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MICROPROPAGATION OF *CYMBIDIUM IRIDIOIDES* D.DON

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Abstract

A protocol for the micropropagation of *Cymbidium iridioides* D. Don, a highly collected orchid species of Nepal, has been developed. Complete seedlings were obtained from asymbiotically germinated seeds on Murashige and Skoog, 1962 (MS) medium solidified with 0.8% agar and supplemented with 6- benzyalaminopurine (BAP) at 1 mg l^{-1} and α-Naphthaleneacetic acid (NAA) at 1 mg l^{-1} in 8 wks of primary culture. The shoot tip explants were obtained from *in vitro* grown seedling for the shoot multiplication. Maximum shoot multiplication was observed on shoot tip explants on MS media supplemented with BAP 0.5 mg l^{-1} (8.25 shoots per single shoot). Media supplemented with 1 mg l^{-1} indole-3-butyric acid (IBA) was the ideal condition for root formation. Rooted plantlets were able to grow in *ex vitro* condition after a short period of acclimatization.

Introduction

ORCHIDS ARE economically important group of plants for their various uses in floriculture, medicine, and food industries. The rich diversity and population of orchids in the world is decreasing due to human activities and high population pressure. In Nepal, orchid species are under threat mainly due to: (a) habitat destruction, degradation, and fragmentation, and (b) over-harvesting of selected orchids for commercial trade (Pant *et al.*, 1999). Highly exploited species immediately require *ex situ* conservation by tissue culture method.

The genus *Cymbidium* comprises 70 species worldwide of which 10 species are reported in Nepal (Press *et al.*, 2000). *Cymbidium iridioides* is an epiphytic orchid species, which has high ornamental and medicinal value. It is considered to be extra-exotic due to its long lasting beautiful color pattern and extended flowering period from autumn to early winter. Juice from its crushed leaves is used for clotting of blood in deep wound and its paste used as tonic (Subedi, 2002). It is found in the subtropical and temperate zones of Central and Eastern Nepal. This species is reported to be under seriously threatened state due to over exploitation for commercial use (Joshi and Joshi, 2001; Pant *et al.*, 2002).

Seed germination and propagation of this orchid species in nature is very slow due to ecological constraints. The cymbidiums are conventionally propagated through separation of pseudobulbs, but the proliferation rate is very low. A more efficient approach is *in vitro* seed culture. Combination of exogenous growth regulators at suitable concentrations stimulates zygotic embryo to initiate protocorms that develop into plantlets. For mass propagation, regeneration from tissue-cultured explants is superior to seed culture due to year round availability of plant materials and an exponential propagation rate. During the last few years, tissue culture technique has been extensively exploited for the large-scale propagation as well as *ex situ* conservation of cymbidiums (Banerjee and Mandal, 1999; Chang and Chang, 2000; Chauhan *et al.*, 2010; Fonnesbech, 1972; Jamir *et al.*, 2002; Pathak *et al.*, 2001, 2011).

In the present investigation, shoot tip method was used for clonal propagation of *Cymbidium iridioides.*

Material and Methods

Plant Material

The materials used for seed germination were the young capsules (pods) of *Cymbidium iridioides* D. Don, obtained from the tissue culture laboratory, Central Department of Botany, Tribhuvan University, Nepal. The young green capsule was washed with tap water containing a few drops of teepol solution for few min and washed under running water for 30 min. The capsule was surface sterilized by immersing it in the solution of sodium hypochlorite (1%) for 10 min, 70% ethanol for 1 min and finally by rinsing three times with sterile water.

Nutrient Media were prepared by using different concentrations and combinations of NAA and BAP as given in the Tables 1 and 2. The pH of all media was adjusted to 5.7 with NAOH before autoclaving. Agar (0.8%w/v) was added as a gelling agent. Agar was dissolved by boiling the mixture and about 20 ml media was dispensed into each culture tube (150 x 25 mm) and autoclaved at 120°C for 15 min/15lb. The cultures were maintained at 25± 2°C and 200-300 lux under 16 hrs photoperiod.

Seed and Shoot Tip Culture

For the inoculation of seeds, immature green capsule

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Table 1. *In vitro* germination response of *Cymbidium iridioides* immature seeds in MS and B_5 media and their combination with growth regulators.

Medium	Growth regulators [Concentration (mgl^{-1})]	Initation of Germination (wks)	Time taken for development of (wks)					Remarks
			Protocorms	Chlorophyll	1st leaf primordium	1st root primordium	Seedlings	
B_5	-	-	-	-	-	-	-	No germination
MS	-	4	9	28	-	-	-	Germination favored
MS	BAP(1)	4	13	28	-	-	-	Germination favored
MS	BAP(2)	4	13	28	-	-	-	Germination favored
MS	BAP(1) + NAA(1)	4	2	4	5	6	8	Germination growth and development of seedling favored

was cut opened longitudinally and the seeds were scooped out with the help of microspatula and spread over the surface of the MS and Gamborg's (B_5) media with or without NAA and BAP.

For the clonal mass propagation, shoot tips (3-5 mm) were excised from *in vitro* grown seedlings and cultured in the culture tubes containing MS media supplemented with different concentration of NAA (0.5 and 1 mgl^{-1}) and (0.5, 1, 2 mgl^{-1}), either alone or in combinations.

Multiple shoot formation and root initiation was examined at the given cultured condition. Cultures were subcultured into fresh media once every 8 wks.

Rooting of Shoots

Individual shoots with two or three expanded leaves were detached from shoot clump and transferred to MS medium, which was further either supplemented with IBA, IAA, and NAA at 1-2 mgl^{-1}. The regenerated plantlets were transferred to pots containing cocopeat in order to grow into normal plants after a short period of acclimatization. Eight replicates were used for each treatment and the cultures incubated at 25$\pm$ 2°C.

Results

Seed Culture

MS medium with or without growth regulators was found to be effective for the germination of immature seeds whereas B_5 medium was found to be ineffective. (Table 1). Seeds started to germinate after a wk of inoculation. The first visible sign of germination was the swelling of embryos followed by their turning green and emergence out of the bursted seed coats (spherule stage). Within 3 wks of culture, the spherule developed into oval, green, protocorms with marked absorbing hairs all over the surface. The germination of seed and growth of protocorms were variously affected depending on the concentrations and combinations of growth regulators. The protocorms so obtained developed roots and shoots and complete seedlings were obtained after 8 wks of primary culture on MS media supplemented with BAP (1 mgl^{-1}) and NAA (1 mgl^{-1}). Growth of the protocorms was vigorous in this culture condition. On MS medium supplemented with BAP at 1 mgl^{-1} and 2 mgl^{-1}, seed germination was observed only after 13 wks of primary culture while on MS basal medium, the seed germination was observed after 9 wks of primary culture.

Shoot Tip Culture

MS media supplemented with different concentrations and combinations of NAA and BAP was found to be effective for the growth and multiplication of shoots from the shoot tip explants (Table 2). Out of different concentrations and combinations of NAA and BAP tested, MS medium supplemented with BAP (0.5 mgl^{-1}) was found to be optimal for shoot multiplication in *C. iridioides*, where 8.25 shoots were obtained from a single shoot tip after 12 wks of primary culture. Increase in concentration of BAP showed inhibitory effect. Multiple shoots were also developed when BAP was combined with NAA, though the number of shoots was less in number. Explants developed into shoots with the callus mass at the base, on the media supplemented with NAA (0.5 mgl^{-1}) and BAP (2mgl^{-1}) and NAA (1mgl^{-1}) plus BAP (0.5mgl^{-1}). Tahara (1977) reported the similar result in *Calanthe discolor* and *C. sieboldii*.

Rooting of Shoots

MS medium either hormone free or supplemented with different auxins at different concentration were tested for the *in vitro* rooting of microshoots. Of the three auxins *i.e* IBA, IAA, and NAA tested for inducing roots, MS-media supplemented with IBA (1mgl^{-1}) was more effective for rooting; an average of 4.25 roots per shoot were developed in 12 wks of culture. Number of roots increased in further subculture. Increase in concentration of IBA to 2mgl^{-1} resulted in less roots (2.25/shoot)

Table 2. Effect of BAP and / or NAA enriched MS medium on multiple shoot formation in *Cymbidium iridioides.*

↓NAA (mg l⁻¹) \ BAP → (mg l⁻¹)	Number of shoots			
	0	0.5	1.0	2.0
0	1.8±1.06	8.25±3.37	3.8±1.2	5.2±0.3
0.5	5.5±2.12	4.34±0.9	6.5±2.47	4±1.92
1	4.8±0.2	7.67±2.82	6.5±0.35	3.6±1.3

formation. Secondary characters were observed during rooting in *C. iridioides*. Thick hairy aerial roots were observed on the media supplemented with IAA ,(1mgl^{-1}) whereas emerging plantlets with thick roots were observed on the media supplemented with NAA (1mgl^{-1}). The micropropagated plantlets were acclimatized at 25± 2°C using cocopeat and for 2-3 wks and finally transplanted to the small plastic pots containing peat moss and small pieces of brick. Rooted plantlets were able to grow into normal plantlets in *ex vitro* condition after a short period of acclimatization.

Discussion

Each plant species has specific nutritional requirements, for its seed germination and plant regeneration. MS medium with or without growth regulators was found to be effective for the germination of immature seeds of *Cymbidium iridioides* whereas B_5 medium was found to be ineffective. On hormone free MS media and MS media supplemented with different concentrations of BAP, seed germination and development of chlorophyllous protocorms were observed at different culture period. However complete seedlings were observed only when the media was supplemented with NAA (1 mgl^{-1}) and BAP (1 mgl^{-1}). The promotory effect of auxin and cytokinin on seed germination and protocorm development on orchid species has been reported earlier by Mathews and Rao (1980). The interacting influence of cytokinin (BAP) and auxin (NAA) in seedling development in different orchid species was significant in the present investigation as has also been reported by other workers (Hajarika and Sharma, 1995; Jamir *et al*, 2002; Pant and Gurung, 2005; Teng *et al*, 1997). Various workers have investigated the different species of cymbidiums for the germination behaviour of seeds using different media (Bopaiah and Jorapur, 1986; Muralidhar and Mehta, 1986). Similarly different explants have been investigated for the clonal mass propagation of *Cymbidium* species (Chang and Chang, 1998, 2000; Jamir *et al.*, 2002). Shoot tip explants usually proliferate into protocorm like bodies (PLBs) when cultured which eventually go on to form plantlets as well as proliferate PLBs (Arditti, 1977). The result of the present research is analogous with those results. Shoot tips are regarded as the appropriate source for the clonal mass propagation and genetic homogeneity. Presently, eight shoots were developed from a single shoot tip within 12 wks of culture, in *Cymbidium iridioides.*

Though different auxins were tested for *in vitro* rooting, IBA (1 mgl^{-1}) was found to be ideal condition for *in vitro* rooting and its higher concentration resulted in the inhibitory effect. Nayak *et al.* (1997) observed that IBA (2-mgl^{-1}) was effective for rooting in *Acampe praemorsa* (Roxb.). Banerjee and Mandal (1999) found that NAA (2 mgl^{-1}) was most appropriate in inducing 3-4 roots in 2 months in *Cymbidium.* In the present investigation development of roots was observed in 2-mgl^{-1} IBA or in 2 mgl^{-1} NAA as well, but the roots were least in number in NAA supplemented media.

Since this species is highly exploited for commercial use, the *ex situ* conservation of this species is highly recommended. Mass propagation using shoot tip culture can be started in a commercial scale to conserve this species in their natural habitat.

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