

EX SITU CONSERVATION OF FLORICULTURALLY AND MEDICINALLY IMPORTANT ENDANGERED ORCHID, *COELOGYNE CRISTATA* LINDL.

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Abstract

The current study was intended to facilitate *ex situ* conservation of floriculturally and medicinally important endangered *Coelogyne cristata*. The cultures were initiated using asymbiotic seed germination technique on a defined Mitra *et al.*, 1976 (M) medium supplemented with plant growth regulators (PGRs) such as [6-benzyladenine (BA - 4.44 μ M), furfuryl aminopurine (KN - 4.65 μ M), α -naphthalene acetic acid (NAA - 5.37 μ M) and indole, 3-butyric acid (IBA - 4.92 μ M)]. The frequency and onset of germination was markedly influenced by the type of growth regulator used in the nutrient medium. Cytokinins proved optimal for germination and led to fastest development of seedlings within 18.08 \pm 0.50 wks of culture. Simultaneously, the efficacy of sucrose at different concentrations (5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 g^l⁻¹) was also assessed on the germination percentage of seeds. The growth of the seedlings was best on the medium containing 20 g^l⁻¹ of sucrose.

Introduction

BIODIVERSITY CONSERVATION is one of the major global concerns. The survival of each species is necessary for dynamic execution of an ecosystem. The family Orchidaceae is one of the highly evolved angiospermic families. The orchids need amiable atmosphere to sustain in an ecosystem. These are excellent indicators of a healthy ecosystem being site-specific. The present-day scenario alludes to their rare, endangered and threatened status all over the world. Several factors such as deforestation, habitat fragmentation specifically in the tropical regions of the world, excessive exploitation of the soil by the fertilizers, and above all unabated collections from their natural habitats, are accountable for their current status and the genus *Coelogyne* does not seem to be an exception. Integrative multidisciplinary methodologies are required for *in situ* and *ex situ* conservation of the orchid species.

Coelogyne is a genus encompassing almost 190 species of evergreen, epiphytic, pseudobulbous, sympodial orchids, which bear gorgeous blooms. A total of 38 species are found in India, of which 10 are endemic to the subcontinent including *Coelogyne cristata* (Kumar and Manilal, 1994). *C. cristata*, also known as 'snow queen' is a floriferous, evergreen, epiphytic orchid species; this sympodial, pseudobulbous orchid species thrives best in cool and moist climates. It is endemic to the Himalayan ranges and extends from Garhwal eastwards to Sikkim, Khasi and Jaintia hills in subtropical to temperate climates at an altitude of 1700-2300 m (Bose and Bhattacharjee, 1980). This horticulturally important species bear white, fragrant flowers (Fig. 1A)

in pendant or drooping raceme inflorescences (Fig. 1B) and has tremendous potential as a progenitor of meritorious hybrids of international repute. Amongst the noticeable hybrids of this genus, *Coelogyne cristata* var. *intermedia* a primary hybrid between *Coelogyne cristata* \times *Coelogyne flaccida* and another hybrid named *Coelogyne* Linda Buckley raised from *Coelogyne cristata* \times *Coelogyne mooreana* are on records (<http://www.oscov.asn.au/articles3/coelogyne.htm>). Due to its delightful blossoms, *C. cristata* is generally used as pot plant, in the home gardens, and for beautifying landscapes in most parts of the world. Apart from being highly floriferous orchid, it is medicinally important and used extensively in treating epilepsy, and nervous disorders, by native people of the temperate Himalayas. Further, its anti-ageing and anti-stress property makes the species a good health tonic and rejuvenator (Pramanick, 2016). *C. cristata* also known as 'Hadjojen' (bone jointer), harbours a bioactive chemical compound 'Coelogin' which is osteo-protective in post-menopausal osteoporosis (Sharma *et al.*, 2014). Extensive commercial collections of this species, in nature have severely degraded its natural populations. As a consequence, its natural populations have become threatened of survival and the species is included in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2017). Hence, there is an urgent need to mass propagate the species using tissue culture techniques. Presently, an attempt was made to develop an effective *in vitro* propagation protocol for the species with a view to initiate cultures through asymbiotic seed germination technique, thus, assessing the effect of, i) different plant growth regulators, and ii) sucrose concentrations on the



Fig. 1 A-B. *Coelogyne cristata*: A, Flower; B, Drooping racemes of *Coelogyne cristata*.

germination percentage and further development of the seedlings *in vitro*.

Materials and Methods

Plant Collection and Greenhouse Maintenance

Coelogyne cristata plants and their capsules were collected from a commercial grower of Darjeeling district, West Bengal, India. The healthy plants were replanted in pots (27.5 cm × 22.4 cm diameter) containing charcoal pieces, brick pieces, bark pieces as substrate in the ratio of 1:1:1. The sphagnum moss covered the top surface of potting mix. The plants were maintained in the greenhouse under natural light conditions with a 70% relative humidity and 25°C/20°C day/night temperature. The capsules were collected, brought to the laboratory and prepared for inoculations into the medium with and without growth adjuncts.

Culture Medium for In Vitro Seed Germination

The required quantity of M (Mitra *et al.*, 1976) medium (Hi-media, Mumbai, India) was weighed and dissolved in 200 ml of double-distilled water. To raise the volume of medium 1 litre, requisite amount of double-distilled water was added. The growth regulators such as BA (4.44 μM), KN (4.65 μM), NAA (5.37 μM), IBA (4.92 μM), (Hi-media, Mumbai, India) were used individually and in combination, in the medium. In another set of experiment, additional activated charcoal was also incorporated into the medium. Simultaneously, varying concentrations of sucrose (Duarala, India) *i.e.* 5.0, 10.0, 15.0, 20.0, 25.0 and 30.0 g l⁻¹ was also added into the Mitra medium. The pH of the medium was adjusted to 5.8 after adding the growth regulators and organic growth supplements. The medium was dispensed in the test tubes of size (25 mm × 150 mm) and autoclaved at 121°C at pressure of 1.06 kg cm⁻² for 15 min. The autoclaved medium was kept at 37°C to check any

further contamination. The viability of the seeds was estimated by staining in 1% solution of 2, 3, 5- triphenyl-tetrazolium chloride (TTC; pH 6.5) (Hi-media) for 3 days at 32±1°C (Lauzer *et al.*, 1994). The embryos which stained red were considered viable. Almost all the seeds were viable.

Surface Sterilization of Capsules

The capsules were first scrubbed with a soft brush in running tap water to remove any debris. These were rinsed with dish-washing liquid to remove any debris from the surface of the capsules then rinsed thoroughly in water. The capsules were swabbed with ethyl alcohol under a sterile laminar airflow hood and surface sterilized with 0.1% (wv⁻¹) mercuric chloride (HgCl₂; Qualigens, Pvt. Ltd., Mumbai, India) in an aqueous solution containing 1-2 drops of 'teepol' as a wetting agent for 2 min. They were rinsed 2-3 times with sterilized double-distilled water to remove any traces of HgCl₂ left on the surface of capsules. Thereafter, capsules were flamed on a burner and seeds scooped out into a petri-dish by making a longitudinal slit. The seeds were inoculated onto Mitra medium and its combinations with different growth adjuncts.

Inoculations and Incubation Conditions

The inoculations were done under aseptic conditions in a laminar air-flow cabinet. The cultures vessels were incubated at 25 ± 2°C under 12 hr photoperiod of 40 μ mol m⁻² s⁻¹ light intensity provided by white fluorescent tubes (Fluorescent tubes; Philips India Ltd, Mumbai, India). Eight replicates were used for each experiment and to check the reproducibility of the protocol, the experiment was repeated twice.

Per Cent Germination

Nearly after three to four weeks of the inoculations, some of the seeds were scooped out of the test tube

Table 1. *In vitro* asymbiotic seed germination of *Coelogyne cristata* immature seeds on M medium and its combination with growth regulators.

Additive(s)	Initiation of response	Time taken in wks for					
		Spherule	Development of Chlorophyll		Differentiation of		
				Protocorm	1st Leaf	1st Root	Seedlings (wks)
-	4.25±0.00 ^{bc}	6.50±0.10 ^b	8.00±0.03 ^a	12.00±0.25 ^a	18.00±0.03 ^{bc}	22.00±0.50 ^{cd}	24.34±0.60 ^{cd}
AC	3.00±0.27 ^{ab}	8.65±0.07 ^{cd}	11.02±0.12 ^c	13.13±0.02 ^b	22.45±0.95 ^f	24.21±0.60 ^{ef}	26.00±0.22 ^e
BAP	2.25±0.18 ^a	4.00±0.00 ^a	7.05±0.05 ^a	11.00±0.32 ^a	14.06±0.39 ^a	16.02±0.00 ^a	18.08±0.50 ^a
BAP+AC	2.12±0.06 ^a	6.24±0.23 ^b	9.35±0.25 ^b	13.60±0.84 ^b	18.00±0.67 ^{bc}	20.55±0.96 ^c	23.81±0.49 ^c
KN	2.00±0.40 ^a	4.12±0.14 ^a	7.46±0.37 ^a	11.21±0.62 ^a	14.32±0.60 ^a	16.00±0.52 ^a	18.52±0.60 ^a
KN+ AC	2.00±0.00 ^a	5.00±0.67 ^a	9.24±0.42 ^b	12.44±0.04 ^b	17.18±0.89 ^b	18.35±0.12 ^b	22.44±0.43 ^b
IBA	5.00±0.49 ^c	8.45±0.51 ^{bc}	11.00±0.81 ^c	14.21±0.08 ^{bc}	17.00±0.00 ^b	20.23±0.87 ^c	22.00±0.00 ^b
IBA+ AC	6.15±0.00 ^d	9.20±0.00 ^e	12.23±0.11 ^d	16.57±0.43 ^d	20.64±0.90 ^e	23.02±0.45 ^d	27.01±0.01 ^f
NAA	4.20±0.00 ^{bc}	8.01±0.47 ^{bc}	11.00±0.29 ^c	15.11±0.26 ^c	17.21±0.70 ^b	21.00±0.00 ^c	23.33±0.55 ^c
NAA+AC	6.00±0.03 ^d	9.42±0.03 ^e	12.44±0.78 ^d	15.60±0.07 ^c	19.32±0.41 ^{cd}	23.43±0.30 ^{de}	27.00±0.00 ^f
NAA+KN	5.01±0.82 ^{bc}	7.05±0.44 ^{bc}	11.05±0.26 ^c	15.01±0.50 ^{bc}	19.72±0.00 ^{cd}	23.21±0.44 ^e	27.50±0.90 ^f
NAA+KN+AC	6.00±0.27 ^d	8.00±0.12 ^{bc}	13.00±0.51 ^d	17.00±0.06 ^d	21.01±0.50 ^e	24.02±0.94 ^{ef}	28.32±0.77 ^g

Values in a column with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.

with the help of a spatula. These were dispersed in a drop of water on a glass-slide and observed under light microscope. The percentage of seed germination was calculated by employing the following formula:

Germination (%) = (Number of enlarged seeds showing swelling of the embryo \times 100) / Total number of seeds.

Once the spherules were developed, pertinent observations were recorded at the intervals of one week to trace the different stages of development of the cultures. These were observed using a stereozoom microscope (Nikon, H600L, Japan). Sub-culturings were carried out as and when required.

Observations and Statistical Analysis

The cultures were observed regularly under binocular microscope (Olympus SZX10, Japan) and data recorded accordingly. The results were analysed using one-way ANOVA test and were analyzed using Tukey's Multiple Comparison at $p \leq 0.05$ using SPSS (Version 17) software package (SPSS Inc. Chicago, US).

Results and Discussion

Presently, it was made possible to induce the germination in immature seeds from green capsules through *in vitro* asymbiotic seed germination technique on M medium (Table 1) without the intricacies of host-fungus relationship. Through this technique, it has

become possible to bypass the complex fungal requirements of the 'highly reduced' non-endospermic seeds. The asymbiotic seed germination technique ensures better germination percentage, thus saving the time lapse between pollination and seed sowing (Sagawa, 1963). The method of asymbiotic seed germination also ensures better germination rates from immature seeds, rather than those from mature seeds, because of their physiologically active state. Immature seeds possess metabolically awakened embryos and distended testa cells; they also lack dormancy or inhibitory factors (Yam and Weatherhead, 1988). The appropriate stage at which the embryos can be germinated successfully varies with the genus, species, hybrids and the local conditions (Arditti *et al.*, 1982). The technique has been widely and successfully tested in a large number of orchid species of diverse habit and habitats (Anuprabha and Pathak, 2012; Arditti and Ernst, 1993; Buyun *et al.*, 2004; Deb and Temjensangba, 2006; Dutra *et al.*, 2008; Lo *et al.*, 2001; Magrini *et al.*, 2011; Park *et al.*, 2000; Pathak *et al.*, 1992, 2001, 2011, 2016; Pierce *et al.*, 2010; Roy and Banerjee, 2001; Shimura and Koda, 2004; Sabin *et al.*, 2014; Verma *et al.*, 2013; Vij and Pathak, 1988; Vij *et al.*, 1995; Yamazaki and Miyoshi, 2006). A few other explants have also been exploited for *in vitro* propagation purposes by some earlier workers (Arora *et al.*, 2014, 2016; Bhattacharjee *et al.*, 2015; Borah *et al.*, 2015; Chauhan *et al.*, 2015; Hegde, 2012; Hoque *et al.*, 2016;

Pathak *et al.*, 1992, 2001, 2011, 2012; Vij *et al.*, 1994, 1995). Men *et al.* (2003) indicated that this technique ensures better germination percentage, reduces the time lapse between pollination and sowing of the seeds, facilitating easy sterilization of the green capsules, exploits the polyembryonate potential of the seeds, and has proved advantageous for hybrid rescue besides cloning the apomictic species.

The present study explains various morphogenetic events of the *in vitro* seed germination, seedling development of *Coelogyne cristata*, thus providing information about mass propagation and conservation of this species. Almost all seeds were embryonate (Fig. 2A). In the basal medium, the germination of the seeds

initiated after 4.25 ± 0.00 wks of culture. Upon germination, the swollen seeds emerged out of the seed coats through lateral slits and/or terminal pores (Fig. 2B) as globular spherules, which were slightly green in colour. They grew in size while still attached to the seed coats. After about 8 wks, the spherules developed into pear-shaped chlorophyllous protocorms (Fig. 2B) and shoot-tips at the opposite ends (Fig. 2C). The protocorms later developed hairy outgrowths at their lower surfaces (Fig. 2D). Later, these differentiated 1st leaf primordia and later 2nd leaf primordia. The roots initiated soon after at the base of leaf primordia and developed into seedlings after 24.34 ± 0.60 wks of culture (Fig. 2E). Additional activated charcoal invariably impaired the germination frequency, delayed

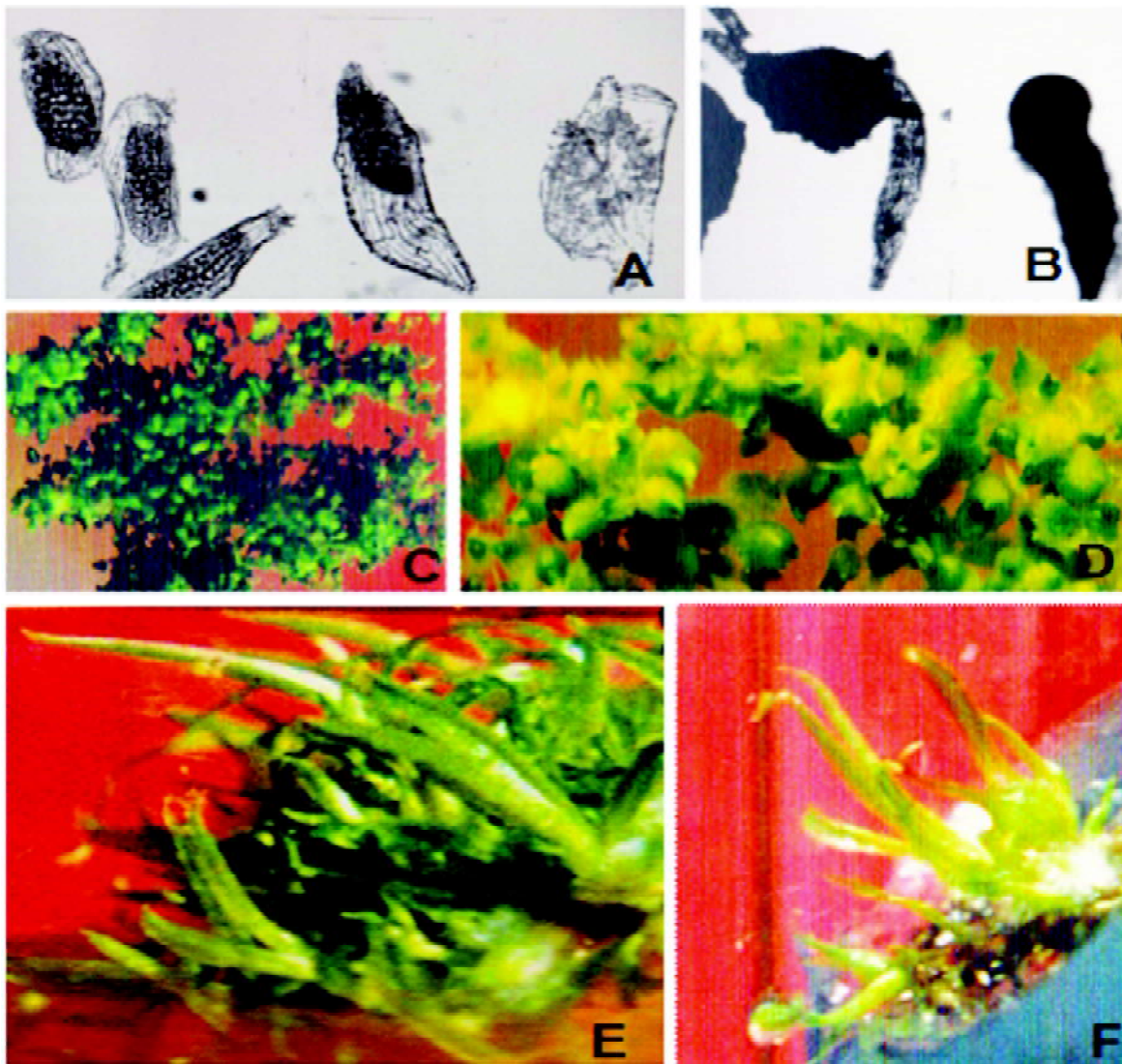


Fig. 2 A-F. *In vitro* asymbiotic seed germination of *Coelogyne cristata* on M medium showing various morphogenetic changes: A, Embryonate swollen seeds emerging out of the seed coat through lateral slit and terminal pore; B, Spherules transforming into pear-shaped protocorm; C, Protocorms with differentiated shoot tips; D, Hairy protocorms; E, Development of seedlings after 24.34 ± 0.60 weeks of culture (M); F, Impaired seed germination and meagre seedling development (M+AC).

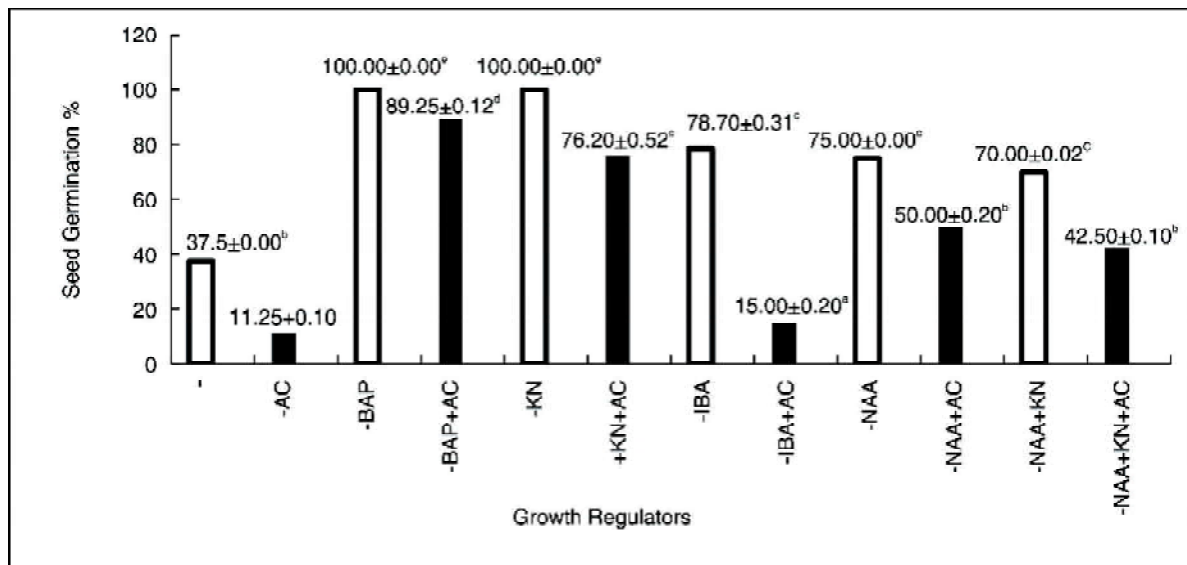


Fig. 3. Effect of growth regulators on per cent seed germination of *Coelogyne cristata* on M medium.

organogenesis and differentiation in the cultures (Fig. 2F). In this study, the seeds germinated readily on basal medium without additional PGRs suggesting that the seeds had sufficient level of endogenous hormones. The results are in accord with similar earlier findings in *Aerides odorata* (Pant and Gurung, 2005), *Coelogyne cristata* (Pant et al., 2008), *Coelogyne flaccida* (Kaur and Bhutani, 2014).

In this experiment, the germination competence was markedly influenced by the chemical stimulus in the nutrient pool (Fig. 3). The seeds in cytokinins (BAP/KN) supplemented medium germinated within 2.00±0.00

wks of culture with 100 per cent frequency and induced early protocorm development and early seedlings development within 18.08±0.50 wks of culture. A perusal of literature reveals that cytokinins had beneficial effect in promoting advanced morphogenetic events of germinating entities and seedling development in *Acampe longifolia* (Kabita and Sama, 2001), *Coelogyne nervosa* (Sonia et al., 2012; Sibin and Gangaprasad, 2016), *Cymbidium iridoides* (Swar and Pant, 2004), and *Cypripedium* species (De Pauw and Remphrey, 1993). Literature study reveals that normally orchid seeds do not require exogenous cytokinin treatment to induce germination. The orchid seeds are cytokinin autonomous

as they contain sufficient endogenous level of cytokinin (De Pauw et al., 1995; Mercier and Kerbauy, 1991). Present species seems to be an exception as it did not contain sufficient cytokinin levels and required exogenous application of the same to accomplish cent per cent germination in the seeds.

Auxins (IBA/NAA) alone and in combination with cytokinins lowered the per cent germination frequency and even delayed seedling development unlike earlier in *Cymbidium bicolor*, where seeds showed cent per cent germination (Verma et al., 2013); *Eulophia dabia* (Sharma and Vij, 1986); *Calanthe discolor* (Miyoshi and Mii, 1995), and

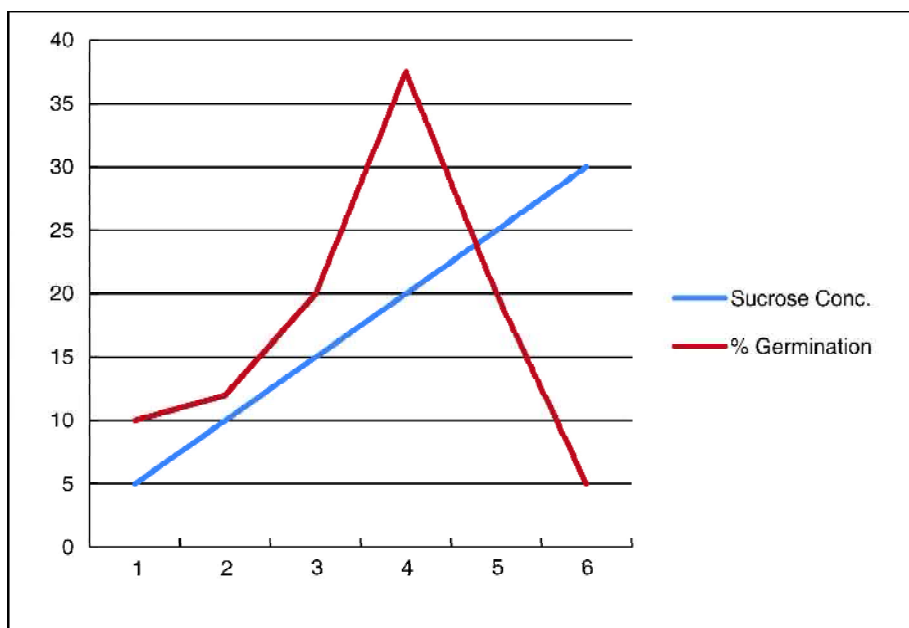


Fig. 4. Effect of different sucrose concentration on per cent seed germination of *Coelogyne cristata* on M medium.

hybrids of *Bletilla*, *Cattleya* and *Cymbidium* (Strauss and Reisinger, 1976).

Activated Charcoal (AC) is reported to promote seed germination, morphogenetic process and healthy growth of the seedlings in numerous orchid species (Pathak *et al.*, 2001; Verma *et al.*, 2013; Vij and Pathak, 1988). On the contrary, presently, activated charcoal invariably reduced per cent germination response and even delayed seedling formation (Fig. 2F) probably due to the adsorption of essential nutrients which were otherwise required for the healthy growth of the seedlings of *Coelogyne cristata*.

Presently, the effect of sucrose was also tested on seed germination percentage by varying concentration from 5 and 30 g l⁻¹. The germination frequency was optimal at its concentration of 20 g l⁻¹ in the medium and other levels of sucrose concentrations reduced per cent germination (Fig. 4). A perusal of literature indicates that the sub-optimal level of sucrose is inhibitory and its supra-optimal level increases the osmotic pressure in the medium, and proves detrimental to the survival of the cultures (Hans and Stephans, 1992) and possibly this could have remained the possible reason for the differential response of seeds to the varying concentration of sucrose in the medium.

Presently, *in vitro* asymbiotic seed germination in *C. cristata* has made possible to study the morphogenetic changes occurring during seed germination till seedling development. All these data suggest that the seeds of *C. cristata* are capable of germinating with maximum germination percentage and early seedling development in simple defined Mitra medium supplemented with cytokinins (BAP/KN). Sucrose at 20 g l⁻¹, efficiently initiated maximum germination response in the cultures. The present study has indicated the potential to assist with the *ex situ* conservation of the threatened species by producing large number of viable seedlings.

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