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Micropropagation of *Cymbidium iridioides*

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Abstract

Seeds of *Cymbidium iridioides* D. Don, a highly collected orchid species of Nepal, has been developed were cultured on Murashige and Skoog, 1962 (MS) static media solidified with 0.8% agar. Complete seedlings were obtained from asymbiotically germinated seeds on MS media supplemented with 6- benzyalaminopurine (BAP) 1 mg/l plus α-naphthaleneacetic acid (NAA) 1 mg/l in eight weeks of primary culture. The shoot tip explants were obtained from *in vitro* grown seedling for the shoot multiplication. Maximum number of shoots were developed from shoot tip explants on MS media supplemented with BAP 0.5 mg/l (8.25 shoots per single shoot). This ability did not decline even after a year of subculture. For rooting of shoots the media was supplemented with various auxins. Media supplemented with 1mg/l indole-3-butyric acid (IBA) was the ideal condition for root formation. Thus obtained complete plantlets were able to grow in community pots after a short period of acclimatization. This result suggests that the methodology might be applied as an alternative to minimize the over exploitation of the natural population of this species.

Key words: micropropagation, protocorms, orchid, explants, *In vitro*

Introduction

Orchids are economically important group of plants for their various uses in floriculture, medicine, food, and as glycosidal value. 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.* 2007). Highly threatened species immediately require *ex situ* conservation.

The genus *Cymbidium* comprises 70 genus worldwide of which 10 species are reported in Nepal (Press *et al.* 2000). *Cymbidium iridioides* D. Don. (Fig. 1) 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 range and characteristics flowering from autumn to early winter. Juice from its crushed leaves is used for clotting of blood in deep wound and paste used as tonic (Subedi, 2000). 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 and medicinal use (Joshi & Joshi, 2001; Pant *et al*. 2002).

Fig. 1. Flower of *Cymbidium iridioides*

Seed germination and propagation of this species in nature is very slow due to ecological constraints. The *Cymbidiums* are conventionally propagated through separation of pseudobulbs; however 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 have been extensively exploited for the large-scale propagation as well as *ex situ* conservation of *Cymbidiums* (Fonnesbech 1972, Benerjee & Mandal 1999, Chang &Chang 2000, Jamir *et al*. 2002, Pant & Pradhan 2011)**.**

The main aim of this study is to produce maximum number of plantlets *in vitro* which can be cultivated for various purposes.

Methodology

Plant material

The materials used for seed germination were the young pods of *Cymbidium iridioides* D. Don, obtained from the plants grown in Central Department of Botany, Tribhuvan University. The young green pod was washed with tap water containing few drops of teepol solution for few minutes and washed under running water for 30 minutes. The pod 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.

Media were prepared by adding different concentration and combination of NAA and BAP as given in the Table 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 ^{0}c for 15 min/15lb. The cultures were maintained at 25± 2°C and 200-300 lux under 16 hours photoperiod.

Table 1. *In vitro* germination of immature seeds of *C. iridioides* D. Don

Medium	Growth adjuncts	Conc.	Germination	Time take in weeks for					Remarks
				Development of		Differentiation of			
				Protocorms	Chlorophyll	1st leaf primordia	1st root primordia	Seedlings	
B5	–	–	–	–	–	–	–	–	No germination
MS	–	–	4	9	28	–	–	–	Germination favored
MS	BAP	1mg/l	4	13	28	–	–	–	Germination favored
MS	BAP	2mg/l	4	13	28	–	–	–	Germination favored
MS	BAP + NAA	1mg/l +1mg/l	4	2	4	5	6	8	Germination growth and development of seedling favored

Culture condition: - MS medium, 25± 2°C, 24 weeks, 8replicates were used in each concentration

Table 2. Effect of BAP and NAA on multiple shoot formation rate of *Cymbidium iridioides*

BAP ⇒ NAA ⇓ (mg/l)	0	0.5	1	2
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

Culture condition: - MS medium, 25± 2°C, 24 weeks, 8 replicates were used in each combination.

Seed and shoot tip culture

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

For the clonal mass propagation, shoot tips about 3-5 mm in size were excised from *in vitro* grown seedling and cultured in the culture tubes containing MS media supplemented with different concentration of NAA (0.5 and 1 mg/l) and (0.5, 1, 2 mg/l), either alone or in combination. Multiple shoot formation and root initiation was examined at the given cultured condition. Cultures were subcultured into fresh media once every 8 weeks.

Rooting of shoot

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 1-2mg/l. The regenerated plantlets were transferred to pots containing cocopit in order to grow into normal plants after a short period of acclimatization

Results and Discussion

Seeds culture

MS medium with or without growth regulators was found to be effective for the germination of immature seeds whereas B5 medium was found to be ineffective (Table 1). In MS media seeds started to germinate after a week 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 three weeks of culture the spherule developed into oval, green, protocorms with marked absorbing hairs all over the surface (Fig. 2a). The germination of seed and growth of protocorms was variously affected depending on the concentrations and combinations of growth regulators. Growth of the protocorms was vigorous on MS media supplemented with BAP (1mg/l) + NAA (1 mg/l) (Fig. 2b). The protocorms so obtained developed root and shoot and complete seedlings were obtained after 8 weeks of primary culture. On MS medium supplemented with BAP 1 mg/l and 2 mg/l, seed germination was observed only after 13 weeks of primary culture while on MS basal medium the seed germination was observed after 9 weeks of primary culture.

Fig. 2. Germinating seeds developed *in vitro* in BAP (1mg/l) + NAA (1mg/l)
a: Spherule stage
b: Vigorously growing protocorms

Shoot tip culture

MS media supplemented with different concentration and combination of NAA and BAP was found to be effective for the growth and multiplication of shoots from the shoot tip explants. Effect of different concentration of NAA and BAP on shoot tip explants has shown in Table 2. Among the different concentration and combination of NAA and BAP tested, MS media supplemented with BAP (0.5 mg/l) was found to be the appropriate condition for shoot multiplication in *C. iridioides*, where 8.25 shoots were obtained from a single shoot tip after 12 weeks of primary culture (Fig. 3). 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 mg/l) + BAP (2mg/l) and NAA (1mg/l) + BAP (0.5mg/l). Tahara (1977) reported the similar result in *Calanthe discolor* and *C. sieboldii*.

Fig. 3. Multiple shoot developed in from shoot tip explants in MS media with BAP (1mg/l) + NAA (0.5 mg/l).

Rooting of Shoot: Hormone free MS media and MS Media supplemented with different auxins at different concentration were tested for the *in vitro* rooting of microshoots. Of the three auxins *viz* IBA, IAA and NAA tested for inducing roots, MS-media supplemented with IBA (1mg/l) was more effective for rooting**.** In this condition an average of 4.25 roots per shoot were developed in 12 weeks of culture. Number of roots increased in further subculture. Increase in concentration of IBA to 2mg/l resulted decrease in root (2.25/shoot) formation.

Secondary characters were observed during rooting in *C. iridioides*. Thick hairy aerial roots (Fig. 4) were observed on the media supplemented with IAA (1mg/l) whereas emerging plantlets with thick roots were observed on the media supplemented with NAA (1mg/l**)** (Fig. 5).

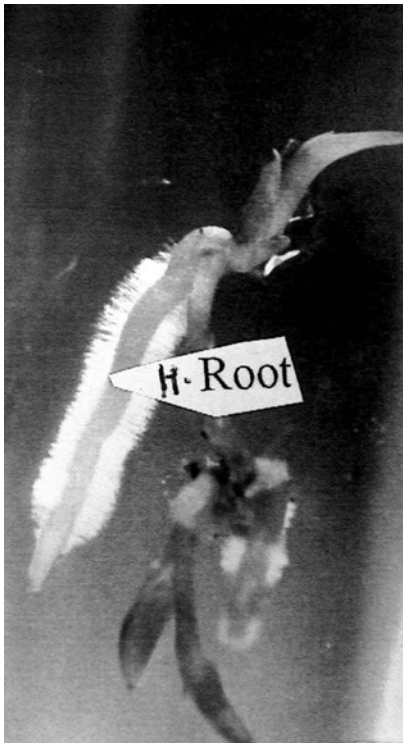

Fig. 4. Secondary rooting characters (Thick hairy aerial roots) on MS media with IAA (1mg/l)

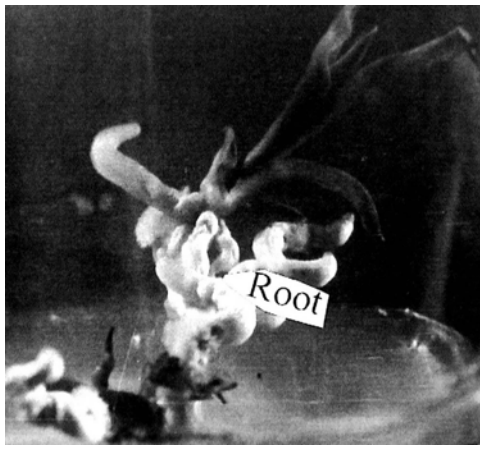

Fig. 5. Bunch of spongy white roots developed on MS media with NAA (1mg/l)

The micropropagated plantlets were acclimatized at 25± 2°C using cocopit and for 2-3 weeks 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 (Fig. 6).

Fig. 6. Acclimatized plants of *Cymbidium iridioides*

Each plant species has specific nutritional requirements for its seed germination and plant regeneration in *in vitro* condition. MS medium with or without growth regulators was found to be effective for the germination of immature seeds of *Cymbidium iridioides* whereas B5 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 protocorm was observed at different culture period. However complete seedling was observed only when the media was supplemented with NAA (1 mg/l) + BAP (1 mg/l). The promontory effect of auxin and cytokinin on seed germination and protocorm development on orchid species has 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 & Sharma 1995, Teng *et al.* 1997, Jamir *et al.* 2002, Pant & Gurung, 2005). Various workers have investigated the different species of *Cymbidiums* for the germination behaviour of seed using different media (Muralidhar & Mehta, 1986; Bopaiah & Jorapur, 1986). Similarly different explants have been investigated for the clonal mass propagation of *Cymbidium* species (Chang & Chang, 1998, 2000, Jamir *et al.* 2002). Shoot tip explants usually proliferate into so-called 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. We applied shoot tip method for the clonal mass propagation, in which an average of eight shoots was developed from a single shoot tip within 12 weeks of culture.

Though different auxins were tested for *in vitro* rooting IBA 1 mg/l was the most effective condition for *in vitro* rooting and its higher concentration resulted the inhibitory effect. Nayak *et al.* (1997) observed the 2mg/l IBA was effective for rooting in *Acampe praemousa* (Roxb.). Bannerjee *et al*. (1999) found that 2 mg/l NAA was most appropriate in inducing 3-4 roots in 2 months in *Cymbidiums*. In the present investigation development of roots was observed in 2-mg/l IBA or in 2 mg/l 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 applied in a commercial scale to conserve this species in their natural habitat.

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