Cryopreservation of Christmas bush (*Ceratopetalum gummiferum* Ms.) by encapsulation-dehydration method.

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Abstract

Shoot tips of Christmas bush (*Ceratopetalum gummiferum* Ms.) derived from *in vitro* cultures were successfully cryopreserved by the encapsulation-dehydration method. The excised shoot tips were placed for 1 day on hormone free Murashige and Skoog (MS) medium with 0.3 M sucrose. After encapsulation, the beads were precultured on MS liquid medium with 0.5-0.75 M sucrose on the agitator shaker. Beads containing the shoot tips were then dehydrated in the Laminar Air Flow cabinet before being preserved in liquid nitrogen. Our result gives us an easy protocol to preserve *in vitro* propagated Christmas bush.

Key Words

Christmas bush, *Ceratoetalum gummiferum* Ms, cryopreservation, encapsulation-dehydration, germplasm storage, *in vitro* preservation, rapid freezing

Introduction

Christmas bush (*Ceratopetalum gummiferum* Ms.) is a native to New South Wales. It is usually propagated by cuttings or by seeds. Cuttings can be difficult to obtain and take a long time to root. Seed propagation can result in undesirable variation in calyx colour, size and time to flowering (Armstrong *et al.*, 1999; Armstrong and Johnson, 2001). Tissue culture has an advantage over the other propagation systems, and provides rapid clonal propagation. It can also provide material used for germplasm storage (Engelmann, 1997).

In vitro techniques are used for both conservation and storage of plant genetic resources. *In vitro* storage can be performed for short and long term conservation (cryopreservation). Cryopreservation proved to be an amenable method for long-term preservation of biological material where the minimum space is used (Dussert *et al.*, 1991; Engelmann, 1997; Chang and Reed, 2000; Turner *et al.*, 2001).

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The development of a simple and reliable method for cryopreservation allows for much more widespread use of cryopreserved cells, meristems and somatic embryos (Matsumoto and Sakai, 1995). Engelmann (1997) reported that cryopreservation has been developed for almost 80 different plant species cultivated in various form and for different parts of plants, and more have been developed in recent years.

Cryopreservation using alginate coated shoot tips has been applied to several important fruit crops, such as apple, pear and grape (Niino and Sakai 1992; Matsumoto and Sakai, 1995). The success of encapsulated methodology depends upon desiccation tolerance, and the ability to circumvent ice formation during cooling and warming steps in the process (Matsumoto and Sakai, 1995). This study was conducted to define a successful potential methodology for cryopreservation of *in vitro* grown Christmas bush.

Materials and Methods

Plant Material

In vitro plant material of Christmas bush was obtained from the University of Technology, Sydney (Armstrong and Johnson, 2001). Cultures were maintained on MS (Murashige and Skoog, 1962) medium containing 2.2 µM benzyl adenine (BA) and 0.5 µM indole acetic acid (IAA) and 3% sucrose. Medium was solidified with 0.7% gelcarin (GP812 Xarma pty Ltd) . The pH was adjusted to 5.8 with NaOH prior to autoclave. Cultures were maintained at 24± 2°C under 16 hour light with white fluorescent light at 50 µmol m^{-2} s^{-1}. Subculture was performed every 4-5 weeks to establish enough stock plants for experiments.

Cryopreservation

Encapsulation-Dehydration Procedure

Shoot tips 2-3 mm long were encapsulated in 3% alginate beads (medium viscosity) according to the methods of Fabre and Dereudder (1990). Encapsulated apices were pregrown for various durations in liquid medium, with sucrose concentrations from 0.08-1.0 M. Desiccation was performed by placing the beads on sterile Petri dish in the Laminar Air Flow cabinet.

After the desiccation, beads were transferred to MS medium free of hormone (control desiccation) or placed in a 2.0 mL sterile cryovial tube (10 beads/tube), and immersed rapidly in liquid nitrogen (LN) where they were stored at least for 24 hours. For each treatment three replicates of 20 beads were used to plunge in the liquid nitrogen. Thawing was done by placing the cryovial tube under laminar airflow. Survivors was defined as the shoot tips that turned green and produced normal growth about 4 weeks after planting.

Beads were then transferred to Petri dishes containing medium without hormones. Shoot tips were placed under 25µmol m^{-2} s^{-1} white fluorescent light, and then transferred to white fluorescent light at intensity of 50 µmol m^{-2} s^{-1}. Shoots were transferred to new medium for further growth and development. The description of the encapsulation and dehydration procedure is shown in Figure 1.

Moisture Content

The beads were weighed and dried in the oven at 90 °C for 16 hours. A standard curve was drawn using beads with shoot tips, which were precultured for 24 hours in liquid medium with 0.5 M and 0.75 M sucrose, and desiccated for various durations (1-9 hours). Four replicates of five beads were used to measure the moisture content after each desiccation period.

Freezing Experiments

The first experiment investigated the survival of shoot tips after cryostorage in liquid nitrogen and preculture with 0.5 M sucrose for 3 days. The second experiment investigated the survival of shoot tips after cryostorage in liquid nitrogen and preculture with 0.75 M sucrose for 1 day

Results

The survival rate of encapsulated shoot of Christmas bush precultured on 0.3 M sucrose solid medium for 1 day was 100%. Alginate-coated shoot tips resumed growth more slowly than the control (excised shoot tips), but they started to regrow normally after 3 days. Encapsulated shoot tips which were not precultured with sucrose (Table 1) delayed the regrowth of shoot tips. Encapsulated shoot tips precultured with 0.3 M sucrose for 1, 3 and 5 days and 0.5 M sucrose for 1 day had no effect on survival or appearance (Table 1).

Regrowth of encapsulated shoot tips was 100% when precultured with 0.5M sucrose for 3 days. Alginate-coated shoot tips with 24.3% moisture content gave highest survival after LN when shoot tips were precultured with 0.5 M sucrose for 3 days (Table 2). Beads, which were precultured on different sucrose concentrations for different durations, gave no survival after freezing at a moisture content of more than 29.9% (Tables 2 and 3). Pregrowth was obtained after freezing in LN at different moisture content (15.3-29.9%) with the optimal pregrowth at around (20%-24.3%). Optimal regrowth of frozen shoot tips was 52% when shoot tips were precultured with 0.5M sucrose for three days.

Table 1: Effect of sucrose concentration and pregrowth duration on the regrowth of encapsulated Christmas bush shoot tips.

Sucrose Concentration (M).		Pregrowth duration (day) 1	3	5
0.08 Control	Regrowth%	100	100	100
	Day to regrowth	3^+	3^+	3^+
	Appearance	++++	++++	++++
0.3	Regrowth%	100	100	100
	Day to regrowth	3^+	3^+	4^+
	Appearance	++++	++++	++++
0.5	Regrowth%	100	96	96
	Day to regrowth	4^+	4^+	7^+
	Appearance	++++	++++	++++
0.75	Regrowth%	95	66	33
	Day to regrowth	6^+	8^+	8^+
	Appearance	++++	++	++
1.0	Regrowth%	66	0	0
	Day to regrowth	9^+	0	0
	Appearance	+	-	-

Appearance: ++++ very good, ++ good, + weak (yellow), - no regrowth.

Table 2: Survival of shoot tips after cryostorage in liquid nitrogen (LN) after preculture with 0.5 M sucrose for 3 days.

Desiccation duration (hour)	Beads Moisture content%	Survival% -LN	Survival% +LN
0	84	100	0.0
1	78	100	0.0
3	50	86.7	0.0
5	29.9	72.2	35
6	24.3	57	52
7	22.8	56	31
8	20.2	46	18
9	17	16	10

Table 3: Survival of shoot tips after cryostorage in liquid nitrogen (LN) after preculture with 0.75 M sucrose for 1 day.

Desiccation duration (hour)	Beads Moisture content%	Survival% -LN	Survival% +LN
0	80.1	100	00
1	72	64	00
3	40	60	00
5	25	52	21
6	20	45	33
7	19.2	46	24
8	18	33	16
9	15.3	22	9

Discussion

Cryopreservation of Christmas bush by encapsulation dehydration has never been reported before. Cryopreservation has been successfully applied to plant species, such as apple, pear and mulberry (Niino and Sakai, 1992). From our preliminary result, we found that at 1.0 M sucrose concentration, the survival of shoot tips after freezing in liquid nitrogen was low. For successful cryopreservation it is necessary to dehydrate shoot tips sufficiently to avoid lethal intracellular freezing during cooling (Faber and Dereuddre, 1990; Matsumoto and Sakai, 1995; Niino and Sakai, 1995). Suzuki *et al.* (1997) reported that the optimal concentration of sugar in the preculture medium was 0.5 M with 48 hour preculture, which supports our results with the highest survival of 52% at 24.3% moisture content.

A high level of sugar during preculture was reported to be important in improving survival of cryopreserved meristems (Faber and Dereuddre, 1990; Matsumoto and Sakai, 1995; Niino and Sakai, 1995). However, Matsumoto and Sakai (1995) indicated that the sucrose molarity increases markedly during the drying process and reaches or exceeds the saturation point of the sucrose solution, resulting in a glass transition during cooling to –196 °C. The sugar accumulation maintains the stability of membranes under severe dehydration conditions. Our result proved that sucrose is

necessary to produce a high rate of shoot survival after cooling in liquid nitrogen following dehydration, which is similar to previous findings (Niino and Sakai, 1992; Suzuki *et al.*, 1997).

Our results demonstrate that the encapsulation dehydration method can be used for preservation of Christmas bush, and that encapsulated shoot tips can subsequently regenerate into plantlets. This procedure is easy to use, rapid, effective, and allows the establishment of a germplasm bank. Further study is necessary to find conditions for higher survival rates.

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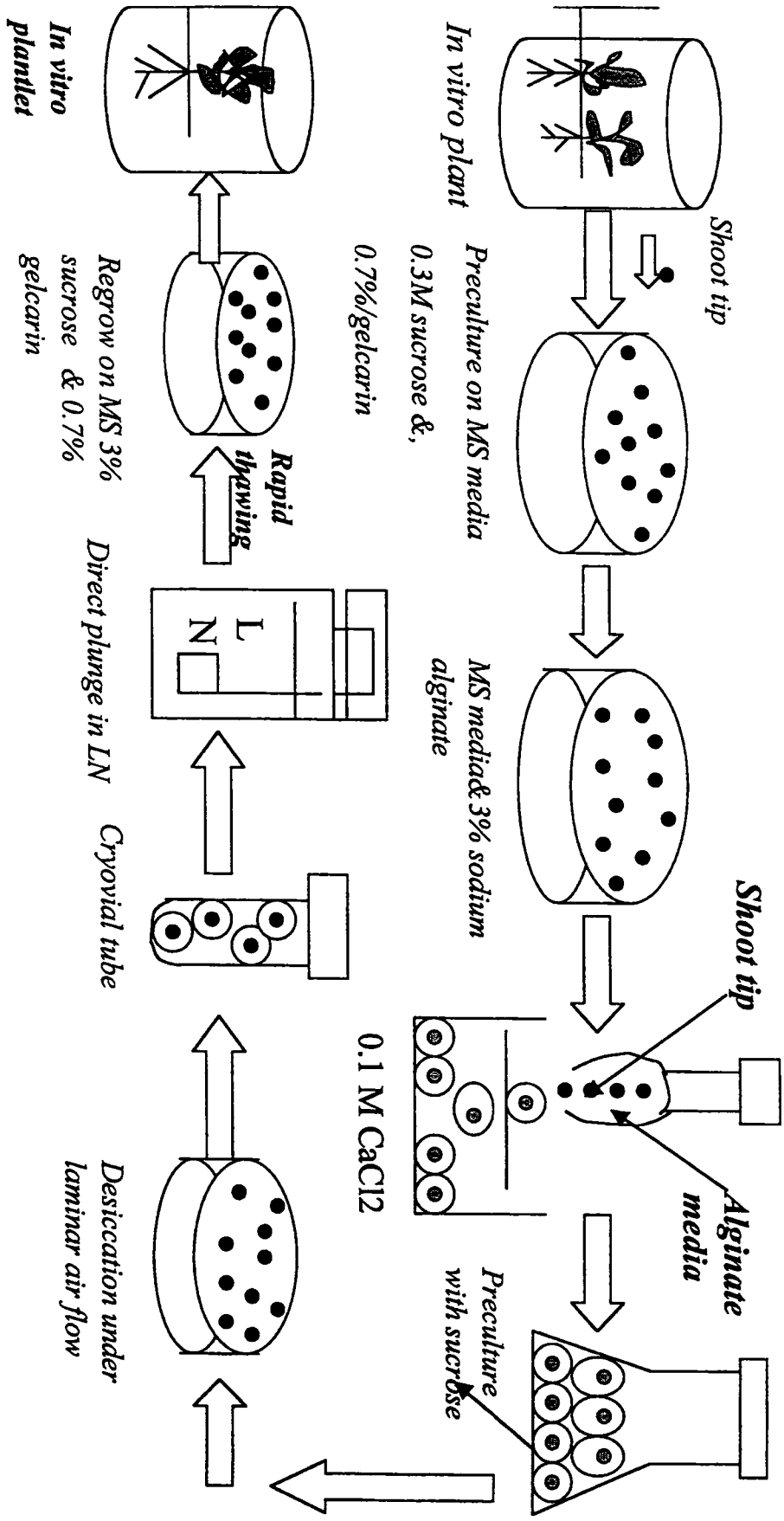

Figure 1: Cryopreservation of Christmas bush by the encapsulation dehydration method.

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