ASYMBIOTIC SEED GERMINATION AND PLANTLET DEVELOPMENT OF *COELOGYNE FUSCESCENS* LINDL., A MEDICINAL ORCHID OF NEPAL

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Abstract: *Coelogyne fuscescens* Lindl. is one of the commercially threatened epiphytic orchid of Nepal. It has reported to have medicinal properties as its pseudobulb is used to reduce the abdominal pain. Germination of the orchid seeds in nature is difficult without the symbiotic association of the Mycorrhizal fungi, *Rhizoctonia* species. Therefore, the present research was focused to study the effect of growth hormones on asymbiotic seed germination and their subsequent development into plantlets. Immature seeds of this orchid species cultured in MS media supplemented with different concentrations of 6 – Benzylaminopurine, BAP (0.5 mg/l-2mg/l) and α-Napthalene Acetic Acid, NAA (0.5mg/l), singly and in combination showed various responses on germination and seedling development. MS medium supplemented with BAP (1.0 mg/l) plus NAA (0.5 mg/l) was found to be the most favourable condition for the early seed germination and protocorm development while medium supplemented with NAA (0.5 mg/l) was found to be favourable for their further differentiation into plantlets.

Keywords: Seed*; in-vitro* culture; MS media; tissue culture; protocorms.

INTRODUCTION

Orchids are one of the most beautiful ornamental plants of Nepal. Nepalese orchids are very popular due to their shape, size, habit, habitat, colourful flowers, shining green leaves and variously shaped pseudobulbs, so are highly used for horticultural purpose (White and Sharma, 2000). Beside their ornamental values many orchid species have medicinal as well as edible values (Baral and Kurmi, 2006; Pant, 2011).

Orchid seeds are very unique than the seeds of other flowering plants. They are very minute, dust like and produced in large numbers within a capsule or pod but lacks storage tissue. Therefore orchid seeds require mycorrhizal association for their germination in nature which is a very slow process (Mitra,1986). This problem can be overcome by asymbiotic germination of orchid seeds using *in vitro* culture techniques. During last few years, this technique has been highly exploited for mass propagation of many threatened orchid species (Stewart and Kane, 2006; Hossain, 2008; Pant *et al*. 2011).

Twelve species of *Coelogyne* has been recorded from Nepal (Raskoti, 2009). Among them, *C. fuscescens* Lindl. is one of the important ornamental species because of its coconut fragrance beautiful flower which blooms during October to November and persist for about 20 days. Commonly it is called the 'Ocher Yellow Coelogyne'. Its flower is apple green or light yellow in color. Its pseudobulb looks like banana so the local people also know it with the name of 'Ban-kera'. Local people use this species for its medicinal properties. Paste and juice of its pseudobulbs are used for abdominal pain and burns (Pant & Raskoti, 2013). Deforestation and uncontrolled collection from wild for ornamental and medicinal purposes are decreasing the population of this species in nature. So, conservation of this species is an urgent need. Plant tissue culture techniques provide an alternative tool for their rapid propagation *in vitro*. The present research highlighted on asymbiotic seed germination and plantlet development of *C. fuscescens.*

MATERIALS AND METHODS

The materials used for the present investigation were the young capsules of *Coelogyne fuscescens* Lindl. collected from the Thulswara V.D.C – 1, Kaski, Nepal.

Culture media

Murashige & Skoog (MS) medium (1962) was taken as a basal medium either alone or in combination with different plant growth regulators for this investigation. Media was fortified with 3% sucrose and different concentrations of BAP and NAA (Table 1). The pH of

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medium was adjusted at 5.8 by using 0.1N NaOH and 0.1N HCl before gelling with 0.8% difco-bacto agar. Agar was dissolved by boiling the medium. About 20ml of molten medium was dispensed in each sterile culture tube and covered by aluminium foil before autoclaving at 1.05kg/cm^2 at 121ºC for 20 min. After autoclaved, the cultures were maintained at 25(±2)^{o}C under 16/8 hrs photoperiod.

Sterilization and inoculation of seeds

Before inoculation, the laminar airflow chamber was sterilized by cleaning it with cotton soaked with 70 % alcohol. All the requirements for inoculation i.e. 70 % ethyl alcohol, sodium hypochlorite solution(1%), sterile distilled water, aluminium foils, petridishes, surgical blade, spatula, beaker, forceps, culture tubes with media were exposed under ultraviolet (UV) radiation for 45 minutes to remove the possible contaminants presenting on them and around the transfer area. After UV exposure, the blower was switched on for 15 minutes. Then, the laminar air-flow chamber was ready for inoculation.

Since the immature capsules of the orchids were collected from the plants grown in natural habitat, they need proper sterilization before their culture. For their sterilization capsules were dipped in detergent water for few minutes then washed in running water for 1 hour. The capsules were then rinsed with distilled water. Inside the laminar airflow, capsules were dipped in 70 % ethyl alcohol for 2 minutes and surface sterilized in 1 % sodium hypochlorite solution for 15 minutes. Finally, the capsules were washed with sterile water for five times. The capsules were cut longitudinally into two equal halves and the seeds were inoculated on MS medium alone and in various combination and concentrations of hormones (BAP + NAA). Sterile spatula was used to scope out and spread the seed on agar medium and observed regularly. All the cultures were maintained at 25 (±2) °C temperatures under 16/8 hours photoperiods.

RESULT AND DISCUSSION

The germination of the orchid seed is rather difficult in comparisons to the germination of other angiospermic seeds, because they lack endosperm, radical and leaf rudiments. In nature they need the mycorrhizal symbiosis for the germination which takes very long time. So tissue culture technique proves itself a reliable and promising method for the conservation of orchids.

Immature seeds of *C. fuscescens* was used for the *in vitro* seed germination and seedling development. Seeds were cultured on MS medium supplemented with various concentration of NAA (0.5mg/l) and BAP (0.5-2mg/l) and without hormone supplement. Almost all conditions favoured seeds germination. MS medium supplemented with BAP (1.0 mg/l) + NAA (0.5 mg/l) was found to be good culture condition for the earlier germination of seeds whereas MS medium supplemented with NAA (0.5 mg/l) was the most favourable condition for germination as well as seedling development of *C. fuscescens*. The most appropriate condition for the germination of the seeds was concluded on the basis of time taken for the germination, efficiency of germination and growth and development of the seedlings.

After germination, seeds underwent further differentiation to form seed clumps, protocorms which finally gave rise to complete plantlets. The protocorms of *C. fuscescens* were found to be indistinct, grey in colour from which green plantlets were raised.

Culture conditions: MS medium, 25 ± 2°C, 16/8 hrs photoperiod, 32 weeks, 4 replicates were used in each combination.

In vitro seed germination of orchids is greatly influenced by several factors such as age of the seeds, nutrient media, organic carbon sources, different adjuncts and quality and quantity of plant growth regulators (Pongener and Deb, 2009). MS medium supplemented with various concentrations of plant growth regulators (BAP and NAA) was effective for *in vitro* seed germination of *C. fuscescens*. The first visible sign of germination i.e. swelling of embryo was started after 6 weeks of culture and about 90 % of seeds were germinated on MS + BAP (1mg/l) + NAA (0.5 mg/l). In this condition, swelling embryo later developed into green spherules protocorms after 10 weeks of culture and finally the shoots were initiated after 13 weeks of culture. In spite of vigorous germination of the seeds the root primordia were not seen in this condition even after 32 weeks of culture. This condition was followed by MS + NAA (0.5mg/l) and MS + BAP (0.5mg/l) + NAA (0.5 mg/l) where the germination started after 9 and 10 weeks of culture respectively. Germination and differentiation were also satisfactory in hormone free MS media.

Combined treatment of BAP and NAA proved to be beneficial in promoting germination and shoot development in all tested conditions. Lower concentration of BAP either alone or in combination with NAA was found to be effective for germination while its higher concentration took longer period. It might be due to the genetic constitution of the species and interaction between the growth stimulating substances. Though all the tested conditions favoured seed germination, the complete seedling was developed only on MS + NAA (0.5 mg/l). These results showed that media fortified with only NAA was appropriate for giving shoot and root development in this species.

Initiation of germination, protocorm formation and subsequent growth and development of seedlings varies

Table1: Effect of growth regulators supplemented in MS medium for seed germination and seedling growth of *C. fuscescens* Lindl.

Media	Growth Hormones	Concentration of hormones(mg/l)	Observation taken in weeks			
			Initiation of germination	Protocorm formation	Initiation of shoots	Initiation of roots
MS	BM	-	10	13	17	-
"	BAP	0.5	12	15	19	-
"	BAP	1.0	15	18	21	-
"	BAP	1.5	13	15	19	-
"	BAP	2.0	18	22	28	-
"	NAA	0.5	9	12	16	23
"	BAP+NAA	0.5 + 0.5	10	12	15	-
"	BAP+NAA	1 + 0.5	6	10	13	-
"	BAP+NAA	1.5 + 0.5	12	14	17	-
"	BAP+NAA	2.0 + 0.5	17	20	22	

with the species and the medium employed (Reddy *et al*, 1992, Baskar & Narmatha, 2010). In present investigation, earlier germination, protocorm formation and shoot development was observed on MS medium supplemented with BAP (1mg/l) + NAA (0.5 mg/l). Similar findings were reported by various researchers in different orchids. Swar and Pant (2004) reported that MS medium supplemented with NAA (1 mg/l) and BAP (1 mg/l) was the most effective condition for the germination of *Cymbidium iridioides*. Jamir *et al.* (2002) found that asymbiotic germination was best in Nitsch medium supplemented with NAA and Kinetin at 1 mg /l each in *C. iridioides*. Kabita and Sharma (2001) observed that hundred percent germination was obtained on MS medium supplemented with NAA (0.1 mg/l) and Kn (1 mg/l) in *Acampe longifolia* Lindl.

However the earlier germination on MS + BAP (1mg/l) + NAA (0.5 mg/l) of this species was not effective for initiation of root. Complete plantlet was observed on MS medium supplemented with NAA (0.5 mg/l). Hence this condition was regarded as the best condition for germination and plantlet development of *Coelogyne fuscescens* which is in accord with the findings made by Shrestha (2005) who successfully achieved asymbiotic germination of *C. ovalis* Lindl. on MS medium supplemented with NAA (1 mg/l). Luan *et al.* (2006) also found high germination rate on ½ MS medium containing NAA (0.5 mg/l) and 20% CW in *Dendrobium supperbum, D. wardianum, D. nobile* and *D. primulinum.*

CONCLUSION

Coelogyne fuscescens is one of the beautiful fragrant orchids highly exploited for ornamental and medicinal purpose. In the present investigation, MS media supplemented with NAA (0.5 mg/l) was the appropriate condition for seed germination, protocorm formation and seedling development of this species. Hence, the present

investigation might be useful for *ex situ* conservation of this species and also fulfil the commercial demand of this species by using *in vitro* seed culture techniques.

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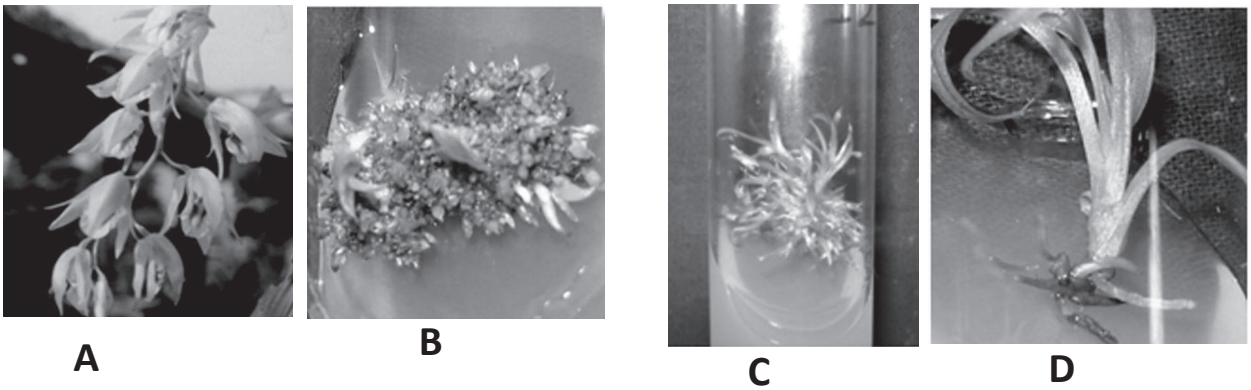

Fig.1. Asymbiotic seed germination and plantlet development of *Coelogyne fuscescens.* **A. Flower of** *C. fuscescens* **in natural habitat, B. Small protocorms, pseudobulbs and leaves developed on MS + BAP (0.5 mg/l) + NAA (0.5 mg/l), C. Multiple shoot developed on MS + BAP (1 mg/l) + NAA (0.5 mg/l), D. Complete plantlet developed on MS + NAA (0.5 mg/l).**

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