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Slow-growth conservation- cryopreservation and Analysis of genetic stability of *in vitro* regenerated *Coelogyne Nervosa* A.Rich A endemic orchid

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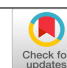
ABSTRACT



Coelogyne Nervosa A. Rich., an endangered medicinal epiphyte (lithophyte) is a strict endemic of the Western Ghats distributed across Karnataka, Kerala and Tamil Nadu. KC medium supplemented with 2,4-D is best suited for direct embryo induction from 80 days old protocorms. Hormone-free KC basal medium supported the conversion of somatic embryos into complete plantlets. For the medium-term storage experiment, somatic embryos were placed on storage media containing various growth inhibitors (such as different levels of chlormequat, maleic hydrazide and paclobutrazol). They were stored for ten months to assess the percentage of survival. The surviving shoots were transferred to the recovery medium for eight weeks. At 8°C, the highest survival rate was observed with ABA at 0.2 mg L^{-1}. For cryopreservation: droplet-vitrification best results were obtained when the somatic embryos, after 20 days of subculture were immersed in an osmoprotectant solution. Osmo-protected samples were immersed in PVS2 (15% ethylene glycol and 15% DMSO) at 0°C for 30 min. Pre-prepared Somatic embryos were plunged into cryovials for one day. Regrowth levels were as high as 70%. DNA samples extracted from leaves of the mother plant, somatic embryos without storage, regenerates of slow growth preservation and cryopreservation. The samples were analysed by DNA based molecular marker – inter simple sequence repeats (ISSR) and found no genetic changes during the cryopreservation process.

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INTRODUCTION

India is known to have 34 species of *Coelogyne*. *Coelogyne Nervosa* A. Rich, an endangered medicinal epiphyte (lithophyte) (Synonyms: *Coelogynecorrugata* Wight., *Pleione Nervosa* (A.Rich.) Kuntze., *Pleionecorrugata* (Wight) Kuntze) is an endemic of the Western Ghats distributed across Karnataka, Kerala and Tamil Nadu. The regions of distribution in Karnataka include Kodagu, Kotagiri, Madikeri, Brahmagiri range, Kerala: Silent Valley, Eravikulam National Park, Neyyar Wildlife Sanctuary. Tamil Nadu: Doddabetta, Coonoor, Bikkapathimund Reserve Forest, Pandiar, Mukurthi National Park, Naduvattom, Anamalais, Pykara, Nil-

giris. An attempt was made towards the seed germination of *C. Nervosa* using MS, KC and VW medium supplemented with various growth adjuvants and growth regulators were previously reported (Abraham *et al.*, 2012). This plant species requires immediate attention for its protection, large scale systematic cultivation and conservation.

MATERIALS AND METHODS

Collection of fresh seeds pods

The immature capsules of *C. Nervosa* (naturally pollinated) were obtained from the National Orchidarium, Botanical Survey of India, Yercaud, Salem, Tamil Nadu, and were used for asymbiotic seed germination.

Establishment of *in vitro* asymbiotic seed culture was done using previously developed protocol.

In our previous studies, five different basal media such as Murashige and Skoog (1962) medium and Schenk and Schenk and Hildebrandt (1972) were tested to select a suitable medium for asymbiotic seed germination of *C. Nervosa.* Among the five different basal media used, Knudson C medium was found to be most effective in inducing a germination frequency of 95% in *C. Nervosa*, whereas, Linsmaier and Skoog and Lindemann orchid medium induced 80 % seed germination.

Standardisation of protocol for medium-term conservation of *C.nervosa* A.Rich.

Development of somatic embryos

Plant materials

The 80 day-old protocorms, (before the emergence of first leaves) were used as explants for further *in vitro* studies.

Preparation of media

Knudson (1946) basal medium with or without growth regulators such as IAA (2.9, 5.7 and 11.4 μM), IBA (2.46, 4.92 and 9.84 μM), 2,4-D (2.26, 4.52 and 9.03 μM), BA (2.22, 4.44, 8.88 and 13.32 μM) and Kin (2.32, 4.64 and 9.29 μM) either individually or in combinations were used. Both half and full strength hormone-free KC basal medium with 3% sucrose and 0.8% agar was used for the differentiation of PLBs and their conversion into plantlets. The pH of the medium was adjusted to 5.6 with either 1N NaOH or 1N HCl and autoclaved at 1.06 kg pressure for about 20 minutes at 121°C. Five replicates were used for each treatment maintained at 25$\pm$2°C in culture room under a 12 h photoperiod of 50 μmol m-2 s-1 irradiance provided by white fluorescent tubes and with a relative humidity of 70%.

Slow growth conservation

For the medium-term storage experiment, somatic embryos were placed on storage media containing various growth inhibitors (such as different levels of chlormequat (CCC), maleic hydrazide(MH), paclobutrazol (PP333) or abscisic acid (ABA). They were stored for ten months to assess the percentage of survival. The surviving shoots were transferred to the recovery medium and were maintained for a recovery period of 8 weeks.

Cryopreservation: Droplet-vitrification

Somatic embryos, after 20 days of subculture were immersed in an osmoprotectant. Somatic embryos were then placed and stored in liquid nitrogen for one day.

Analysis of genetic stability

DNA samples extracted from leaves of the mother plant, somatic embryos without storage, regenerates of slow growth preservation and cryopreservation. The samples were analysed by DNA based molecular marker – inter simple sequence repeats (ISSR).

RESULTS AND DISCUSSION

Direct embryogenesis

Somatic embryos developed directly from seed-derived protocorms of *Coelogyne Nervosa*. KC medium supplemented with various concentrations and combinations of 2,4-D (2.26, 4.52 and 9.03 μM) and BAP (2.22, 4.44 and 8.88 μM) were found to be efficient in inducing embryogenesis from protocorms. KC medium fortified with 2,4-D was found to be the best one for 100% embryo induction. The embryo induction frequency and the number of embryos per explant decreased with increasing concentrations of 2,4-D (4.52 and 9.03 μM), with an average maximum of 5.8 $\pm$0.37 embryos observed on MS medium containing 2,4-D (9.03 μM). Addition of BAP (2.22, 4.44 and 8.88 μM) to KC medium supplemented with 2,4-D (4.52 μM) did not enhance the effects of 2,4-D. The time required for embryo induction was longer, and the frequency of direct embryogenesis was lower on MS medium supplemented with 2,4-D (4.52 μM) and BAP (8.88 μM).

The embryos developed on KC medium supplemented with 2,4-D (2.26 μM) exhibited uniformity in maturing on transfer to half-strength hormone-free MS medium when compared to the embryos induced on MS medium supplemented with higher concentrations of 2,4-D individually and in combination with BAP. Around 80% of the embryos developed on KC medium containing 2,4-D (2.26 μM)

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elongated and subsequently developed into young protocorms with dense absorbing hairs. However, the embryos induced on KC medium supplemented with 2,4-D (4.52 and 9.03 μM) individually and in combination with BAP (2.22, 4.44 and 8.88 μM) exhibited delayed maturation. Around 10% of the embryos developed on MS medium supplemented with 2,4-D (4.52 μM) and BAP (4.44 μM) survived the transfer to half strength hormone-free KC medium.

Many terrestrial and epiphytic orchids have been successfully propagated using *in vitro* asymbiotic seed germination techniques (Malmgren, 1992; Chou and Chang, 2004). Species-specific media for germination of seeds have been reported in many orchids (Arditti and Ernst, 1984; Johnson *et al.*, 2007).

Slow growth conservation

For the medium-term storage experiment, somatic embryos were placed on storage media (hormone-free KC basal medium) containing various growth inhibitors such as different concentrations (0.1- to 0.5 mg/l) of chlormequat, maleic hydrazide, paclobutrazol or abscisic acid individually. They were incubated at three temperature levels such as 25°C, 8°C, and 4°C with eighth and irradiance of 50±10 μmol m−2 s−1 for ten months. After ten months of storage, the percentage of survival was recorded. The surviving shoots were transferred to multiplication medium and maintained for a recovery period of 8 weeks. At 8°C, the highest survival rate was observed with a storage medium containing ABA at 0.2 mg L^{-1}. Slow growth under low temperature is a successful technique (Peng *et al.*, 2015).

Cryopreservation: Droplet-vitrification

Somatic embryos were stored as per the method in a tank of liquid nitrogen in the liquid for one day and were transferred to firm KC basal medium for recovery at 25°C. Regrowth levels were as high as 70%.

Zhang *et al.* (2015) identified a droplet-vitrification protocol for the cryopreservation of petunia cultivars by using orthogonal array tests and one-factor experiments. Similar reports are available for strawberry (Pinker *et al.*, 2009) and carnation (Sekizawa *et al.*, 2011).

Analysis of genetic stability

DNA samples were extracted from leaves of the mother plant, somatic embryos without storage regenerate of slow growth preservation and cryopreservation and stored in −80°C, respectively. DNA samples were diluted to 50 ng μL−1. DNA amplification, band separation, and DNA marker profile visualisation were performed using ISSR primers. There were no genetic changes with the marker system. Similar results were obtained by Zhang *et al.* (2015).

CONCLUSION

In the present study, we have developed an advantageous protocol for clonal propagation, medium-term conservation and cryopreservation of the endangered medicinal orchid *C. Nervosa.*

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Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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