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In vitro Mass Propagation of an Epiphytic Orchid, *Cymbidium aloifolium* (L.) Sw., through Protocorm Culture

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Authors' contributions

This research work was carried out in collaboration between all authors. Author TR performed the experiments, analysed the data and wrote the first draft of manuscript. Author SP assisting the experiment, literature searches and finalize the manuscript. Author BP designed the study and editing the manuscript. All authors read and approved the final manuscript.

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ABSTRACT

Aim: To develop a protocol for *in vitro* propagation of *Cymbidium aloifolium,* a threatened orchid highly used for medicinal purpose through protocorm culture.
Place and Duration of Study: Tissue culture Laboratory, Plant Biotechnology Unit, Department of Botany, Tribhuvan University, Kirtipur, Nepal, between November 2013 to December 2014.
Methodology: Small, green and globular protocorms with 0.1-0.3 cm diameter were subjected to grow individually on solidified Murashige and Skoog (MS) basal medium and MS medium supplemented with various concentration of plant growth regulators, 6-Benzylaminopurine (BAP, 0.5; 1; 1.5; 2 mg/l) or α-Naphthalene Acetic Acid (NAA, 0.5; 1 mg/l) or their combination. Six replicates were used for each concentration. The data for development of shoot and root from each protocorm culture were recorded in every two weeks for upto six month.
Results: Almost all conditions favoured multiplication but MS medium fortified with BAP (1 mg/l) and NAA (1 mg/l) resulted in maximum induction of rootless healthy shoots with an average value of 8-9 shoots per culture. On this medium, shoot multiplication was initiated after 9 weeks of culture

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whereas MS medium fortified with BAP (2 mg/l) and NAA (0.5 mg/l) was found to be most effective condition for the shoot multiplication along with well developed roots.
Conclusion: MS medium supplemented with high concentration of BAP and low concentration of NAA was found to be efficient for maximum multiplication of shoot and root. The *in vitro* developed healthy rooted plantlets of *C. aloifolium* were successfully acclimatized in green house on potting mixture containing cocopeat and moss in the ratio of 2:1. On this condition, nearly 70% of the plantlets were successfully survived. Hence, this protocol might be useful for mass propagation and *ex situ* conservation of this orchid through protocorm culture.

Keywords: In vitro; protocorm; MS media; growth regulator; micropropagation.

1. INTRODUCTION

Cymbidium aloifolium (L.) Sw., an orchid having high medicinal and ornamental values is enlisted as one of the threatened species of Nepal [1]. It is one of the most popular and desirable species of the genera *Cymbidium* which are epiphytic in nature and remains attached to the bark of old trees like *Castanopsis indica, Shorea robusta* etc. These plants make great houseplants, and are also popular in floral arrangements and corsages. It blooms from April to June and flower persists for approximately 20 days. It can be found in an altitude ranging from 300m to 2900m [2,3]. *C. aloifoilum* was the first species of *Cymbidium* known in Europe which was described by Linnaeus (1753) as *Epidendrum aloifolium* in Species Plantarum [4].

Apart from the floricultural value, it has some traditional medicinal properties as they have rich contents of alkaloids, glycerides and other useful phytochemicals [5]. Paste of pseudobulb and leaves is used as tonic and used over fractured or dislocated bones. The leaves are also extensively used for styptic properties in the treatment of boils and fever and also for the treatment of otitis and inflammatory conditions [6]. The roots are used as medicine to treat paralysis and chronic illness [7]. Since, the species has great commercial potential and are mostly exported to international market, their conservation and preservation has become an urgent need. Thus, tissue culture technique is a potential alternative method for mass scale propagation and conservation of this threatened orchid. Therefore, the present investigation was conducted in an attempt to preserve this medicinally important orchid by developing a protocol for rapid *in vitro* mass propagation through protocorm.

2. MATERIALS AND METHODS

Two month old *in vitro* grown normal protocorms of diameter 0.1-0.3 cm developed on hormone free MS medium were sub-cultured individually (Fig. 2A) on Murashiage and Skoog (MS) [8] medium alone and MS medium supplemented with different concentration and combination of BAP (0.5-2.0 mg/l) and NAA (0.5-1.0 mg/l) for growth and proliferation of shoot of *Cymbidium aloifolium*. 30 g/l sucrose was added to the mixture of medium for carbon source. The medium was solidified with 8 g/l agar-agar (Qualigens, India) and boiled on heater until the agar is completely dissolved. The pH of the medium was adjusted to 5.6 with 0.1N NaOH or HCl. Hormone containing media were prepared by adding the required concentration of phytohormone each with 6 replicates. The media was autoclaved at pressure of 15psi and temperature of 121ºC for 20 minutes. The cultures were maintained in control room and exposed to artificial light i.e. fluorescent light (Philips, India) with light flux of 3000 Lux in a light/dark cycle of 16/8 hours at 25±2ºC. They were observed in every two weeks and the obtained results were recorded.

2.1 Statistical Analysis

Significance of treatment effects on shoot multiplication were analyzed using one way analysis of variance (ANOVA, $p \leq 0.05$) and comparison between mean values of treatments were made by using SPSS ver. 11.5 (SPSS Inc., USA).

3. RESULTS AND DISCUSSION

The regeneration capacity of protocorm explants has been influenced by different plant growth hormones in media. Protocorm that has been obtained from the seeds grown on hormone free MS medium were sub-cultured on MS medium alone and MS medium supplemented with different concentrations of BAP (0.5; 1; 1.5; 2 mg/l) or NAA (0.5; 1 mg/l) or their combinations (Fig. 1). In present study, it was found that of the various concentrations of hormones tested,

treatment with BAP (2 mg/l) and NAA (0.5 mg/l) in the medium gave earliest response to proliferate protocorm after 5 weeks of culture (Fig. 2B). This medium concentration also proved to be best for multiplication of shoots (4.5 shoots per culture), leaves (8.17 leaves per culture) and well developed roots (3.33 roots per culture) of *C. aloifolium*. However, the maximum multiplication of shoots without root was best favoured on MS medium supplemented with BAP (1 mg/l) and NAA (1 mg/l) (8-9 shoots per culture) after 9 weeks of culture (Fig. 2C). This medium was followed by MS+BAP (1 mg/l). On this condition, the protocorm was first developed into clump of hairy buds which was later developed into shoots.

The result of present study was supported by the findings made by Matsui et al. [9] who reported that NAA alone had no effect but the combination of BAP and NAA induced greatest effect on development and differentiation of PLB's (protocorm like bodies) in *Cymbidium.* It might be due to their physiological process or interaction between the two hormones. Similarly, Parmar and Pant [10] also reported that MS medium supplemented with BAP (1 mg/l) and NAA (1 mg/l) was most effective for the seedling development of *Coelogyne stricta*. In comparisons to growth of *C. aloifolium* on individual auxins, MS medium supplemented with 0.5 mg/l NAA gave maximum number of shoot (5.17 shoots per culture) and root (1.23 roots per culture) (Fig. 2D) than MS medium supplemented with 1 mg/l NAA. All combinations of hormones gave effective results however increasing the concentration of NAA up to 1 mg/l suppressed the root development.

In present study, higher concentration of BAP when employed in the medium individually as well as combined with NAA led to reduce the multiplication and elongation of shoots, leaves and roots. Induction of maximum number of shoots at low concentration of BAP in the medium was reported in species of *Cymbidium* and *Cattleya* by Nagaraju et al. [11]. Shimura and Koda [12] reported that the intensity of BAP was crucial for vegetative growth of *Cymbidium* sp. Statistical analysis showed that growth parameters studied had no significant effect on different concentration of hormones. Das et al. [13] found that protocorm cultured on MS medium supplemented with 1mg/l BAP and 1mg/l IAA was effective for proliferation of shoot (5.1 shoots per plantlet) and MS medium supplemented with 1 mg/l BAP and 0.5 mg/l IAA was proved to be best for multiplication of root (3.9 roots per plantlet) in *Cymbidium devonianum*.

Similarly, the result of present study was also analogous to multiple shoot proliferation from protocorm of *Dendrobium* [14,15], protocorm of *Aerides crispum* [16], protocorm of *Cleisostoma racemiferum* [17] and protocorm proliferated into plantlets of *Vanda helvola* [18]. Protocorm apices elongate to form rhizomes that

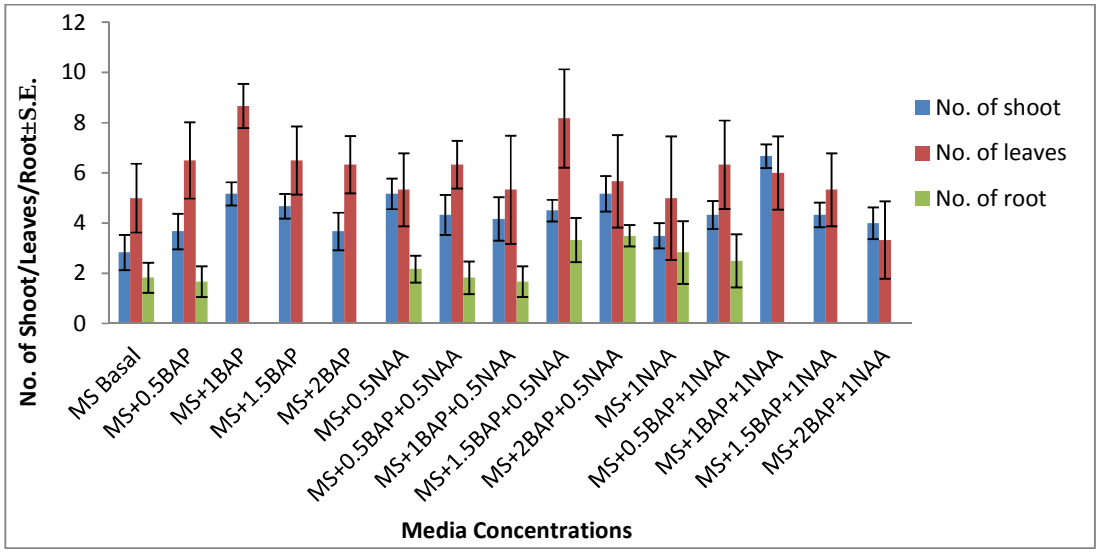

Fig. 1. Average number of shoot, leaves and root produced per responsive protocorm cultured on MS medium along with the addition of different concentrations of BAP and NAA

Fig. 2. Shoot multiplication of *Cymbidium aloifolium* through a protocorm culture: (A) A single protocorm cultured on MS basal medium; (B) Development of callus from protocorm (C) Multiple shoots developed on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l NAA; (D) Development of long shoots and roots on MS medium supplemented with 0.5 mg/l of NAA; (E) Multiple shoots and roots developed on MS medium supplemented with 2.0 mg/l BAP and 0.5 mg/l NAA; (F) Development of shoots on MS medium supplemented with 1.5 mg/l BAP (G) Shoots developed on MS medium supplemented with 2 mg/l BAP and 1mg/l NAA; (H) Acclimatized plantlet of *C. aloifolium*

continue to grow to give branches with several nodes. After the elongation of the rhizome, the terminal bud grows upward and differentiates into shoots and roots, which was similar to the

findings of Chang and Chang [19]. The variation in time taken and response on induction of shoot of orchid might be due to the difference in the orchid genotype and the physiological age of explants [20]. Similarly, MS medium supplemented with increasing concentration of BAP (0.5 mg/l to 2.0 mg/l) was found to be effective for induction of large number of shoot of *Esmeralda clarkei* [21].

The combined effect of high concentration of BAP (2 mg/l) and low concentration of NAA (0.5 mg/l) was effective for the multiplication of shoot and root (Fig. 2E). Pradhan et al. [22] found the similar result on *Dendrobium densiflorum*. Hossain [23] also reported that MS medium fortified with BAP (2 mg/l) and NAA (1 mg/l) proved to be best for induction of secondary protocorms and subsequent seedling development of *Dendrobium aggregatum*. MS basal medium responded average number of shoot production (3-4 per culture) as compared to other combinations tested but with longest shoots length (2.18 cm) (Fig. 2F) which was supported by the findings made by Sheela et al. [24] in *Dendrobium*. On MS basal medium, protocorms first turned brownish at the tips, turned hairy and directly gave rise to elongated shoot without undergoing multiplication. The present study also induced the embryonic yellowish green hairy callus from the single protocorm (Fig. 2G). Callus induction was vigorous from protocorm. This finding was consistent with Huan and Tanaka [25] who observed embryogenic callus induction and plant regeneration in *Cymbidium* from longitudinally bisected segments of PLB's within one month on VW medium supplemented with NAA (1.0 mg/l). The *in vitro* well developed healthy rooted plantlets of *C. aloifolium* were successfully hardened in green house on potting mixture containing cocopeat and moss in the ratio of 2:1 (Fig. 2H). On this condition nearly 70% plantlets survived. Similar results were found on acclimatization of artificial seed derived plantlets of *C. aloifolium* [26].

4. CONCLUSIONS

Hence, the present study revealed that protocorm culture can be successfully employed for rapid multiplication by suitably adjusting the nutrient environment. Thus, the developed protocol will significantly contribute to the mass propagation, conservation as well and meet the commercial demand of this wild threatened orchid, *C. aloifolium*.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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