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Research Article – Plant Biotechnology

Tissue culture studies on *Cymbidium ensifolium* (L.) Swartz

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Abstract

An efficient protocol for seed germination and micropropagation of *Cymbidium ensifolium* (L.) Swartz. was established. Four nutrient media were used for seed germination and early protocorm development: Murashige and Skoog (MS), half –strength MS, Knudson 'C' (KC), and Vasin and Went (VW); combinations and alone of four plant growth regulators i.e. 6-benzylaminopurine (BAP), kinetin (KN), α-napthalene acetic acid (NAA), and indole-3-butyric acid (IBA) were studied. MS medium was found as most ideal for seed germination (98±0.48) and lowest in VW (71.12 ±0.42). 3 months old protocorm were sub cultured on fresh MS medium supplemented with different concentrations of BAP, KN, NAA, and IBA alone and in combination. After 30 days highest secondary protocorms (21.25±0.63) were observed in MS medium containing BAP (4.0μM). MS medium supplemented with 8μM IBA induced the maximum roots per shoot. After 16 days of transfer to green house the survival rate was 88%.

Keywords: *Cymbidium ensifolium* (L.) Swartz, MS, protorm, micropropagation, BAP

Introduction

Orchid is one of the richest heritage of natural the shape of flower. The genus was established by Sir W. resources of Bangladesh. The climate of Bangladesh is Jones in 1795 and his type species is *Cymbidium ensifolium* (L.) Swartz. One of the important vegetational places in the world. There are about 80 species plants, native to China the Bangladesh lies in the transition of three important Himalayas, Bangladesh, Indonesia and northern Australia. vegetational zone of the world. Hooker (1895) first Vanda is monopodial orchids and most are epiphytic. In reported about 1250 species from the Bangladesh region Bangladesh, it can be found on trunks and branches of of the British India (Lokho, 2013). Sometimes it grows as lithophyte on rocks. All Orchids are nature's most extravagant group of *Vanda* enjoy the light and with sufficient sunlight the may flowering plants distributed throughout the world from bloom two or three times a year. The inflorescences tropics to high alpine (Rao, 1998). Orchids belong to the family appear from the axis of the leaves. Flowers are long lasting Orchidaceae is one of the most diversified and largest and appear in cluster along the spike. The flower of among the flowering plants. It is an attractive monocot orchids has great aesthetic value. Family with its large number of species and natural This genus is one of the five most horticulturally hybrids.

There are various estimates about the number of important orchid genera, because it has some of the most genera, species and varieties. The number of genera magnificent flower to found in entire orchid family. This varies from 500-800 and the number of species from 15000-has contributed much to the work of hybridists producing 35000 according to various repots (Anonymous, 2008; Arditti, 1979; Arditti *et al.,*1982; Rao and Sridhar, 2007; Kotia *et al.,* 2010; Aktar *et al.,* 2008). However, some flowers for cut flower market. *Cymbidium ensifolium* is one of conservative statement accounts about as many as 750 the few botanical orchids with blue flower (actuall a very genera with about 17000 species constituting the family bluish purple), a property much appreciated for producing orchidaceae (Aktar *et al.,* 2007; Rao and Sridhar, 2007). interspecific and inter generic hybrids.

Commercial cultivation of orchids both for pot plant and cut flower production has been developed into sizeable industries in many countries as the lovely flower fetch a very high price in the international market and the sale of flower run a million of dollars. The techniques of plant organ, tissue and cell culture are now established in many research laboratories throughout the world and rare being used in different areas of plant science. These techniques are widely used for mass scale propagation of commercially important plant, development of virus free plants, induction of somaclonal variation and also in execution of genetic engineering programmes. The research in the field of tissue culture is still continuing for the development of new protocols, refinement of the media and increasing the efficiencies of the techniques. Orchid seeds usually germinate in symbiotic association with mycorrhizal fungus, which supplies food to the germinating undifferentiated orchid embryos. Therefore, the presence of mycorrhizal fungus is prerequisite for germination of orchid seeds.

The distribution of orchids is related to availability of mycorrhizal fungus. As a result, orchid generally propagate in nature very slowly. Now a day, commercial orchid are predominantly propagated by tissue culture technique. Kundson (1922) first succeeded in development of in *vitro* germination technique of some orchid *viz*. *Cattleya, Laelia* and *Epidendrum* seeds on agar medium containing sugar. From the commercial point of view, rapid multiplication is

necessary and accordingly in 1950's more refined techniques of *in vitro* germination of some orchid seeds have been developed and established. In 1960's very rapid *in vitro* clonal propagation of commercially important orchids such as *Cymbidum sp; Phalaenopsis sp; Dendrobium sp.* Was achieved by meristem, organ and tissue culture ((Maridass *et al,* 2008; Nimisha and Yadav, 2012). Different explants including shoot tip (Orchid Society of Alberta, 2012; Kumar *et al.,* 2007; Kumar *et al.,* 2011), leaf stalk (Orchid Society of Alberta, 2012), leaf segment (Parthibhan *et al.,* 2012), young leaves (Pyati *et al.,* 2002), inflorescence (Rao, 1998), root tip and rhizome segments have been used successfully for commercial propagation of orchids. Based on these techniques in 1980's a number of commercial farms have been set up in different countries especially in South East Asia. Million of ornamental orchid seedlings are produced in these countries and sold in local and foreign markets. An attempt was taken to develop a suitable *in vitro* protocol for seed germination of indigenous orchid, *Cymbidium ensifolium.* both for their commercial uses and conservation purposes.

Materials and Methods

Plant materials

The immature capsule of *Cymbidium ensifolium* was used as seed source which was collected from Rajshahi, Bangladesh. The plantlets of *Cymbidium ensifolium* were grown through *in vitro* condition for further used as the source of explants for experiment purpose.

Seed sterilization and in vitro seed germination

At first, the capsules were washed by running tap water and surface sterilized by detergent. Then capsules were treated with 0.2% (w/v) HgCl2 solution for 10 minutes and finally dip in 70% ethanol for 10-12 seconds. Then washed them with sterile distilled water and sterilized capsules were cut longitudinally by a sterile surgical blade.

Around 100 mg seeds were cultured per vessel (Fig. 1). Various media *viz.* MS (Murashige and Skoog, 1962), B5 (Gamborg *et al*., 1968) and PM (PhytamaxTM, Sigma, USA) were used under this study. The basal media (MS and B5) amended with 3% (w/v) sucrose and the PM was amended with 2% (w/v) sucrose. The pH for all medium was adjusted at 5.6- 5.8. Inoculated vessels were maintained in the culture room under a period of 16 hrs light and dark for 8 hrs at 25±2°C. After two weeks of inoculation, some of the seeds were taken out and dispersed in one drop of water on a glass slide and examined its developments under a light microscope. Once the spherules were formed, then protocorm like bodies (PLBs) developmental stages were recorded in every week up to 8-10 weeks.

PLBs development and seedlings elongation

To evaluate the regeneration efficiency of protocorms, the PLBs were transferred to half strength MS medium supplemented with different concentration and combination of auxins and cytokinins. The protocorms, which developed from the germinated orchid seeds were isolated aseptically and transferred into fresh culture vessels containing the same germinating medium. Further subculture of the protocorm was done at an interval of 15- 20 days. Prior to each subculture the density of seedlings per vessel was reduced. For estimating rapid elongation of shoots, germinating seedlings were transferred into different types of shoot elongation media. The elongation media were based on agar solidified MS medium supplemented with BAP (0.5-2.0 mgl-1), NAA (0.5 mgl-1), Kin (0.5 mgl-1) and IAA (1.0 mgl-1).

Multiple shoot induction

For multiple shoot development the *in vitro* growing seedlings were used when it raised up to 4-5 cm in height. These shoot segments were transferred into shoot induction and elongation media for development of adventitious shoots. Subculture of these explants was done at an interval of 4 weeks.

Rooting and acclimatization

Newly developed adventitious shoots when reached a height of 2-3 cm were transferred into rooting medium for root development. For efficient root induction we used three plant growth regulators *viz.* IAA, IBA and NAA in ½MS medium. The well rooted *in vitro* grown plantlets were hardened successfully in the potting mixture containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition following by the methods of Rahman *et al*. (2009).

Shoot elongation and rooting

After culture for 8 weeks, the adventitious shoots regenerated from explants were transferred to hormone-free MS medium for shoot elongation. Three basal media were assessed for growth and development efficiency: Murashige and Skoog (MS), ½ MS and Knudson C (KC) [17]. When the shoots reached 0.5-1.5 cm in height, they were transferred onto basal MS medium supplemented with 0.1-2.0 mg l-1 BAP (6- Benzylaminopurine) and Kn (Kinetin or 6-Furfurylaminopurine) [18]. For the effect of sucrose at concentrations of 1%, 2%, 3%, 5% or 7% (w/v) were separately added into the MS medium for shooting and rooting.

Culture conditions and data analysis

Uniform culture conditions were applied in all experiments.All experiments were conducted 3 replicates with 500 ml conical flasks in each (containing 80 ml medium) and 7-14 explants were cultured in every 500 ml conical flask. The pH of the media was adjusted to 5.7 before autoclaving. The media was autoclaved for 15 min at 121°C. Cultures were incubated at 25 ± 2°C under a 16 h photoperiod with cold white fluorescent light mixed with incandescent light at 55.6 mol m-2 s-1.

In-vitro rooting

The matured sub-cultured plants were subjected to rooting using Rooting Culture Medium. *In-vitro* rooting was successful with MS media supplemented with 2 mg BAP, 1.5 mg IAA, 50 ml CM and 500 mg AC.

Ex-vitro rooting

The basal ends of healthy shoots from the shoot multiplication medium were dipped in an auxin solution, 10 ml of IAA(200 PPM)for 15 mints then planted in small pots containing solrite (Garden Soil, Rock Dust and Peat Moss) sprayed with bavistin to avoid fungal infection. *In-vitro* rooted plants in the pot trays containing potting mixture maintained under mist chamber and covered with perforated plastic cups.

Hardening

Roots were treated with 500 PPM Bavistin, a systemic fungicide for 2-3 minutes. For ex-vitro rooting induction, shoots were given treatment with 200 PPM NAA. Plantlets were transferred to thumb pots containing soil, and solrite and were kept in the green house. Plantlets were also subjected to high humidity conditions of green house for healthy growth. Successfully established plantlets were subsequently transferred to field condition method.

Results and Discussions

Asymbiotic seed germination in sterile conditions is one of the methods of conservation and propagation of orchids (Maridass et al, 2008). The highest seed germination percentage was obtained in B5 medium (96.7% ±0.25) followed by NN (94.4% ± 0.30), MS (94.1% ±0.33) and the least was recorded in KC (29.7% ± 0.46) (Table 1, Fig. 2). A balanced supply of both organicand inorganic nutrients is needed for the development of orchid seeds (Nimisha and Yadav, 2012). Plant growth regulators, i.e.,cytokinins are reported to play an important role in orchid seed germination (Orchid Society of Alberta, 2012), however, in our study, the seeds of *Cymbidium ensifolium* germinated in medium devoid of growth regulators. This could be due to the presence of sufficient endogenous growth regulators needed for the initial stages of germination (Kumar *et al.,* 2007). The presence of nitrogen in the form of potassium nitrate in B5 medium could have accounted for the high germination percentage of *Cymbidium ensifolium* seeds. Also, the presence of the vitamin, thiamine in higher amount in B5 might have influenced the germination of these seeds (Kumar *et al.,* 2011; Parthibhan *et al.,* 2012). The largest protocorm volume was recorded in MS (29.95x 10–4 mm3) and the smallest in KC (1. 8 x 10–4 mm3). This might be because MS medium is rich in both micro and macro nutrients.

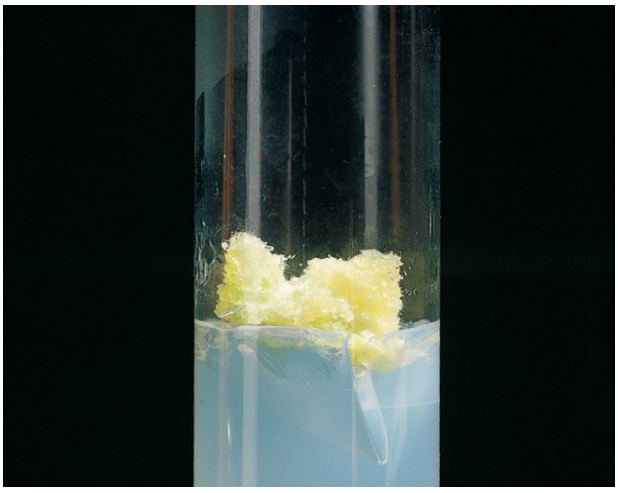

Fig. 1. Explant of *Cymbidium ensifolium*

The presence of nitrogen in the medium is also reported to influence the growth and differentiation of cells (Pyati *et al.,* 2002). Further development of protocorms into seedlings can be attributed to the efficient assimilation and utilization of nitrogen in the form of ammonium nitrate present in the MS medium. The growth of the seedlings *viz*., shoot number (1.90 ± 0.11), shoot length (1.17 ± 0.04), number of leaves (2.80 ± 0.09), root number (3.05 ± 0.09) and root length (0.80 ± 0.009) was also found to be highest in MS medium. In B5 and NN media, the protocorms formed shoots and roots but growth stopped after 90 days. This might be attributed to the inhibitory influence of nitrogen in the form of ammonium sulphate in B5 medium or mixtures of vitamins present in both B5 and NN media on seedling growth (Parthibhan *et al.,* 2012; Pyati *et al.,* 2002; Rao, 1998).

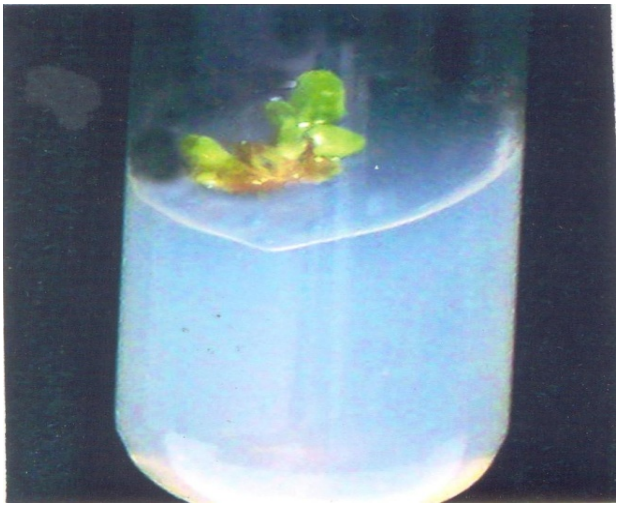

Fig. 2. Callus formation of *Cymbidium ensifolium*

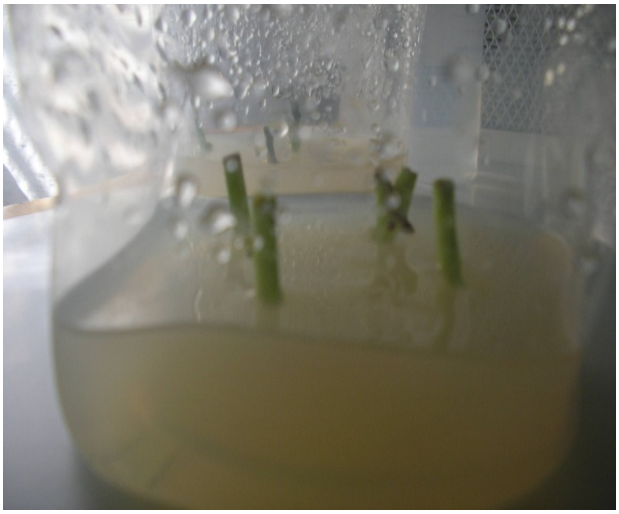

Fig. 3. Shoot and root formation of *Cymbidium ensifolium*

Fig. 4. Mother plant of *Cymbidium ensifolium*

The poor response in terms of seed germination and growth of the protocorms in KC medium could have been due to the lower amount of nutrients and vitamins present in KC medium which were not sufficient for complete development of the seedlings (Arditti *et al.,* 1982). However, Nongrum *et al.* reported enhanced seed germination of *Coelogynae ovalis* and *C. nitida* in KC medium. Nath *et al.* reported a similar finding where *Vanda coerulea* failed to develop beyond the protocorm stage. There are also similar reports of inhibition of seed germination of epiphytic orchids in KC medium (Rao, 1998). The differential response of orchid seeds to different nutrient media is due to specific requirement of the species. Plantlets were hardened in a compost mixture comprising brick, charcoal, decaying litter in a ratio of 1:1:1 and a layer of moss on top. During the process of hardening it was observed that the transferred plants initially shed their leaves then produced new leaves. According to Preece and Sutter plantlets when transferred, must produce new leaves to adjust to new conditions in order to enable effective photosynthesis and growth of the *in vitro* - raised plants. Plantlets hardened in a compost mixture comprising brick, charcoal, decaying litter in a ratio of 1:1:1 and a layer of moss on top showed 71% survival (Fig. 3). The high moisture content retention ability of the layer of the moss on top proved to be beneficial for the successful transplantation of the *Cymbidium ensifolium* plants. The protocol developed in the present study can be used for *in vitro* mass scale propagation of *Cymbidium ensifolium* through asymbiotic seed germination wherein a maximum of around 200 plants can be produced from 100 seeds in basal MS medium. Thus, the protocol is suitable for *ex situ* conservation and propagation of this threatened medicinally important orchid species

Conclusion

From these studies it can be concluded that the MS medium is most suitable for *Cymbidium ensifolium* (L.) Swartz, seed germination. This study also revealed that a low concentration of 2mg BAP and 5 mg NAA was found to be more suitable for plantlets and multiple plantlets. MS medium supplemented with 2 mg BAP, 1.5 mg IAA, 50 ml CM and 500 mg AC was found to be suitable for *In-vitro* Rooting. The result clearly shows that the seeds of *Cymbidium ensifolium* (L.) Swartz, can be used for micropropagation of the plant effectively. This protocol can be used to produce viable, uniform and healthy plants of *Cymbidium ensifolium* (L.) Swartz, to restore the significantly decreasing no. of populations of this beautiful rare orchid in nature and mass scale propagation for its commercial use.

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