***In vitro* mass multiplication of *Cymbidium iridioides* D.Don– a medicinal orchid from Arunachal Pradesh, India**

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Abstract

The paper describes *in vitro* mass multiplication of *Cymbidium iridioides* D.Don., an epiphytic medicinal orchid from Arunachal Pradesh, through seed culture and early seedling development. Murashige and Skoog (MS, 1962) medium supplemented with 6 – Benzylaminopurine [(BAP) 0.5 mgL⁻¹] plus α – Naphthaleneacetic acid [(NAA) 0.5 mgL⁻¹] proved to be the best for initiating seed germination. It favored high regeneration frequency, early response, and high proliferation rate in protocorm formation. Plantlets with roots (4 – 7 roots per plant) were obtained on MS medium supplemented with BAP (0.5 mg/l), NAA (0.5 mg/l) and Activated Charcoal (1 gm/l). Vigorous growth of *in vitro* seedlings was observed on MS medium supplemented with 10 % coconut water (v/v) plus 10 % banana pulp (w/v). *In vitro* raised *Cymbidium iridioides* seedlings recorded 94 % survival in greenhouse conditions.

Key words: *Cymbidium iridioides*, Seed culture, Mass propagation, Medicinal orchid.

INTRODUCTION

Orchids are nature's most extravagant group of flowering plants distributed throughout the world from tropics to high alpine areas (White & Sharma 2000). They exhibit incredible range of diversity in shape, size and color of their flowers and, hence, are important aesthetically, medicinally and also regarded as ecological indicators (Joshi *et al.* 2009). Several orchid species are cultivated for their various economic uses especially in floriculture. Orchids are grown primarily as ornamentals and are valued as cut flowers because of their exquisite beauty and their long lasting blooming periods (Hew *et al.* 1997) and are linked to culture and customs in many societies including traditional tribes. At the same time, many species of orchids are used as herbal medicines and food (Khasim & Rao 1999; Kasulo *et al.* 2009). Although, large populations of orchids are still confined in their natural habitat in many parts of the world, their number in the wild is seriously affected due to their high demand and excessive anthropological disturbances. Many orchid species are threatened due to loss of their natural habitat and indiscriminate collection.

The North-east Indian state Arunachal Pradesh is also housing a large number of naturally occurring orchids and has provided an exhaustive list of 972 species for NE India of which 577 species are known to grow in Arunachal Pradesh (Rao 2018).

Cymbidium iridioides D.Don (PLATE - I) is an epiphytic, sub-tropical orchid species, which has high ornamental as well as medicinal values. Juice from its crushed leaves are used for clotting of blood (stops excessive bleeding) in deep wounds and leaf-paste is used as tonic (Subedi 2011). It is considered to be extra-exotic due to its long lasting beautiful color range and characteristics flowering from September to November. In NE India, so far, the species

has been recorded from Sikkim, Arunachal Pradesh, Meghalaya, Nagaland, Mizoram and Manipur (Rao 2018). It is found in the sub-tropical and temperate zones of Arunachal Pradesh. This species is reported to be over exploited for commercial and medicinal uses (Joshi & Joshi 2000; Pant *et al.* 2002). Consequently, its populations are depleted, became rare and is now restricted to very narrow pockets in the natural habitats. Herbal medicines are the precursors of many common drugs and are getting preferences in the primary health care systems (Jawahar *et al.* 2008) so are extremely important in biotechnological industries and research for the production of pharmaceutical compounds (Chand *et al.* 1997). *In vitro* culture techniques offer viable systems for true-to-type rapid mass multiplication of plants and for germplasm conservation of RET, aromatic and medicinal plants (Arora & Bhojwani 1989; Sharma *et al.* 1991; Sudha & Seeni 1994; Sahoo & Chand 1998; Karuppusamy & Pullaiah 2007). The main objective of the present study is to establish a protocol for *in vitro* mass propagation of *Cymbidium iridioides* through asymbiotic seed germination to produce maximum number of plantlets for its cultivation and conservation.

PLATE - I: A. Flowers of *Cymbidium iridioides* D.Don; **B.** Seven months old capsules

METHODOLOGY

The explants used for the present investigation were seven months old mature un-dehiscent pods of *Cymbidium iridioides* collected from the plants under cultivation at Sessa Orchid Nursery, Sessa, West Kameng district of Arunachal Pradesh, India (PLATE - II).

Surface sterilization and Inoculation:

Green, mature and undehiscent capsules of *Cymbidium iridioides* were used as explants for the present investigation. Green pods were first cleaned with Labolene (detergent) and thoroughly washed in running tap water till the detergent was washed off completely. After that, green

Pods were surface sterilized sequentially by immersing in 10 % Sodium hypochloride (NaOCl_2) solution for 20 minutes; absolute ethyl alcohol for 10 minutes and finally rinsed thoroughly three times with sterile distilled water. The sterilized capsules were dipped in absolute ethyl alcohol for 2 minutes and then passed through the spirit flame for three times. The capsules were cut longitudinally with the help of a sharp sterile surgical blade to extract the seeds. The seeds were scooped out from the capsules and inoculated by spreading thinly as possible over the surface of the culture medium in conical flasks (250 ml), with each flask containing 40 ml of semi-solid medium.

Culture medium and Culture Conditions:

In the present study, full and half strength of Murashige and Skoog (MS, 1962), modified Knudson C, (MKc; 1946) and Vacin & Went (Vw; 1949) medium supplemented with or without plant growth hormones viz, BAP (0.5 mg/l), NAA (0.5 mg/l) and organic additives viz. 10 % coconut water (v/v) and 10 % rippened banana (w/v) were added for *in vitro* seed germination and seedling development (Table 1). All culture media were gelled with 0.8 % (w/v) agar and pH were adjusted at 5.4 – 5.8 by using 0.1N NaOH or HCl prior to autoclaving at 121°C for 45 minutes. All cultures were incubated at $23 \pm 2^\circ\text{C}$ under 16/8 hrs photoperiod of 3000 to 3500 lux light intensity (40 W Fluorescent tubes; Philips India Ltd., Mumbai, India). The experiments were repeated twice. The observations were made regularly and data were recorded accordingly (Table 1).

RESULTS

The results of the culture cum propagation experiments under different composition of media has been presented in Table 1 below.

Table 1. *In vitro* seed germination and seedling development of *Cymbidium iridioides* D. Don

[Abbreviations used: MKC = Modified Knudson C (MKc, 1946); V&W= Vacin& went (Vw, 1949); MS = Murashige and Skoog (MS, 1962); $\frac{1}{2}$ MS = Half strength macronutrients; BAP = 6-Benzylaminopurine; NAA = α -Naphthalene acetic acid; AC = Activated charcoal; Rb = Ripe banana; Cw = Coconut water; PGR = Plant growth regulators]

Basal Medium	PGR (mgL^{-1})		Germination (in week)	% of germination	Additives	Seedling (in month)	Observation/remark
	BAP	NAA					
MKc				00			No germination
V&W				00			No germination
V&W			10	30	10% Cw		Germination favoured
MS			08	70			Germination favoured
$\frac{1}{2}$ MS			08	65			Germination favoured
MS	0.5	0.5	06	96			Germination, growth, & development
MS	0.5	0.5			1 gm AC	2	Plantlets developed roots
MS					10% Cw + 10% Rb	4	Vigorous growth of seedlings

PLATE II. Different stages of *in vitro* seed germination and seedling development of *Cymbidium iridioides* D.Don: **A.** Mature seeds turned into small spherules to protocorms on full strength MS + BAP (0.5 mg/l) + NAA (0.5 mg/l); **B.** Protocorms with primordial leaf on same fresh primary culture media; **C.** Development of small shoots on MS + BAP (0.5 mg/l) + NAA (0.5 mg/l) + 1 gm/l Ac; **D.** Vigorous growth and development of plantlets on MS + 10% Cw + 10% Rb; **E.** Deflasked plantlets; **F.** Acclimatized seedlings

DISCUSSION

Varied response of *Cymbidium iridioides* seeds cultured on V&W, MKc, V&W supplemented with 10 % coconut water (v/v), $\frac{1}{2}$ MS, MS and MS basal media supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l), was recorded (Table 1). MS medium with or without growth regulators was found to be effective for the germination of mature seeds whereas

MKc and V&W without additives were found to be ineffective. In MS medium seeds started germination after two weeks of inoculation. In half and full strength of MS basal media, the visible sign of germination was observed as the swollen yellowish green spherule like protocorms developed within 8 weeks of primary culture. The first primordial leaf was developed in 12 weeks of culture, and by 16 weeks, roots were developed when protocorms with leaf primordial were sub-cultured on the same fresh basal media. Although, MS, $\frac{1}{2}$ MS and V&W supplemented with 10% coconut water (v/v) (Table 1) favoured seed germination, the rate of seed germination was less in comparison to MS medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) where the initiation of seed germination was observed within 6 weeks of primary culture and around 96 % of seeds were successfully germinated. The germination of seeds and growth of protocorms were variously affected depending on the concentrations and combinations of growth regulators. Growth of protocorms were vigorous on MS media supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l) (PLATE - II, Figure A). Protocorms obtained on germination medium were subcultured onto $\frac{1}{2}$ MS, Vw and MS supplemented with BAP (0.5 mg/l), NAA (0.5 mg/l) and 1 gm Activated charcoal for further development. Though proliferation and plantlet development of protocorms were observed in all three tested media, MS basal medium fortified with optimum cytokinins and auxins proved more effective. Such promoting effects of cytokinins and auxins for regeneration of PLBs and multiplication thereof have been reported earlier by Gangaprasad *et al.* (1999) and Vij *et al.* (2000). Vigorous growth and further proliferation of plantlets were observed when small plantlets from the primary cultures were transferred to full strength MS basal medium supplemented with 10 % coconut water(v/v) and 10 % ripe banana Pulp (w/v) (FPLATE - II, Figure D). Well-rooted plantlets were planted in poly bags containing mixtures of decomposed wood and dried leaf-mould with top soil and transferred to a Green House for acclimatization (PLATE - II, Figures E & F). About 96 % of *in vitro* raised seedlings survived and established in the green house conditions.

In the present studies, a combination containing cytokinins and auxin [BAP (0.5 mg/l) + NAA (0.5 mg/l)] proved to be the best. The requirement of synergistic combinations of cytokinins and auxins for stimulating proliferation and further differentiation is well reported in orchid literature (Kanika & Vij 2002; Mathews & Rao 1985; Park *et al.* 2000; Pathak & Vij 2001; Seeni & Latha 1992, 2000; Tanaka & Sakanishi 1997; Vij *et al.* 2002). A successful attempt was made to compare the *in vitro* seed germination and their subsequent differentiation of *C. iridoides* on $\frac{1}{2}$ MS, MS, Vw, MKc and MS fortified with BAP (0.5 mg/l) and NAA (0.5 mg/l). Although $\frac{1}{2}$ MS, MS and Vw basal media with 10% Cw (v/v) showed seed germination, higher percentage of germination in shorter period was observed on full strength MS basal medium supplemented with BAP (0.5 mg/l) and NAA (0.5 mg/l). Hormone free full and half of media took longer period for germination. Hence, in conclusion, MS medium was found to be the best in terms of vigorous growth, production of large protocorms and time requirements especially when medium was supplemented with optimum growth hormones. Thus, this protocol might be useful for selection of best condition for *in vitro* mass multiplication for *ex-situ* conservation as well as large scale cultivation of *Cymbidium iridoides* D. Don - a valuable medicinal orchid species.

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