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MICROPROPAGATION OF *CYMBIDIUM ALOIFOLIUM* (L.) SW., A MEDICINAL ORCHID BY ARTIFICIAL SEEDS TECHNOLOGY

SHREETI PRADHAN[1], BABU LAL TIRUWA[1], BIJAY R. SUBEDEE[2] AND BIJAYA PANT[1]✉
[1]Central Department of Botany, Tribhuvan University, Kirtipur
[2]Research Center for Applied Science and Technology, Tribhuvan University, Kirtipur
bijayapant@gmail.com

ABSTRACT

Artificial seed technology is a rapidly growing area of research in plant cell and tissue culture. Application of this technology opens an alternative route for mass scale production, efficient delivery of cloned plantlets and fulfils the increasing demand of local growers. An attempt was made to produce artificial seeds and their subsequent regeneration of a highly valuable medicinal orchid of Nepal i.e. *Cymbidium aloifolium.* Artificial seeds were obtained through encapsulation of protocorms in calcium alginate beads. Protocorms were encapsulated by using 3% sodium alginate and 0.2 M calcium chloride solution. Murashige and Skoog (MS) medium (1962) was used as the basal medium for *in vitro* germination and seedling development of artificial seed. In *Cymbidium aloifolium*, 20-25 days old *in vitro* grown protocorms were used for production of artificial seeds. Artificial seeds were inoculated on two different culture conditions of MS medium i.e. MS solid & MS liquid with four different treatments i.e. strength of 1.0, ½, ¼ and MS media supplemented with plant growth regulators viz. BAP (0.5 mg/l) and NAA (0.5 mg/l). Highest percentage of germination (100%) and plantlet conversion was found on hormone free full strength (1.0 MS) of MS liquid medium after 13-14 weeks of culture. Plantlets regenerated from artificial seeds with well developed shoot and root systems were successfully acclimatized in potting mixture of cocopeat, litter and sphagnum moss in a ratio 2:1:1.

Keywords: Protocorms, MS media, artificial seed, encapsulation, orchid

INTRODUCTION

Orchids, one of the most fascinating creations of nature, are also one of the most widely distributed groups of flowering plants. Nepal harbors about 457 spp of native orchids belonging to 102 genera, out of which 18 species are endemic to Nepal (Rajbhandari, 2014). Thirteen species of *Cymbidium* have been reported from Nepal. *Cymbidium aloifolium* (L.) Sw., an epiphytic orchid grows on trees trunk which has high medicinal value. The plant is reported to have emetic and purgative properties. Paste of its parts (pseudobulb and leaves) is used as tonic and used over fractured or dislocated bones (Hossain *et al.,* 2009; Pant & Raskoti, 2013). Plant can be found in an altitude ranging from 300 m to 2900 m (Rajbhandari & Dahal, 2004). It blooms from April to June and flower persists for approximately 20 days. The flower is yellowish with central radial stripes. Pod of *Cymbidium aloifolium* (L.) Sw. is large and greenish remains hanging arising from a very small pseudobulb enveloped by leaf bases.

Plant tissue culture technique provides a new dimension for efficient conservation and commercialization of numbers of rare and useful orchid species (Vij *et al.,* 2004). Orchid

produces huge number of seeds which are very minute and non-endospermic which rarely germinate in nature, they require an appropriate mycorrhizal fungus association for successful seed germination (Gutiarrez-Miceli *et al.,* 2008; Pradhan *et al.,* 2013). The fungal association is believed to provide physiochemical stimulus required for growth initiation (Sharma and Tandon, 1990; Ovando *et al.,* 2005). Orchid seeds can be germinated *in vitro* without fungal association. Seed culture will help in the production of quality planting materials in larger scale.

Production of artificial seeds provides a new vista in *in vitro* plant propagation technology because it offers many useful advantages on a commercial scale. Artificial seed technology not only produce the artificial seeds but also used for their conversion into plantlets under *in vitro* and *in vivo* conditions. This technology is thus useful for multiplying large number of rare, threatened and endangered plant species which are difficult to regenerate through conventional methods and natural seeds.

The present study was aimed to standardize the protocol for propagation of *Cymbidium aloifolium* through artificial seed technology.

MATERIALS AND METHODS

Source of explants

Immature capsules of *Cymbidium aloifolium* were harvested from nature. Capsules were first washed under running tap water for at least 30 minutes to remove the soil or other particle attached to it. Then, capsules were surface sterilized under laminar air flow cabinet by using 70% alcohol for 2 minutes followed by 1% sodium hypochlorite solution for 15 minutes and were subsequently rinsed in sterile water for at least three times. After sterilization, capsules were dried on Whatsman filter paper and dissected longitudinally with the help of sterilized surgical blade to expose the powdery seeds. Seeds were scooped out and spread thinly over the surface of MS basal medium under aseptic condition. Protocorms were started to develop after 10 weeks of culture of seeds and 20-25 days old protocorms were selected as primary explants for present study.

Culture medium

In vitro germination and subsequent development of artificial seed was carried out on two different conditions of MS (Murashige and Skoog, 1962) medium i.e. solid and liquid. Each media consists of different strength i.e. full (1.0), half (1/2) and quarter (1/4) and media supplemented with different concentration of plant growth regulators viz. 0.5 mg/l BAP and 0.5 mg/l NAA (Table 1). MS medium was fortified with 3% sucrose as carbon source with and without solidified with agar. The pH of MS media was adjusted to 5.8 before autoclaving. About 16- 20ml of media was dispensed into each culture tube (150 X 25mm, Borosil) and autoclaved at pressure 15 psi and temperature of 121^{o}C for 20 minutes. All cultures were maintained at 25±2^{o}C under 500 lux illuminance for 16/8 hrs. (light/dark) photoperiod using cool white light (Philips, India).

Encapsulation and Inoculation

Protocorms were used as crucial explants to produce artificial seeds. For encapsulation of protocorms, 3% sodium alginate was used with 0.2 M calcium chloride for complexation. Individual protocorms were separated and mixed in 3% sodium alginate matrix. This solution

was poured into drop by drop on calcium chloride solution by using wide mouth and left it for at least 30 minutes to harden the alginate beads. After that, the beads now called as artificial seeds or synthetic seeds, were washed with sterile water for three times and dried on blotting paper. Artificial seeds were then inoculated on different strength of MS solid and liquid medium i.e. full strength (1.0), half strength (1/2) and quarter strength (1/4) and full strength of medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA for their germination and regeneration (table 1). The cultures were kept at 25 ± 2°C under 16/8 hrs photoperiod from cool-white-light in culture room.

Hardening

Plantlets regenerated from artificial seeds of C*ymbidium aloifolium* after attaining the height of 3-4cm *were* used for hardening process. Plants with well developed shoots and roots were taken out from the culture vessels and washed thoroughly with running tap water to remove the traces of agar without causing harm to roots. These plants were treated with 0.1 % fungicide (Bavistin) solution for 5 minutes and again washed with sterile water. After washing, they were blot dried and finally acclimatized in pot containing potting mixture of cocopeat, litter and sphagnum moss in a ratio 2:1:1. The potted plants were watered once a day and fertilized at weekly intervals with a foliar spray of a mixture of nitrogen, phosphorous and potassium (20:10:10). The potted plants were covered with a perforated plastic bag to maintain the humidity and kept under greenhouse until the seedlings were established. They were observed regularly.

STASTICAL ANALYSIS

Each treatment consisted of at least 6 explants and each experiment was repeated twice. Statistical analysis was done using an analysis of variance (ANOVA) one way classification system. The data obtained were analyzed using application software-Microsoft excel. The significant difference between the MS medium and MS medium supplemented with different growth hormones were analysed at $P \leq 0.05$ using SPSS version 16.0 (SPSS Inc. USA).

RESULTS

Cymbidium aloifolium (L.) Sw. is one of the most important orchid, used for medicinal as well as ornamental purposes. In present study, *in vitro* production, germination and propagation of artificial seed was carried out by using 20-25 days old protocorms. Zhang *et al.* (2011) reproted that protocorms were the best propagator for artificial seed production in *Dendrobium candidium*. The production of artificial seed was done with the help of sodium alginate (3%) and calcium chloride (0.2M). Finally, the rounded bead of artificial seeds of *cymbidium aloifolium* were formed (fig. 1, A).

In present study, it was found that artificial seed was started to germinate *in vitro* after 5-6 weeks of culture in all the condition of MS liquid medium (fig. 1, B, C) whereas it took 7 weeks of culture on MS solid medium. During germination, artificial seeds become swollen after absorbing the nutrients, which produced elongated, green shoots followed by root formation. First shoot primordia was initiated from 7 weeks of culture while first root primordia was started to develop from 9 weeks of culture on full strength of liquid MS medium. In present study, complete seedling with healthy shoot and root were developed on hormone free MS liquid medium after 14 weeks

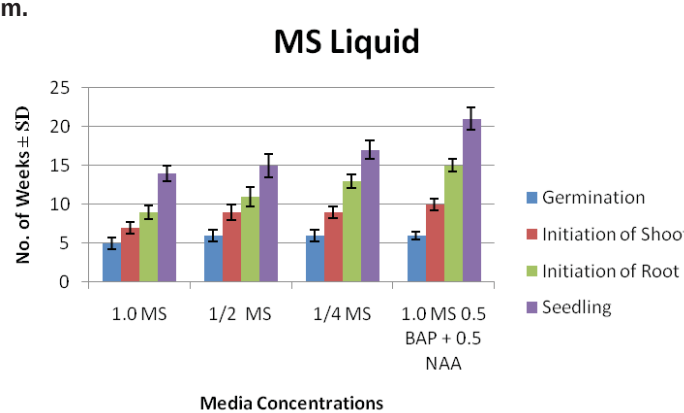
of culture (fig. D). On MS solid medium, first shoot and root primodia were initiated after 9 and 15 weeks of culture on hormone free full strength of MS medium rather than 1/2, 1/4 strength and hormone supplemented MS solid medium. Well developed plantlet with healthy shoot and root were also observed after 18 weeks of culture on hormone free full strength of MS solid medium in comparison to other concentrations of MS solid medium (fig. 1, E).

DISCUSSION

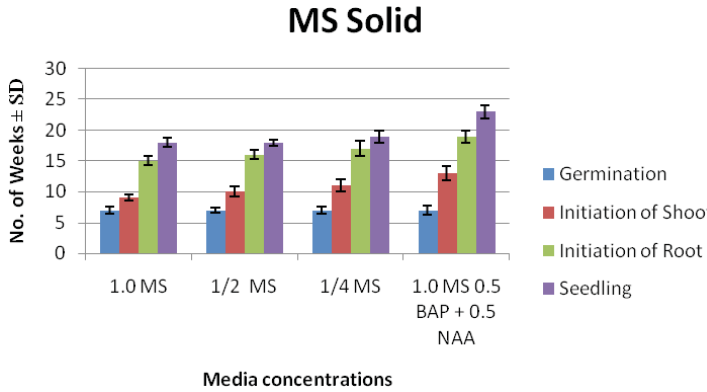
In present study, *in vitro* germination and propagation of artificial seed of *Cymbidium aloifolium* was carried out on two conditions of MS basal media i.e. MS solid and MS liquid medium supplemented with various concentrations of BAP and NAA. The MS basal medium was found to be the most appropriate condition for both solid and liquid condition than that of ½ MS, ¼ MS and MS supplemented with hormone BAP & NAA. The most appropriate condition for the germination and multiplication of the artificial seed was concluded on the basis of time taken for the germination, efficiency of germination and growth and development of seedlings.

Among different concentration of both MS liquid and MS solid medium, MS liquid medium alone was found to be the most effective condition for earlier germination and propagation of artificial seed of *Cymbidium aloifolium* which took 14 weeks of culture to develop healthy plantlets. In present study, 100% conversion of plantlet from artificial seed was found on different strength and homone concentration of both MS solid and liquid medium (graph 1& 2). The present finding was supported by Corrie & Tandon (1993) who reported 100% conversion of encapsulated Plb's into plantlet under *in vitro* condition in *Cymbidium giganteum*. Similarly, Sarmah *et al.* (2010) produced *Vanda coerulea* synseeds by encapsulating PLBs regenerated from the leaf base with a 94.9 % conversion frequency. Nagananda *et al.* (2011) encapsulated the PLBs of *Flickingeria nodosa* and achieved 95% conversion after 3 months' storage at 4°C. Gantait *et al.* (2012) obtained 96.4% conversion in alginate- encapsulated *Aranda* × *Vanda* PLBs with 3% sodium alginate and 75 mM calcium chloride. The present findings were dissimilar to the reports obtained by other researcher. Datta *et al.* (1999) found that modified Knudson medium supplemented with BAP (1mg/l) and NAA (1mg/l) was the most effective condition for germination of artificial seed of *Geodorum densiflorum* (Lam.) Schltr.

GRAPH 1. *In-vitro* germination and seedling development from artificial seeds of *Cymbidium aloifolium* (L.) Sw. in different strength and homone concentration of MS liquid medium.



GRAPH 2. *In-vitro* germination and seedling development from artificial seeds of *Cymbidium aloifolium* (L.) Sw. in different strength and homone concentration of MS solid medium.



Plantlets after attaining the height of 3-4 cm with well developed shoot and root were selected for hardening. These plantlets were acclimatized in earthen pots containing cocopeat, litter and sphagnum moss in 2:1:1 ratio and covered with perforated plastic bags to maintain require humidity (fig. E). About 80% plantlets were survived in this condition (fig. F)

In present study, it was concluded that full strength of MS liquid medium alone is the most effective condition for *in vitro* germination and propagation of artificial seed of *Cymbidium aloifolium* rather than other different strength and hormone supplemented MS liquid and MS solid medium. The present research might be useful to establish the protocol for alternative method of propagation of *Cymbidium aloifolium* by using artificial seed.

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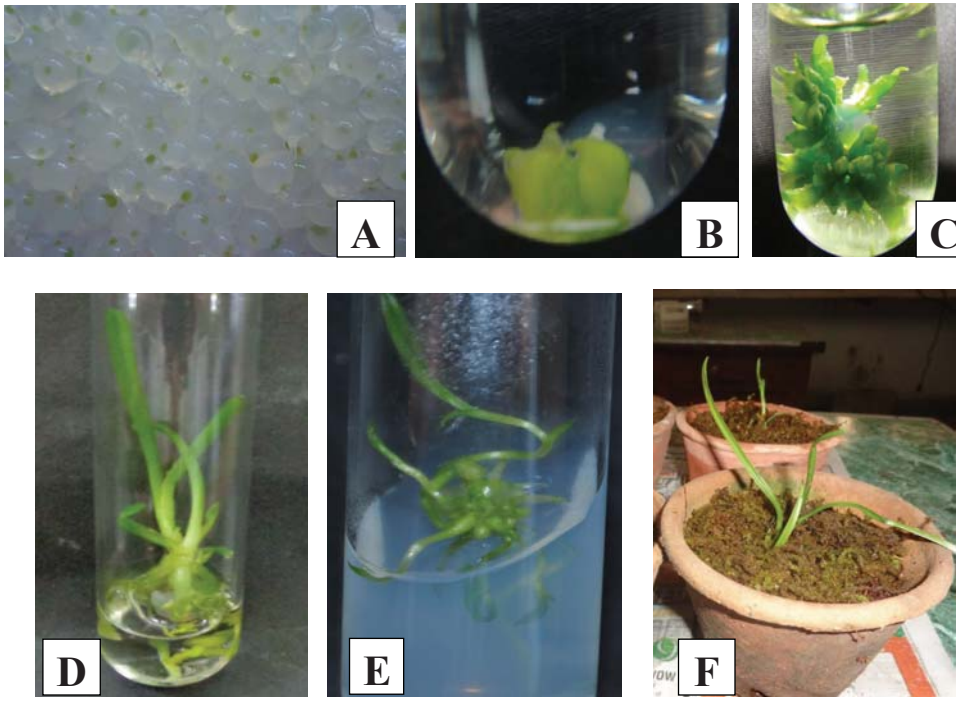

FIG.1. Production, germination and propagation of artificial seed containing protocorms of Cymbidium aloifolium. (A) 3% artificial seed (B) Germinated artificial seeds gave rise to shoot bud on ¼ MS liquid medium. (C) Multiple shoot buds on ½ MS liquid medium. (D) Development of healthy roots and shoot buds on hormone free 1.0 MS liquid medium. (E) Multiplication of shoots with well developed roots on hormone free 1.0 MS solid medium. (F) Hardening of in vitro plantlets on potting mixture of cocopeat, litter and sphagnum moss of 2:1:1 ratio.