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CRYOPRESERVATION OF *CALLERYA SPECIOSA* (CHAMP.) SCHOT THROUGH DROPLET-VITRIFICATION

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Abstract

Cryopreservation is a practical method for the safe preservation of plant genetic resources. Impregnation with cryoprotective agents and the use of suitable explants are key factors for a successful procedure. It is, however, often difficult to work with "hairy" explants because of their high surface tension preventing an efficient treatment with cryoprotectant. Axillary shoot segments with one bud of *Callerya speciosa* were successfully cryopreserved by droplet-vitrification. The results showed that 1 mm shoot segments with one axillary bud excised from *in vitro* shoots could be used as explants for cryopreservation. The best pretreatment was performed with loading solution (LS), applied for 240 min at 25°C on a shaker at 150 rpm. This was followed by a plant vitrification solution (PVS2) treatment at 0°C for 60 min and rapid freezing in liquid nitrogen. Especially, rotation at 150 rpm proved to be essential to obtain high regrowth rates up to 60%.

Key words: axillary buds, hairy explant, shake

INTRODUCTION

Callerya speciosa (Champ.) Schot is a climbing plant and is used for landscaping and as ornamental plant in the parks. It has high drought tolerance and huge milky inflorescence with more than hundreds of flowers. The flowering phase can maintain about 3 months. During the few years, *C. speciosa* suffers from intensive exploitation and is now being endangered in China (Wang et al. 2005). The development of efficient conservation methods plays therefore an important role in maintaining the biodiversity and avoiding the genetic erosion. However, traditional methods for conserving plant genetic resources like field collections are not only costly, but also risky because of insect attack, diseases, and environmental stresses (Engelmann and Takagi 2000, Reed 2005). To date, *C. speciosa* is preserved mainly using *in vitro* conservation because it has recalcitrant seeds (Huang et al. 2008, Qiu-Yin et al. 2009, Ying-Nan et al. 2010). *In vitro* conservation needs to subculture the accessions once a month or a year according to the culture conditions. The subculturing process is labor-intensive and provides opportunity for accessions to become infected by fungal of bacterial contaminants. Furthermore accessions maintained *in vitro*, even under slow-growth conditions, are liable to somaclonal variation.

Cryopreservation, which is the storage of living cells and tissues at ultra-low temperature in liquid nitrogen (-196°C) provides an effective means of storing biological material for the long term. Compared with other available storage approaches, cryopreservation offers some advantages, such as genotype stability, minimal requirement of space, and maintenance (Engelmann 1997, Martinez et al. 1999, Sakai et al. 2000, Gonzalez-Arnao et al. 2008). Since at -196°C, cell divisions, metabolic, and biochemical processes are completely stopped, plant material can be stored for unlimited periods of time (Engelmann 2004, Panis and Lambardi 2005).

Cryopreservation of vegetative tissues involves five stages: (i) Establishment of *in vitro* cultures and their conditioning, (ii) appropriate cryoprotectection, (iii) exposure to liquid nitrogen, (iv) rewarming, (v) regeneration (Bajaj 1995). Due to the differences in size and water content of explants, (i) and (ii) stages need to be optimized for full dehydration. The removal

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of available water is necessary to reduce the damage caused to the cells by ice formation (Grout and Henshaw 1980, Benson 1999).

Droplet-vitrification is now one of the most commonly used methods in cryopreservation (Sakai et al. 1990). It derived from the droplet-freezing technique.

Compared to other vitrification methods, regeneration rates after cryopreservation using droplet-vitrification rates are considerably higher, suggesting that the formation of intracellular ice crystal during cooling and rewarming (Panis et al. 2005) is limited. In recent years, droplet-vitrification was successfully applied to apple (Condello et al. 2011), thyme (Marco-Medinaet al. 2010), olive (Sánchez-Romero et al. 2009), taro (Sant et al. 2008) and bananas (Panis et al. 2005). So far, the application of droplet-vitrification to plants has been limited to a few cases only including *Bletilla striata* (Thunb.) Druce (Jitsopakul et al. 2008), *Rubia argyi* (H.Lév. & Vaniot) Hara ex Lauener & Ferguson (Kim et al. 2010), and *Limonium serotinum* (Rchb.) Pignatti (Barraco et al. 2011). Cryopreservation of plants with hairy shoots has not been reported yet. Cryopreservation using droplet-vitrification employed Loading solution (LS) (Panis et al. 2005) and Plant vitrification solution 2 (PVS2) (Sakai et al. 1990). The LS and PVS2 both contain sticky materials, such as glycerol and sucrose. When the shoots with hairs were put in LS and PVS2, higher surface tension will inhibit the solution to the shoot tip and resulted incomplete loading and dehydration.

The aim of this study was to develop an efficient droplet-vitrification cryopreservation technique for the long-term storage of *C. speciosa* germplasm using hairy axillary shoot segment with one bud.

MATERIALS AND METHODS

Plant material

Before cryopreservation, *in vitro* axillary shoots were cut into 1-3 cm segments with 1-2 axillary buds and transferred to MS (Murashige and Skoog 1962) medium supplemented with 2.2 µM BA in 50 ×10 mm glass bottles at 25 ± 2°C in dark and under 35 to 41 µmol m^{-2} s^{-1} photosynthetic photon flux density with a photoperiod of 16/8 h day/night. The pH was adjusted to 5.8 prior to autoclaving. After 40 days, robust *in vitro* shoots with diameter of 0.8-2.0 mm were obtained.

Droplet-vitrification procedure-Loading

Shoot segments, each containing an axillary bud, 50-100 mm in length and 1.0-1.2 mm in diameter, were excised from the robust *in vitro* shoots. The leaf was cut off from each segment and the two stipules were removed under a binocular microscope. About 30 shoot segments (1.5 mm in length) were transferred to 30 ml LS in plastic tube, sterilized with ethylene oxide, and kept in the dark for 40, 150, 240, 300, and 420 min, respectively, with or without shaking (at 25°C and 150 rpm). The filter sterilised LS contained 2 M glycerol and 0.4 M sucrose dissolved in MS medium (pH 5.8). According to the vitrification procedure, explants were transferred after excision to LS in dark for 40, 150, 240, 300, and 420 min, respectively, and then dehydrated with PVS2. In order to increase the penetration of the loading solution, horizontal oscillation at 150 rpm was introduced.

Droplet-vitrification procedure-explant dehydration with PVS2 and cooling in liquid nitrogen

After loading, the LS solution was replaced by ice-cooled plant PVS2. PVS2 consisted of 30% (3.26 M) glycerol, 15% (2.42 M) ethylene glycol (EG), and 15% (1.9 M) DMSO (Sakai et al. 1991). All these compounds were dissolved in MS medium that contained 0.4 M sucrose. The pH was adjusted to 5.8 before filter sterilization. Axillary buds were immersed in PVS2 solution for 40, 50, 60, 80 or 100 min at 0°C with or without shaking at 10 rpm. At 0°C, low shaking speed produced less tiny bubbles than high speed for the solution had high viscosity.

Two min before the end of the PVS2 treatment, explants were transferred to a droplet of PVS2 solution (25-30 µl) on a sterile strip of aluminum foil (5 mm × 20 mm) that was kept at 0°C during handing (for this it was transferred to a glass dish placed on top of a frozen cooling element). After the PVS2 treatment, for each treatment, one-third of the explants were transferred directly to the filter-sterilized unloading solution (US) (control) while the strips with the remaining explants were plunged into liquid nitrogen (LN) with a fine forceps and kept in LN for 30 min.

Droplet-vitrification procedure-rewarming and unloading

The filter-sterilized unloading solution (US) consisted of 1.2 M sucrose dissolved in MS medium. Aluminum foil strips were rinsed in 25 ml US in a 9 cm diameter Petri dish at room temperature. After a few seconds, shoot segments were released into the medium. Shoot segments were maintained for 15 min in US, then placed onto two sterile filter papers covering semi-solid plant growth regulators-free MS medium, containing 0.3 M sucrose (regrowth medium), less carrageenan (5 g l^{-1}), and transferred to a dark culture room at 25°C.

Regrowth

After 2 days, all explants were transferred onto regrowth MS medium supplemented with 2.2 µM BA, without filter papers. The first week of culture was always in the dark. In one set of experiments, shoot segments were placed in the dark for 1, 2, 3, 4, 5, 6

or 7 days before their transfer to light conditions. The regrowth medium was supplemented with IAA (0.6 μM), GA_3 (0.1 μM), BA (0, 0.9, and 2.2 μM), and NAA (0.5 μM) (Table 2). The regrowth rate was investigated 6 weeks after thawing.

Statistical analysis

All experiments followed a completely randomized design and results are presented by mean percentages with their standard error (SE). Each level of every design had three repeats and each of them had 3 units. Each unit had 3 Petri dishes with 15 explants in each Petri dish (10 for treatment and 5 for control). Statistical difference between mean values of post-warm regeneration was assessed by analysis of variance (ANOVA) with Duncan's multiple range test ($p < 0.05$). Prior to analysis, the original percentage data were subjected to arcsine transformation.

RESULTS

Response of C. speciosa to droplet-vitrification procedure of cryopreservation

Young shoots of *C. speciosa*, originated *in vitro*, are covered by bushy trichomes (Fig. 1 A-C). These trichomes are difficult to remove because the apical meristem that is less than 0.3 mm in size is also covered with them. Removing all the trichomes caused very low regrowth rates (less than 10%). Four to five weeks after cryopreservation, four types of reaction were observed: (i) white axillary buds without blackening caused by dying accompany, (ii) callus not regenerating into shoots, (iii) axillary bud regrowth accompanied by callus growth, and (iv) axillary buds regrowth without callus. The regenerated shoots had the same phenotype like the control (Fig. 2 A-D). Only few of the explants regrew after cryopreservation (less than 10%). This could be due to the fact that *C. speciosa* explants are covered with bushy trichomes, preventing the LS and PVS2 to reach the shoot meristem due to the surface tension.

Effect of rotation during loading on the regrowth rate

Horizontal oscillation at 150 rpm when loading increased the regrowth rate. The optimal LS (25°C, 150 rpm) exposure time that resulted in the highest regrowth rates in combination with a PVS2 treatment of 60 min was 240 min (59.2%). This regrowth rate was significantly different from the ones due to other time treatments (Fig. 3). This regrowth rate is comparable to that of the control (59.7%). When 240 min loading was combined with different PVS2 treatment lengths, 60 min treatment proved to be optimal (Table 1).

Effect of dark culture time on cryopreservation

The culture of the post-thaw explants was in the same conditions as the shoots except for no illumination. The regrowth of cryopreservation was affected by dark culture time (Fig. 4). Regenerable regrowth increased until 6 days of dark culture. Dark culturing time to 7 days did not cause increasing of regrowth rate comparing with that of 6 days.

Effect of growth regulators on regrowth after cryopreservation

The addition of different growth regulators showed a significant effect on the regrowth rate of *C. speciosa*. Without plant growth regulators on the medium, regrowth rate was very low (33.3% of the control and 6.7% after cryopreservation). Adding BA increased regrowth rate significantly. The highest regrowth rate (100%) of the control was obtained on medium supplemented with 2.2 μM BA but that of the cryopreservation (60%) was obtained on medium supplemented with 2.2 μM BA, 0.6 μM IAA, and 0.1 μM GA_3 and 56.7% was obtained on MS medium supplemented with 2.2 μM BA and 0.5 μM NAA (Table 2).

DISCUSSION

Achieving a vitrified state during cooling, and thus avoiding the lethal effects of intracellular ice crystals formation is crucial for a cryopreservation protocol (Fahy et al. 1984, Panis et al. 2005).

Loading leads to an increase in dehydration tolerance and alleviates mechanical osmotic stress caused by the severe dehydration of subsequent the PVS2 treatment (Charoensub et al. 1999). Therefore, the explants used for cryopreservation must be in complete contact

Table 1. Effect of lenght of PVS2 treatment at 0°C on regrowth rates of *C. speciosa* after cryopreservation with loading at 25°C, 150 rpm for 4 h.

	Control regrowth (%)					Regrowth after LN (%)				
Time of PVS2 treatment (min)	40	50	60	80	100	40	50	60	80	100
Regrowth rate (%)	72.0 ± 0.8 A	66.0 ± 0.7 B	59.7 ± 1.6 B	48.9 ± 1.1 C	44.9 ± 0.6 C	35.3 ± 2.0 d	50.0 ± 0.8 b	59.2 ± 3.5 a	41.9 ± 1.8 c	35.1 ± 1.5 c

Means (± SE) within a row followed by the same letter (capitals for control and small letter for cryopreserved material) are not significantly different according Duncan's multiple range test after arcsin transformation at ($p \leq 0.05$).

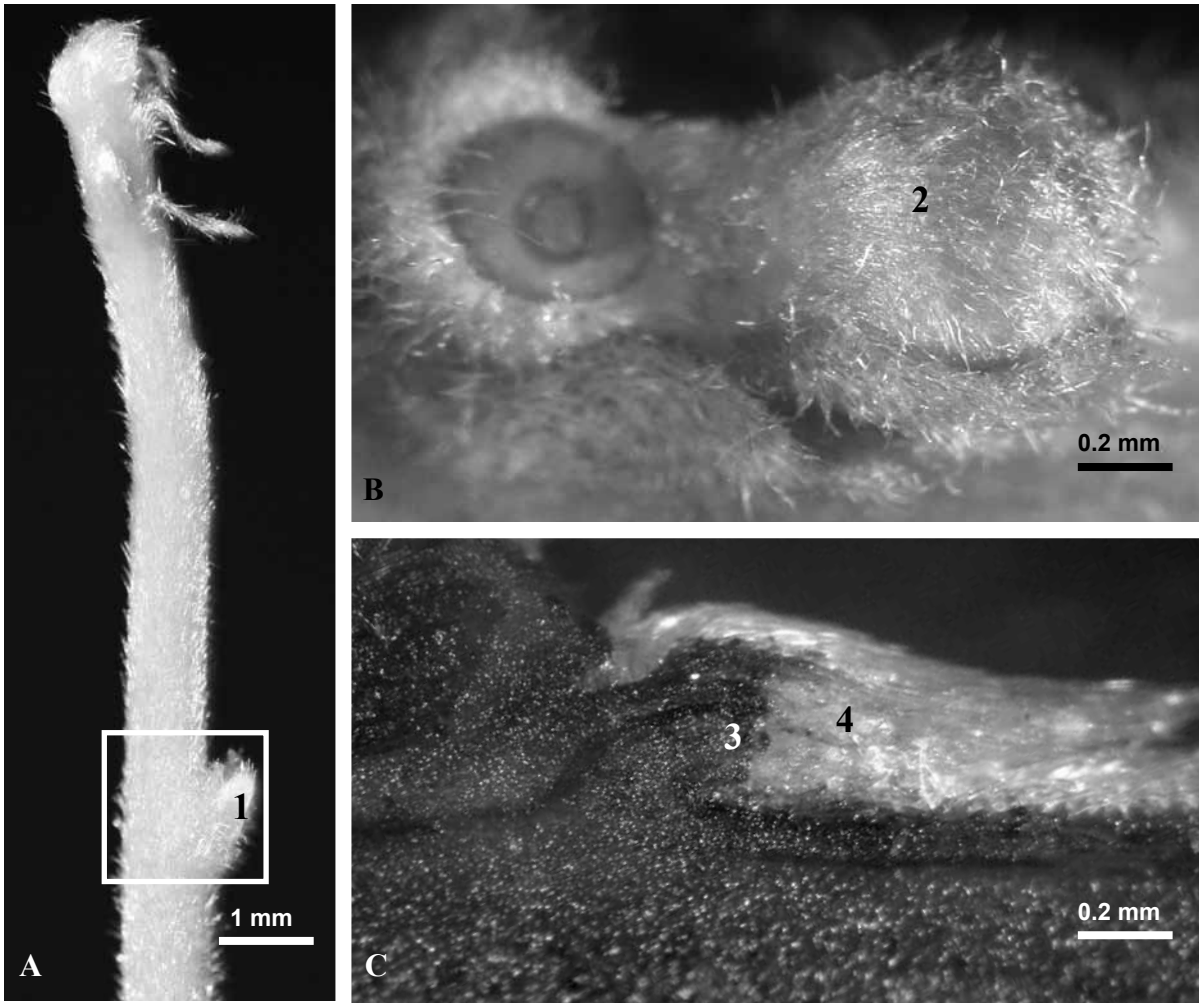

Fig. 1. Morphology of *Callerya speciosa* cultures. A) The *in vitro* explant used for cryopreservation (the framed tissue means the axillary buds which was used for cryopreservation), B) Enlarged bud from part in frame of A where the stipules were removed, C) The axillary buds covered with bushy trichomes. Legend: 1: Stipules of the axillary buds, 2: Bud with leaf primordium, and 3: Shoot apical meristem, 4: Trichome that cover the meristem.

with the LS. A meristem that is tightly covered with leaf primordia such as in banana should be isolated in such way that a partly naked, undamaged apical dome is obtained (Panis 2009). In *C. speciosa*, the apical meristem of shoot tips and axillary buds are covered with bushy hair and are, therefore, difficult to be isolated. The surface tension of the hairs inhibits the LS to reach the apical meristem. Although we prolonged the loading time to 240 min, the regrowth rate was still very low. Therefore, we introduced shaking to remove the tiny air bubbles enclosed between the hairs. Our results proved that shaking during loading is effective for improving the regrowth rate after cryopreservation.

Optimizing the length of treatment with dehydrating solution PVS2 is also of outermost importance. To prevent injury by chemical toxicity or excessive osmotic stress during PVS2 treatment, a precise control of the dehydration procedure is needed (Sakai and Engelmann 2007). Optimal treatment with a vitrification solution provides an adequate dehydration to reduce water content and thus limiting the lethal ice crystal formation in the explants. In addition, the optimal dehydration treatment of different plant species can vary considerably with variable water content and membrane permeability typical for each plant species. It was shown that the dehydration tolerance of different tropical species is different. Cells with more vacuoles have higher chance to form ice crystals (Panis et al. 2005). The dehydration temperature also plays an important role: (i) at room temperature, taro shoot-tips require a 60 min PVS2 treatment while banana meristems were already killed after a 10 min treatment (Sant et al. 2008), (ii) at 0°C, longer treatments can be applied which especially benefits tropical, dehydration sensitive plants, such as banana, *Pelargonium* (Gallard et al. 2008), and *Carica papaya* (Ashmore et al. 2007).

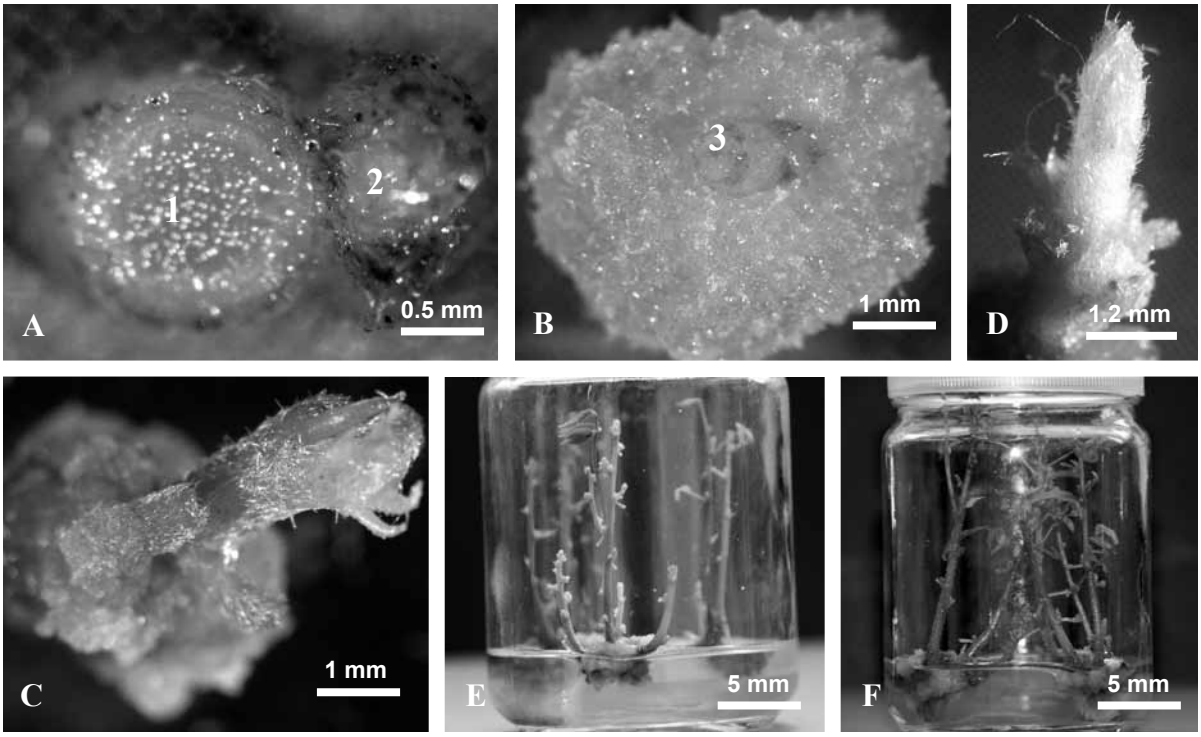

Fig. 2. Reaction of axillary buds (ABs) towards cryopreservation. A) No growth, ABs become white (1: shoot, 2: axillary buds), B) Callus formation (3: no elongated axillary bud), C) Regrowth of ABs accompanied with callus formation, D) ABs regrowth without callus, E) Shoots originated from cryopreserved ABs, F) Shoots originated from the control ABs.

The optimization of the plant growth regulators in the post-warm recovery medium is important for the regrowth of explants (Wang et al. 2000). A medium needs to be optimized for *Populus alba* L. (Lambardi et al. 2000), chestnut (Vidal et al. 2005), *Thymus moroderi* (Marco-Medina et al. 2010), and apple (Condello et al. 2011). For *C. speciosa*, the medium supplemented with 2.2 µM BA, 0.6 µM IAA, and 0.1 µM GA_3 or medium supplemented with 2.2 µM BA, 0.5 µM NAA all resulted in the high regrowth rate (60% or 56.7%) compared to all other combinations (Table 2). However, the highest regrowth rate of the control was obtained on medium supplemented with 2.2 µM BA. This result

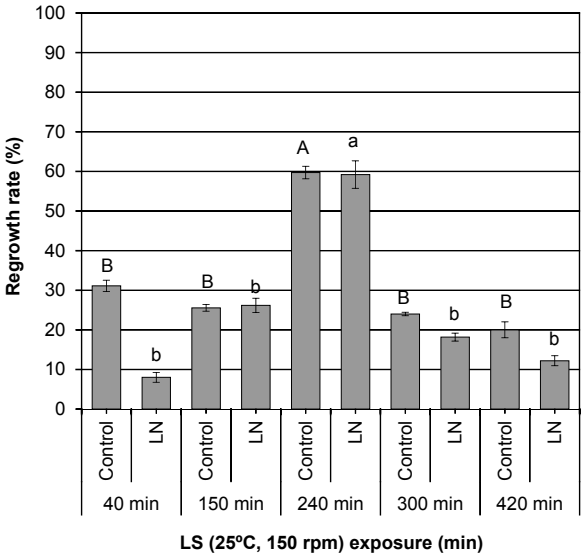

Fig. 3. Effect of treatment time with LS (25°C, 150 rpm) on the regrowth rate (%) of *C. speciosa* without (Control) and with cryopreseration (LN).
Means (± SE) on the columns followed by the same capital (for the control) or small letter (for cryopreserved buds) are not significantly different according Duncan's multiple range test at $p \leq 0.05$.

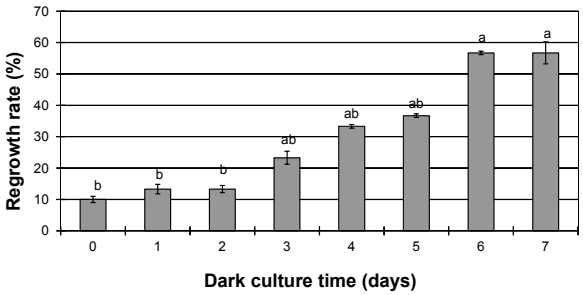

Fig. 4. Effect of dark period (days) on post-thaw regrowth of *C. speciosa*. Recovery was performed on MS containing 2 µM BA.
Means (± SE) on the columns followed by the same letter are not significantly different according Duncan's multiple range test at $p \leq 0.05$.

Table 2. Effects of plant growth regulators on the regrowth rate and callus formation of *C. speciosa* of control and cryopreservation (cryo).

PGR concentration (μM)				Regrowth of control (%)	Regrowth after cryo (%)
BA	NAA	IAA	GA_3		
0.0				33.3 ± 1.2 e	6.7 ± 1.2 d
0.9				80.0 ± 1.0 c	43.3 ± 0.6 b
2.2				100.0 ± 0.0 a	40.0 ± 1.7 b
0.4				93.3 ± 0.6 b	33.3 ± 0.8 c
0.9				66.7 ± 0.6 d	33.3 ± 1.2 c
2.2				80.0 ± 1.0 c	50.0 ± 1.0 b
0.9	0.5			46.7 ± 0.6 d	40.0 ± 1.0 b
2.2	0.5			73.3 ± 1.5 c	56.7 ± 3.5 a
0.9		0.6		33.3 ± 0.6 e	33.3 ± 1.2 c
2.2		0.6		73.3 ± 0.6 c	46.6 ± 2.5 b
0.9	0.5		0.1	86.3 ± 0.6 b	30.0 ± 2.0 c
2.2	0.5		0.1	66.7 ± 0.6 d	46.7 ± 0.6 b
0.9		0.6	0.1	80.0 ± 0.0 c	20.0 ± 0.0 c
2.2		0.6	0.1	66.7 ± 1.5 d	60.0 ± 1.0 a

Means (± SE) within a column followed by the same letter are not significantly different according Duncan's multiple range test after arcsin transformation at ($p \leq 0.05$).

indicated that the sensitivity of explants to plant growth regulators have changed after cryopreservation.

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