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In vitro Multiplication and Protocorms Development of *Dendrobium longicornu* Wall. ex Lindley

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Abstract

Mature capsule explants of *D. longicornu* Wall.ex Lindley were obtained from plants maintained in the nursery of the Plant Biotechnology Laboratory, Department of plant Resources, Thapathali, Kathmandu. *Dendrobium longicornu* Wall.ex Lindley seed were germinated on the MS basal media. The seed showed development of protocorm like bodies (PLBs) after four to six weeks of inocubation. The PLBs then started to germinate in the basal MS media. The PLBs showed development of distinct roots and shoots in media supplemented with NAA, BAP and KN along with coconut water. The germinated microshoots were rooted in media supplemented with auxin, NAA. The rooted microshoots were acclimatized in green house and transferred in moss substrate for strengthening roots. Rotted plantlets were transfered in pot.

Keywords: *Dendrobium longicornu Wall.ex Lindley, In vitro, tissue culture, MS media, PLBs, microshoot BAP, NAA, KN, IAA.*

Introduction

The orchid family is regarded as one of the largest, most diverse and distinctive families in the flowering plant kingdom with estimates of about 20,000 to 35,000 species in the world (Dressler, 1993). They are found in wide array of ecological conditions, except in marine environments and habitats with extreme cold throughout the year. The plants are terrestrial, epiphytic, lithophytic and saprophytic in habitat.In Nepal, nearly 388 orchid's species within 99 genera are reported (Acharya, 2008). Orchids are well known not only for their ornamental value, but also for their uses in herbal medicine (Sumner, 2000). The use of orchids as medicine has a very long history and the Chinese were the first to use them as herbal (Bulpitt, 2005). Many of the orchids are expensive and difficult to cultivate because the germination of the seeds is not possible due to the shedding of the fruits before the attainment of maturity, lack of mycorrhizal association, inadequate nutrition etc. Most of the orchids contain few-celled embryo at the time of seed maturation and its proper development takes place only during the germination of seeds. However, as the seeds do not have sufficient reserve food material (lacks endosperm) to take care of the growth of embryo during germination (Richardson et al., 1992), they have to depend on some external source for nutrients so as to make their undifferentiated embryo to develop into a protocorm. Therefore, only 2-5 % of seeds germinate in the environment, which is very less in comparison to time.The orchid seeds are the tiniest in the plant kingdom. They are extremely light, more or less fusiform and are produced in millions in each capsule. The seeds at maturity are released by the longitudinal slits in the fruits and are dispersed to long distances by wind. A minute seed of orchid travelling long distances through wind cannot afford to carry enough food supplies for independent germination and as soon as the seed lands on a substratum where conditions are favourable for germination, it starts germinating but is unable to grow further in the absence of a suitable fungus and dies for the lack of food. In nature this mycorrhizal association with an orchid seed is not common and thus a high proportion of seeds fail to survive. In order to overcome this association and produce the seedlings of the desired orchid in mass scale, mature seeds can be asymbiotically germinated on a suitable culture medium under controlled conditions in the laboratories (Sharma, 1998). Micropropagation is particularly useful for conservation of germplasm.

The genus *Dendrobium* belong to family Orchidaceae. In Nepal 24 species of *Dendrobiums* are found. Since a few year back, the huge quantities of *Dendrobiums* are consumed as tea and have been found to be beneficial for developing immunity power in the body system. *Dendrobius* are fast diminishing due to over exploitation of orchid flora for export, trade and increasing deforestation. Mostly orchid traders of Nepal also exported orchids by collecting from natural habitats.On account of this, a few species are extremely scant or perhaps already extinct and many more are facing the danger of being extinct.

Different species of *Dendrobium* orchids such as *Dendrobium fimbriatum*, *Dendrobium amoenum*, *Dendrobium densiflorum* have been already developed tissue culture protocol through meristem, shoot tips and seed culture for mass propagation by different researcher of Department of Plant Resource as well as in Botany Department of Trivuban University .*Dendrobium longicornu* Wall.ex Lindley known as the 'Long-horned *Dendrobium*', is an endangered and medicinally important epiphytic orchid. (Chowdhery, 2001). It is medicinally important and extracts are used to treat fever and coughs (Manandhar, 1995). Keeping in mind the conservation and protection of the orchids from extinction, present work was undertaken for large scale "*in vitro*" propagation of *Dendrobium longicornu.*

Materials and Methods

Dendrobium longicornu plants were collected from Makwanpur district.(Fig.1) All the experiments were carried out aseptically in the clean bench of the laminar flow. Before using the clean bench the laminar cabinet was thoroughly wiped with cotton soaked in 70% ethanol. Forceps, needles, surgical blades etc. were inserted in glassbead sterilizer for sterilization. The collected capsules were washed thoroughly with detergent soap under tap water. The capsule were dipped in 70% ethanol and surface flamed. This process was repeated 3 times after which the capsules were rinsed with sterile distilled water and dried in a laminar airflow cabinet before dissection. The flamed capsule were then dissected longitudinally into two half with a help of surgical blade. The seeds were scooped out from sterilized capsules and inoculated, spreading as thinly as possible over the surface of the culture medium (Murashige & Skoog, 1962).The seeds were germinated and formed protocomes. Protocoms were inoculated into MS medium with the combination of different concentration of plant growth regulators Benzyl amino purine (0.5 mg/l , 1.0mg/l , 2.0 mg/l, 2.5 mg/l and 5.0mg/l) and Naphthalene acetic acid 1.0 mg/l (Table no.1) and Kinetin 0.5 , 1.0. 1.5, 2.0 and 3.0 mg/l and Banzyle amino purin 1.0 mg/l for shoot proliferation from the protocormes (Table 2). 3% Sucrose was used as carbon sources and media were adjusted to pH 5.8 using Sodium hydroxide (NaOH) before autoclaving. The media were solidified with 0.8% agar and were autoclaved at 121° C. The culture bottles were were incubated at 25±2°C under 16h photoperiod. The inoculated seeds were examined regularly every week. After third subculture microshhoot were transferred into 1.0 mg/l NAA for in vitro rooting (Fig.5). The rooted cultured bottles with 4-5 cm long microplants were taken out from incubation room to the green house and allowed to remain for 7-10 days for acclimatization. After 2 weeks of acclimatization plantlets were taken out from the bottle and wash throughly to remove all the media attached at the base of plantlets. The microshoot were then transferred into the pot containing pine bark and coal (Fig.6).

Results and Discussion

In the *in vitro* regeneration study, the orchid seeds of *D. longicornu* Wall.ex Lindley germinated successively in MS hormone free medium. After 4-6 weeks of inoculation protocorm like bodies are found to have formed from the seeds. The development of shoots from the protocorms in medium was observed after 8 weeks of inoculation (Fig. 2). The protocoms inoculated in 2.0 mg/l BAP and 1.0 mg/l NAA showed higher multiplication of shoots than other hormones concentration (Fig.3) .

Table 1: Effect of different concentration of BAP and NAA along with coconut water on shoot proliferation from protocoms

S.N.	MS + Growth Hormone mg/l+10%coconut water		Shoots multiplication from protocorms after 8 weeks	Condition of shoots	Remarks
	BAP	NAA			
1	0.5	1.0	Shoot formation	Not good	
2	1.0	1.0	Shoot formation	Good	
3	2.0	1.0	Shoot formation	Very good	Healthy long shoot
4	2.5	1.0	Shoot formation	Satisfactory	
5	5.0	1.0	Protocomes remain same	Not good	

This table show that the healthy long shoot with very good condition of shoots were developed from protocormes in MS media containing 2.0 mg/l BAP and 1.0 mg/l NAA along with coconut water. (Fig.3).Without coconut water the growth of plantlets was not found to be good condition. Shoot multiplication also observed in the media containing 1.0 mg/l BAP and 1.0 mg/l NAA along with coconut but multiplication rate was less in comperision to 2.0 mg/l BAP and 1.0 mg/l NAA.(Table.1)

This table showed that all the concentration of BAP and KN was responded by the shoot formation from the protocormes but number of shoot multiplication was found to be higher in media containing BAP 1.0 mg/l and KN 1.5 mg/l along with10% coconut water (Fig.4). Without coconut water the growth of plantlets was not found to be satisfied.

The development of orchid seeds requires a balanced supply of both organic and inorganic nutrients Arditti & Ernst, (1982). The seeds require a nutrient rich medium which is ubiquitous in MS medium containing optimal macro and micronutrients, vitamins, inositol, glycine etc. This has proved beneficial for seed germination as already suggested by (Devi et al., 1999).The seedling tips of *Dendrobium fimbriatum* Hook. have been cultured in MS medium supplemented with cytokinin.

Niroula & Rajbhandary (1985) propagated *Dendrobium fimbriatum* Hook. from seedling tips. Shrestha & Rajbhandary (1988) regenerated *cymbidium gradiflorum* through meristem culture and shoot tip culture. They used 5.0 mg/l BAP and 1.0 mg/l NAA along with 10% coconut water for shoot multiplication..Shrestha & Rajbhandary (1993) successfully developed the protocol of clonal propagation of *Dendrobium densiflorum* through meristem culture. Rajkarnikar & Niroula (1994) used 5.0 mg/l BAP and 1.0 mg/l NAA for micropropagation of *Dendrobium fimbriatum* Hook. through shoot tip explants. Rajbahak et al. (2005) used axillary bud as an explants for in vitro multiplication of *Vanilla planifolia* Andrews. Rajkarnikar (2010) propagated *Dendrobium amoenum* Wall.ex Lindl. *through* seed culture as well as shoot tip culture. Shrestha & Rajbhandary (1994) studied the invitro germination of native and exotic seed of orchid.

High concentration of nitrogen (60.05mM) i.e.

Table 2: Effect of different concentration of BAP and Kinetin along with coconut water on shoot proliferation from protocoms.

S.N.	MS + Growth Hormone mg/l+10% coconut water		Shoots multiplication from protocorms after 8 weeks	Condition of shoots	Remarks
	BAP	KN			
1	1.0	0.5	few protocormsdeveloped into shoots.	Satisfactory	
2	1.0	1.0	few protocorms developed into shoots.	Good	
3	1.0	1.5	rate of multiplication high	Very good	shoot elongation good
4	1.0	2.0	few protocorms developed into shoots.	Satisfactory	
5	1.0	3.0	few protocorms developed into shoots.	Not good	

ammonium nitrate & potassium nitrate present in MS medium was necessary for the optimal germination of seeds. The nutrient requirements for asymbiotic seed germination of *Dendrobium longicornu* was optimized by Dohling et.al. (2008).The nutrient requirement of orchid seeds in terms of quality as well as in form may vary at different stages of development (Arditti and Ernst, 1984). The importance of ammonium or nitrate ions (individually or in combination) during the *in vitro* germination of orchid seeds as a source of nitrogen is well established. The growth regulators (Auxin and Kinetin) in the medium play the role of mycorrhiza which forms symbiotic association with non-germinating seeds in nature and bring about changes in the physiology which induces germination in the seeds and protocorm development (Kumaria and Tandon, 1991). Kinetin helps in shoot regeneration and auxin induces root development in shoots to make it a complete plant. The effect of auxin and kinetin vary from orchid to orchid (Arditti and Pridgeon, 1977). The promotory effect of growth regulators such as IAA and KN on seed germination and protocorm development in orchid species were studied by Kano (1965), Mathews and Rao (1980). Healthy growth of orchid protocorms in medium containing balanced supply of organic and inorganic nutrients has been reported by some workers (Arditti and Ernst, 1982). Initiation of seed germination, protocorm development and subsequent growth and development of seedlings seems to vary with the species and the medium employed (Arditti and Pridgeon, 1977). The work on tissue culture of *D. aphyllum* in MS medium showed that the *D. aphyllum* seed responded successively in different kinetin concentrations (Mazumdar & Talukdar, 2007). In *D. longicornu*, the maximum number of shoots generated from each explant was recorded in medium supplemented with 30 µM NAA. (Vij and Kaur, 1998) also reported similar results where NAA-enriched medium favoured multiple shoot bud formation in *Malaxis acuminata*.

Above disscussion indicated that different researcher used different growth hormones for the initiation of shoot formation from the protocoms in *Dendrobium* species. Some researcher used high concentration of BAP 5.0 mg/l for shoot multiplication. In our research work germination of seed was found best in hormones free MS medium. After germination , protocorms like bodies were formed from the germinated seed which were globular in shape and green in colour and transferred into the different concentration of BAP and NAA along with coconut water. Among them BAP 2.0 mg/l and NAA 1.0 mg/l along with 10% coconut water was found to be best for shoot initiation from the protocoms. In absence of coconut water rate of shoot multiplication was found to be low. 1.0 mg/l NAA was found to be best for in vitro root initiation.

Conclusion

In vitro multiplication of orchids makes an effective contribution to saving rare species from extinction. The method uses seed as an explants and germinated *in vitro* on basal MS solid medium for protocormes development and for further shoot multiplication BAP 2.0 mg/l and NAA 1.0 mg/l along with 10% coconut water showed high multiplication rate. Kinetin 1.5 mg/l and BAP 1.0 mg/l along with 10% coconut water also showed shoot multiplication but the number of shoot and condition was best in BAP and NAA media. NAA 1.0 mg/l was best for healthy root initiation. The survivability of the micropropagated plantlets on being transferred to pots depends on their proper acclimatization. *Dendrobium longicornu* is epiphytic in nature and the substratum should reflect this by combining water-holding capacity with good drainage.

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Photo Plate

Dendrobium longicornu Wall. ex Lindley

Fig. 1 Flowering plant with capsule

Fig.2 Newly formed seedling from protocorms in MS medium

Fig.3 MS medium with 2.0 mg/l BAP+1.0 mg/l NAA+ 10% Coconut water

Fig.4 MS medium with 1.0 mg/l BAP+1.5mg/l kinetin + 10% C

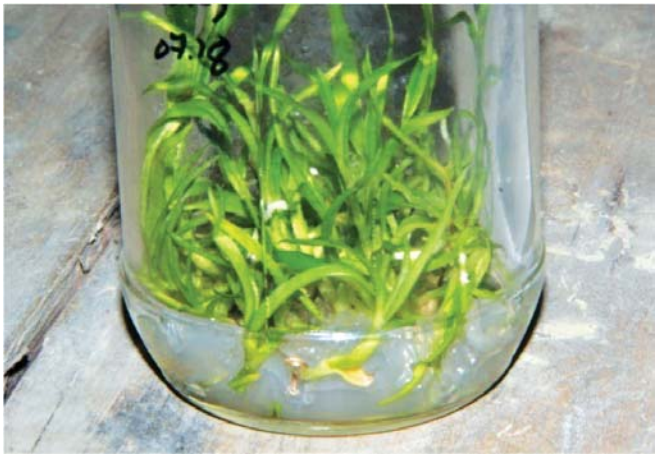

Fig.5 Rooted plantlets MS medium with 1.0 mg/l NAA

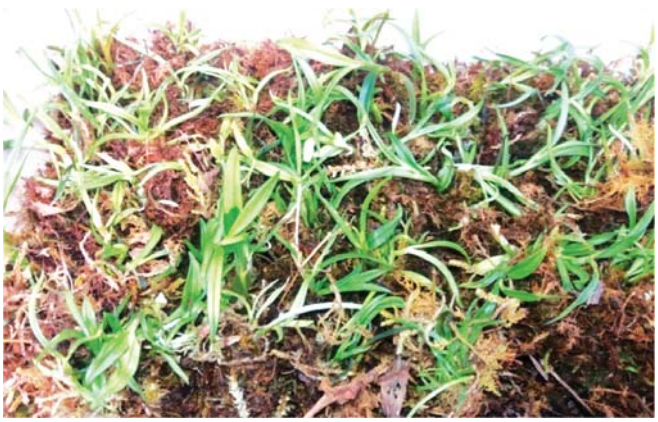

Fig.6 Rooted plantlets transferred mixture of mosscoal and cocopit