stem about 10 mm in length with two leaves were transferred to C-medium and incubated as mentioned above. Subcultures of the plants were performed every 4 weeks.

2.2. Explants

Shoot tips (apical dome with 2–4 leaf primordia) 2–3 mm in length, were excised from 1 to 2 months old in vitro grown plants using two hypodermic needles under a stereo microscope in sterile conditions.

2.3. Encapsulation, preculture, dehydration and cryopreservation

The shoot tips were individually encapsulated in alginate beads by transferring them with a pipette from a 3% solution of sodium alginate in Ca^{2+}-free MS medium to a solution of 1.0 M $CaCl_2$ plus MS mineral salts, where they remained for 20 min with gentle stirring. After 20 min polymerisation the beads (about 3 mm in diameter) were washed with liquid MS medium (pH 5.7) and then incubated in MS medium containing various sucrose concentrations: 0.25, 0.5, 0.75 and 1.0 M for 24 h or 48 h on a rotary shaker (98 rpm) at 24 °C. Subsequently the beads were treated with the vitrification solution PVS2 (Sakai et al., 1990) for different times (up to 4 h) at 24 °C or 0 °C. Following PVS2 treatment, the beads were placed (5 beads/cryovial) in 2 ml cryovials without cryoprotectant medium and directly immersed into liquid nitrogen (−196 °C) contained in a 10 l Dewar flask. Samples remained in liquid nitrogen for 24 h.

2.4. Growth recovery after cryopreservation

Rewarming of samples was performed in liquid MS medium at 24 °C by transferring the beads into 5 ml of liquid MS medium. Recovery of encapsulated, dehydrated and cryopreserved shoot tips took place in Petri dishes (5 cm diameter) on a modified (with 3 g/l agar) C-medium (= semisolid medium) under standard illumination conditions. Encapsulated and dehydrated but not frozen explants were used as control. Encapsulated shoot tips were maintained in Petri dishes until they started to regrow. Thereafter elongated shoots longer than approximately 10 mm were transferred in glass tubes (2.5 cm × 10 cm) on normal solidified (7 g/l agar) C-medium to regenerate whole plants.

2.5. Moisture content of the beads

The moisture content of samples was determined as follows: the fresh weight of 10 beads/treatment was measured after sucrose preculture and dehydration with PVS2. For determination of the dry weight the beads were oven dried at 60 °C until constant weight was attained (48 h). Moisture content of the beads on each treatment was calculated from these values and expressed as the percentage of moisture content over fresh weight. Beads were weighed singly on an analytical balance. Moisture content determinations were related to the entire bead. Controls consisted of beads which were not pregrown in sucrose and were not dehydrated.

2.6. Root formation medium

Shoots (approx. 2–3 cm in length) regenerated from encapsulated, dehydrated and cryopreserved shoot tips were used for rooting and were transferred to MS medium supplemented with indole-3-acetic acid (IAA) in different concentrations (0.1, 0.5, 1.0 and 1.5 mg/l) to improve the formation of roots. After 4 weeks in culture, the percentage of root formation, the number and length of the formed roots were recorded.

2.7. Analysis of results

Shoot regeneration was assessed 30 days after thawing. For evaluation of the regrowth level after cryopreservation, only direct shoot regeneration was considered. A number between 10 and 12 shoot tips were used for each of the three replications per treatment. The results were expressed as the mean ± standard deviation (S.D.). Data were analysed by two-way analysis of variance (ANOVA) using the Tukey test for data comparison.

3. Results

In a preliminary experiment shoot tips were encapsulated in alginate beads and plated on the semisolid C-medium for shoot formation. Encapsulated shoot tips of all tested cultivars resumed growth within 7 days after plating and developed normal shoots without callus formation within 21 days. Encapsulation did not influence survival and regrowth although shoot growth was slower. The shoot regeneration percent for non-encapsulated shoot tips was 100% for all cultivars. For encapsulated shoot tips 97.2 ± 4.8% (for cv. Pallas), 94.4 ± 4.8% (for cv. Pink Candy) and 97.2 ± 4.8% (for cv. Wanessa) resumed growth. There was no significant difference in the regeneration percent between non-encapsulated and encapsulated shoot tips.