

Efficient Plant Regeneration of *Cymbidium aloifolium* (L.) Sw., a Threatened Orchid of Nepal through Artificial Seed Technology

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Abstract

Artificial seed technology is a method of considerable potential for mass propagation and conservation of rare, endangered and threatened species. In the present investigation, artificial seeds were obtained through encapsulation of three weeks old protocorm (3.0 ± 1.0 mm diameter) of *Cymbidium aloifolium* with calcium alginate. Artificial seeds were cultured on liquid Murashige and Skoog (MS) or Knudson (Kn C) medium at different strength ($\times 1.0, 0.5, 0.25$) and full strength (1.0) of both media supplemented with 0.5 mg/l 6-benzyl aminopurine (BAP) and 0.5 mg/l α -naphthalene acetic acid (NAA). Full strength of MS medium without plant growth regulators was found to be the most favourable condition for efficient plantlet regeneration of *C. aloifolium* (9.83 shoot and 2.66 roots per culture). The storage potential of artificial seed was tested at 4°C and room temperature (RT, 21°C \pm 2°C) for up to 90 days on both media and found 83.33% viability at 4°C storage on MS media. Eighty five percent of plantlets regenerated from artificial seeds culture were successfully hardened in a potting mixture of cocopeat, clay and sphagnum moss (2:1:1). Hence, the methodology can be used for propagation and conservation of *C. aloifolium* through artificial seed system.

Keywords

Artificial Seed, Protocorms, *In Vitro*, Germination, Encapsulation, Media

1. Introduction

Artificial seed technology involves the production of embryogenic or non-embryogenic regenerative explants enclosed in a protective capsule (coating) with the ultimate prod-

uct behaving like a seed for many functions including germination and plant regeneration and establishment. The coating protects the explants from mechanical damage during handling and allows germination and conversion into seedling. Artificial seeds have also been referred to as Synthetic seed or Synseed. The concept of Synseed or Artificial seed was first propounded by Murashige in 1977 [1] which was later described by several other investigators due to a recent increase in research interest in Synseed technology [2]-[4]. Previously, the artificial seeds were largely developed by encapsulation of somatic embryo. However, the rate of production of uniform high quality embryo is lower in most species and as a result the preparation of efficient quality artificial seeds has been successful in only few crops like carrot and alfalfa [5]. Bapat *et al.* (1987) proposed the making of Synseed through the encapsulation of *in vitro* derived non-embryogenic propagules such as axillary meristems as in *Morus indica* [6]. This concept was successfully tested in a number of species subsequently [7]-[11].

Cymbidium aloifolium is one of the threatened epiphytic orchids of Nepal with medicinal and ornamental value [12] [13]. It has attractive long-lasting beautiful flowers making it an important floriculture species. *C. aloifolium* is now on the verge of extinction from its natural habitat due to deforestation, over exploitation for trade and absence of pollinators due to indiscriminate use of pesticides or other modifications of the biome [14]. Another reason for its decline is their slow germination rate in nature as they require specific mycorrhizal fungi for successful seed germination [15]. Hence, an efficient propagation strategy is required to save this threatened, economically important orchid. Tissue culture techniques have been widely used for the *in vitro* mass multiplication of several commercially important orchids. Nayak *et al.* (1997); Hossain *et al.* (2009); Nongdam and Chongtham (2011) and Pradhan *et al.* (2013) had already reported the *in vitro* multiplication of *C. aloifolium* by using different explants [16]-[19]. However, little is known about the artificial seed system for *C. aloifolium*. Artificial seeds technology, with its advantage of storage has enormous potential as a mass propagation system for *C. aloifolium* producing field-ready propagules. Therefore, the present investigation was aimed to develop a reliable protocol for production, short to mid-term storage and efficient regeneration of plantlets from artificial seeds containing protocorms of *C. aloifolium*. The present study is the further progress of our previous work in this orchid species [20]. This protocol could be useful for commercial propagation and *ex-situ* conservation of this species.

2. Materials and Methods

2.1. Plant Material

Three weeks old *in vitro* grown protocorms of *Cymbidium aloifolium* was used for the present investigation. Protocorms were obtained from *in vitro* germinated seeds of *C. aloifolium*. Seeds resided in a capsule; a single capsule contains many seeds. In order to produce protocorms, immature capsules of *C. aloifolium*, collected from the wild, were washed under running tap water for 20 minutes, agitated soaked and shaken in detergent water with two drops of tween 20 for 15 minutes and then washed with running

tap water until all the detergent was removed. The capsules were then surface sterilized by dipping it in 70% alcohol for 2 minutes, followed by 1% sodium hypochlorite solution for 15 minutes. They were subsequently rinsed in sterile water for at least three times. The surface sterilized capsules were dried on filter paper under aseptic condition and cut longitudinally using a sterile sharp surgical blade to expose the seeds. Seeds were then inoculated on quarter (0.25), half (0.5) and full strength (1.0) of Murashige and Skoog (MS; Murashige and Skoog, 1962) [21] or Knudson (Kn C; Knudson, 1922) media [22] and full strength (1.0) of MS and Kn C media supplemented with 0.5 mg/l 6-benzyl aminopurine (BAP) and 0.5 mg/l α -naphthalene acetic acid (NAA). The cultures were maintained at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$ under 16/8 h photoperiod from cool-white-light, giving 500 - 1000 lux at culture level. After ten weeks of culture, small green globular protocorms were formed. Three weeks after protocorms started appearing, they were ready for producing artificial seeds.

2.2. Culture Medium and Incubation

Murashige and Skoog (MS) and Knudson (Kn C) media were used as the basal medium for all *in vitro* germination and seedling development from artificial seed are reported here. MS medium is highly enriched with basal salts. It contains macronutrients, micronutrients, iron sources and vitamins however Knudson medium contains low concentration of similar components without vitamins. Both, MS & Kn C liquid media [full (1.0), half (0.5) or quarter (0.25) strength] supplemented with or without plant growth regulators *viz.* BAP (0.5 mg/l) and NAA (0.5 mg/l) were used for this investigation. Basal medium were fortified with 30g/l sucrose and with or without different BAP & NAA (Table 1). The pH of all media was adjusted to 5.8 using 0.1 N NaOH or HCl.

Table 1. Effect of MS & Kn C liquid media on *in-vitro* germination and regeneration of plantlets from artificial seeds of *Cymbidium aloifolium*.

Media	Plant Growth Hormone Concentrations (mg/l)	Germination Mean \pm S.E (weeks)	No. of shoot Mean \pm S.E	Length of shoot Mean \pm S.E (cm)	No. of leaf Mean \pm S.E	Length of leaf Mean \pm S.E (cm)	No. of root Mean \pm S.E	Length of root Mean \pm S.E (cm)
0.25 MS	-	7.00 \pm 0.36	8.16 \pm 1.77	7.56 \pm 0.65	10.66 \pm 1.66	3.63 \pm 0.58	2.16 \pm 0.98	2.50 \pm 0.96
0.5 MS	-	7.00 \pm 0.25	8.83 \pm 2.18	6.78 \pm 1.14	9.00 \pm 1.77	3.18 \pm 0.82	1.33 \pm 0.33	2.30 \pm 1.06
1.0 MS	-	7.00 \pm 0.36	9.83 \pm 2.52	4.7 \pm 1.56	9.16 \pm 3.17	2.63 \pm 1.18	2.66 \pm 1.08	3.03 \pm 1.61
1.0 MS	0.5BAP + 0.5NAA	7.00 \pm 0.36	3.16 \pm 0.30	1.65 \pm 0.36	1.50 \pm 0.61	0.33 \pm 0.13	0.33 \pm 0.21	0.26 \pm 0.16
	<i>p</i> -value	1.0	0.015	0.008	0.014	0.013	0.223	0.237
0.25 Kn C	-	6.83 \pm 0.30	8.66 \pm 1.78	5.61 \pm 0.82	9.16 \pm 1.10	3.23 \pm 0.63	2.00 \pm 0.68	2.95 \pm 0.78
0.5 Kn C	-	6.66 \pm 0.21	9.16 \pm 1.77	4.50 \pm 1.00	10.5 \pm 2.74	2.10 \pm 0.66	2.50 \pm 0.99	2.15 \pm 0.94
1.0 Kn C	-	6.16 \pm 0.16	8.66 \pm 1.82	1.81 \pm 0.70	2.66 \pm 1.42	0.66 \pm 0.39	0.66 \pm 0.33	0.21 \pm 0.14
1.0 Kn C	0.5BAP + 0.5NAA	8.50 \pm 0.88	2.50 \pm 0.75	1.93 \pm 0.37	1.66 \pm 0.71	0.50 \pm 0.15	0.50 \pm 0.34	0.35 \pm 0.22
	<i>p</i> -value	0.034	0.021	0.007	0.011	0.005	0.203	0.064

Culture conditions: -Various strength of MS & Kn C liquid media, $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, 24 weeks, 16/8 hrs. photoperiod, 6 replicates were used in each condition, *p*-values based on one-way ANOVA (df 3.23).

About 20 ml of medium was dispensed into each culture tube (150 × 25 mm, Borosil) and autoclaved at 121°C for 20 min at 1.05 kg/cm² pressure. All cultures were maintained at 25°C ± 2°C under 500 - 1000 lux illuminance for 16/8 h (light/dark) photoperiod using cool white light (Philips, India).

2.3. Encapsulation and Inoculation

Artificial seeds were produced by encapsulation of three weeks old protocorms. Protocorms were mixed in the encapsulation matrix [4 % (w/v) sodium alginate and calcium chloride dihydrate (CaCl₂·2H₂O) solution (0.2M)]. Protocorms were drawn up with sodium alginate and dropped into CaCl₂·2H₂O solution using a sterilized micropipette. The encapsulated protocorms were left in CaCl₂·2H₂O solution for at least 30 minutes for hardening. Each bead contains a single protocorm. The beads, now on called artificial seeds, were washed with sterile water and then blotted dry in sterilized filter paper. Freshly prepared artificial seeds were inoculated on different experimental media for their germination and subsequent development (Table 1). The cultures were kept at 25°C ± 2°C under 16/8 h photoperiod from cool-white-light in culture room with 70% - 80% relative humidity depending upon the temperature. A set of 300 artificial seeds were kept in sterile petridish sealed with parafilm and stored at 4°C as well as at room temperature (21°C ± 2°C) for 90 days. They were sampled monthly and inoculated in full strength liquid MS and Kn C media for germination and plantlet development studies.

2.4. Acclimatization

Culture tubes containing rooted plants were opened and kept in the culture room for one week before transferring to greenhouse. Plantlets from the open culture vessels were removed, washed thoroughly with tap water, then dipped in 0.1% fungicide (Bavistin) solution for 5 minutes and washed with sterile water. They were then planted in earthen pots containing cocopeat, clay or sphagnum moss individually as well as in combination (2:1:1 v/v) for hardening. 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 watered daily and fertilized with 5% Hyponex solution (nitrogen, phosphorous and potassium in a ratio 20:10:10 were prepared in 100 ml) and sprayed once a week. The acclimatized plants were observed regularly.

2.5. Statistical Analysis

The results are expressed as mean ± SE of each treatment. There were 6 replicates per treatment and each experiment was repeated twice. Statistical analysis was carried out by using an analysis of variance (ANOVA) one way classification system. The data were analyzed by using application software-Micros of texcel. The significant difference between the medium and medium supplemented with different growth hormones were analysed at $P \leq 0.05$ using SPSS version 16.0 (SPSS Inc., USA).

3. Results

In the present investigation, three weeks old protocorm were used as primary explants for production of artificial seeds. Though there are several gelling agents such as polyox, agar, agarose, alginate, carboxy methylcellulose, carrageenan, guar gum, etc. for artificial seed production; sodium alginate was selected as a gelling agent for this investigation because of its low toxicity, low cost, quick gellation and bio compatibility characteristics [23].

3.1. Effect of Media on Regeneration of Plantlet from Artificial Seed

Freshly produced artificial seeds through protocorms with mean size 3 ± 1 mm in diameter were inoculated on liquid MS and Kn C media (full, half and quarter strength) with or without 0.5 mg/l BAP & NAA (Table 1). The initiation of artificial seed germination was started after 7 weeks of culture in all the concentrations of MS medium whereas in Kn C medium, germination of artificial seed occurred earlier, from 6-week onwards, on hormone-free full strength formulation.

Germinated artificial seeds developed into plantlets within 15 - 20 days of culture. From the observation (Table 1), it was found that maximum proliferation of shoot (9.83 ± 2.52) and root (2.66 ± 1.08) was found on hormone-free full strength MS medium followed by quarter strength MS. As germination was started at almost same time in all the tested conditions, the poorest development of shoot and root was observed on 1.0 MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l NAA than hormone-free medium. Leaf number and length of shoot and leaf were highest on 0.25 MS and 0.5 MS however; the time taken for their development was longer than hormone-free full strength MS media (data not shown). One way ANOVA showed that among the growth parameters studied only the number and length of shoot and leaf varied significantly among different conditions of MS medium ($p < 0.05$; Table 1). In Kn C medium, the highest number of shoot (9.16 ± 1.77), leaf (10.5 ± 2.74) and roots (2.50 ± 0.99) were observed on 0.5 Kn C medium whereas maximum length of shoot (5.61 ± 0.82), leaf (3.23 ± 0.63) and roots (2.95 ± 0.78) were found on 0.25 Kn C medium (Table 1). One way ANOVA showed that the value of all the growth parameters except number of root and length of root varied significantly among different conditions of Kn C medium ($p < 0.05$; Table 1).

3.2. Effect of Storage Conditions: Temperature and Duration for Viability

In this investigation, the artificial seed of protocorm contained only calcium alginate and were devoid of any other nutrients or plant growth regulators. In both MS and Kn C media, artificial seeds stored at 4°C were green, fresh and remained intact inside the calcium alginate beads until 90 days; whereas those stored at RT became pale and reduced its size. In RT, artificial seeds ruptured slowly after 10 weeks of inoculation and most of them become contaminated; while at 4°C the germination was started within 7 - 8 weeks of inoculation on MS medium. In case of Kn C medium, stored artificial seeds

germinated within 8 weeks of culture at 4°C and 10 - 11 weeks of culture at RT. On MS medium, 83.3% of artificial seeds stored for 30 days at 4°C were viable but it declined substantially with time. A similar trend was evident in Kn C medium as well. On the other hand, the artificial seed of protocorm stored at RT showed only 30% viability on day 30 with no viability or regeneration after 60 days (**Table 2**).

4. Discussions

Artificial seeds offer many advantages and opportunities especially in cross pollinating species where seed production and germination is difficult and expensive [24]. Therefore, the present investigation was carried out for the purpose of germplasm storage, delivery and conservation of the threatened medicinal orchid *Cymbidium aloifolium* through artificial seeds. Both MS and Kn C media have been found suitable for artificial seed germination and regeneration of plantlets. The germination time of artificial seeds was found to be different with media and growth conditions.

Although all tested media were successfully supported the conversion of encapsulated protocorms into whole plantlet with shoot, leaf and root growth showed varied responses. It might be due to the compositional variation of two different media including plant hormones. Many shoot buds were regenerated directly from a single encapsulated protocorm which later developed into a whole plant (**Figure 1(a)** and **Figure 1(b)**). Though earlier germination was observed on hormone-free Kn C medium, healthy and chlorophyllous shoots and roots were developed earlier on hormone free full strength of MS medium (**Figure 1(c)**). It could be due to the low amount of macro

Table 2. Effect of media, storage temperature and duration of storage on germination and plantlet development from artificial seeds of *Cymbidium aloifolium*.

Media	Storage temperature	Duration of storage (days)	Total no. of tested artificial seeds	No. of unruptured artificial seeds	No. of ruptured artificial seeds	Conversion of artificial seeds into plantlets (%)
MS	4°C	30	30	5	25	83.33
		60	30	14	16	53.33
		90	30	17	13	43.33
	RT	30	30	21	9	30.00
		60	30	30	0	-
		90	30	30	0	-
Kn C	4°C	30	30	20	10	33.33
		60	30	23	7	23.33
		90	30	25	5	16.66
	RT	30	30	27	3	10.00
		60	30	30	0	-
		90	30	30	0	-

Culture conditions: MS & Kn C liquid media, 90 days of storage, RT-room temperature (21°C ± 2°C).

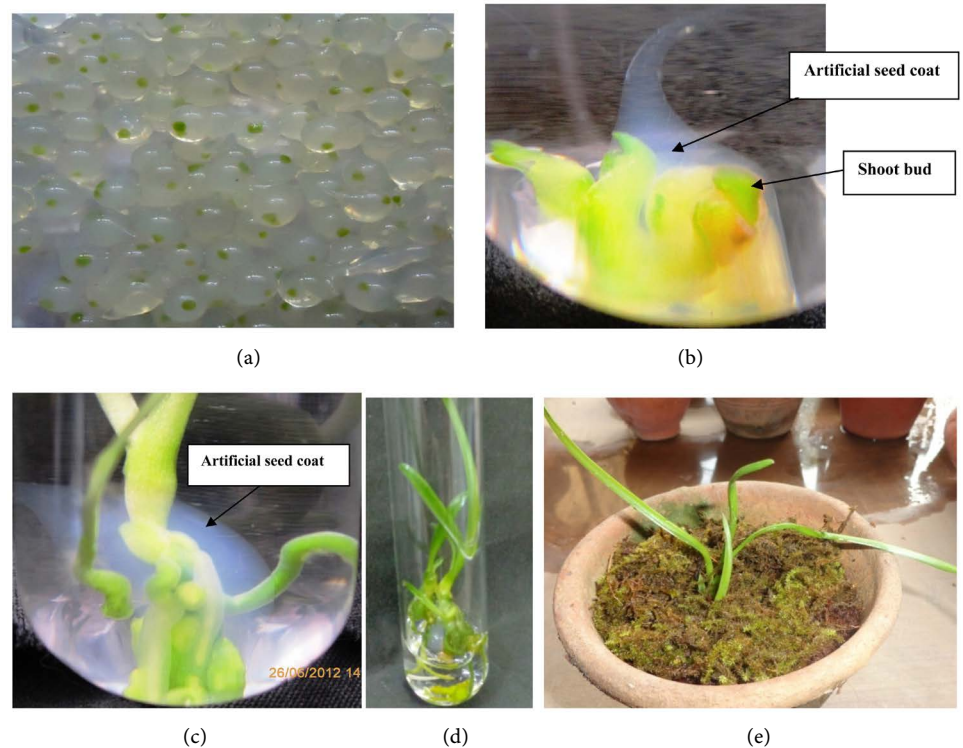


Figure 1. Production, germination and plantlet regeneration of artificial seed containing protocorms of *Cymbidium aloifolium*. (a) Production of artificial seed using 4% sodium alginate and 0.2M calcium chloride solution; (b) Germinated artificial seeds gave rise to shoot bud on 0.25 MS medium; (c) Development of healthy roots and shoot buds on hormone free 1.0 MS medium; (d) Multiplication of shoots with well developed roots on hormone free 1.0 MS medium; (e) Hardening of *in vitro* plantlets on potting mixture of cocopeat, clay and sphagnum moss of 2:1:1 ratio.

and micro nutrients present in Kn C medium. Whereas MS medium was enriched with high concentration of nutritional compounds which made the suitability for efficient regeneration of healthy plantlet from artificial seed of *Cymbidium aloifolium*. Hence, MS medium was significantly more effective for germination and seedling development rather than Kn C medium. In this medium, shoot buds were developed by cracking the alginate beads of artificial seeds of protocorms after 8 weeks of inoculation; complete plant with shoot and root were developed after 14 - 15 weeks of culture (**Figure 1(d)**). The most appropriate condition for the germination of the artificial seed was dependent on the time taken for germination, efficiency of germination, growth and development of seedlings. It can be concluded from this investigation that plants get physiologically stressed in hormone attributed both MS and Kn C media as a result they could not undergo proliferation sufficiently as much as like hormone free MS and Kn C media. In case of lower strength of MS media *i.e.* 0.25 MS & 0.5 MS, plant didn't get sufficient nutrition as they required for their optimum development [19].

The present result was similar to the findings of other researchers: Gupta (2016) in *Renanthera imschootiana* [25], Teixeira da Silva (2012) in hybrid *Cymbidium* [26],

Nagananda *et al.* (2011) in *Flickingeria nodosa* [27], Sarmah *et al.* (2010) in *Vanda coerulea* [28], Mohanraj *et al.* (2009) in *Coelogyne breviscapa* [15] in the aspect of production of artificial seed or Synseed from protocorm or protocorm like bodies for regeneration. However, the present findings are in contrast with the result obtained by other researchers in the aspect of culture of artificial seed in high concentration of hormone attributed medium. Zhang *et al.* (2011) reported that Maltose 4%, 6-BA/NAA 12:1, active carbon 0.3%, sodium alginate 4% and time of ion exchange for 5 minutes were optimum condition for artificial seed production with PLB's in *Dendrobium candidum* [29]. As well, Qin *et al.* (2008) found that modified MS with 4% maltose and BA (1 mg/l) and NAA (1 mg/l) favoured germination and seedling of orchid *Dendrobium huoshanensis* by using 4% sodium alginate [30]. Moreover, Datta *et al.* (1999) found that modified Knudson medium supplemented with BAP (1 mg/l) and NAA (1 mg/l) was the most effective condition for germination of artificial seed of *Geodorum densiflorum* (Lan) Schltr. [5]. Further, Saiprasad and Polisetty (2003) reported 100% conversion of encapsulated PLBs when cultured on MS medium supplemented with BA (4.44 μ M) and NAA (0.54 μ M) on *Dendrobium* and NAA alone on *Oncidium* and *Cattleya* [31]. Finally, Mohanty and Das (2013) found that MS medium supplemented with 2 mg/l BAP was efficient for 100% conversion of encapsulated PLB's into plantlets on *Dendrobium densiflorum* Lindl. ex Wall. [32].

Besides germination and mass propagation of artificial seeds containing protocorms of *Cymbidium aloifolium*, the viability of stored artificial seeds were observed until 90 days in two different temperatures *i.e.* room temperature (RT, 21 °C \pm 2 °C) and 4 °C. In both media, it was found that the viability percentage of artificial seeds of protocorms decreased gradually with increase in storage time. The decline in germination and plant development as a result of prolonged storage of tissue could be due to the inhibited respiration of tissues [32] [33]. The maximum percentage of viability was observed in 30 days stored artificial seed (83.33%) at 4 °C inoculated on MS medium. This result revealed that the germination and regeneration capacity of the stored artificial seeds at 4 °C was quite higher rather than stored at RT. It might be due to the chilling effect of low temperature, which not only store the calcium alginate beads for prolonged period by reducing their growth but also enhance its regeneration capacity when transferred to re-growth medium containing essential nutrients. Here, the storage temperature and duration of storage were played an important role on conversion of artificial seeds into plantlets (Table 2). The present result was supported by the findings obtained by various researchers: Pradhan *et al.* (2014) successfully stored the artificial seed of *Cymbidium aloifolium* up to 28 days on MS medium with 97.5% conversion rate [20]. Nagananda *et al.* (2011) encapsulated the PLBs of *Flickingeria nodosa* and achieved 95% conversion after 3 months storage at 4 °C [27]. Zhang *et al.* (2011) produced artificial seeds of *Dendrobium candidum* with protocormlike bodies as propagator and achieved the germination rate 87.70 \pm 0.46 after 5 days and 14.87 \pm 0.31 after 20 days of storage in refrigerator at 4 °C which was lower compared to the present investigation [29]. Similarly, Datta *et al.* (1999) found 86% viability of artificial seeds of *Geodorum densi-*

florum when stored at 4°C and 44% viability when stored at room temperature for 120 days. Nutrient medium also plays a crucial role on germination and development of artificial seed [5]. In present investigation, MS medium was found to be the most favourable condition for regeneration of plantlet from fresh as well as stored artificial seed containing protocorms of *C. aloifolium*. The use of MS medium only for regeneration of plantlet from stored artificial seed also provides a way to reduce the costs by eliminating the need for plant hormones.

The ultimate objective of producing artificial seed was to recover the whole plantlet from artificial seed under *in vitro* condition. In present investigation, *in vitro* plant with well-developed roots obtained from artificial seeds, cultured on both MS and Kn C media after attaining the height of 4 - 5 cm was selected for acclimatization. They were transferred to earthen pot containing different acclimatization substrates. The combination of cocopeat, clay and sphagnum moss in ratio 2:1:1 was found to be a suitable potting mixture for hardening (Figure 1(e)). Eighty five percentage of seedlings were successfully survived under this condition. Hence, this result suggests that the mixture of cocopeat, clay and moss will be favourable for the acclimatization of epiphytic orchids as in *Cymbidium aloifolium*.

5. Conclusion

Many researchers have successfully produced and propagated artificial seed of many crops but very limited in orchids. The present investigation revealed that full strength of liquid MS medium without plant growth regulators was found to be the most effective condition for germination as well as for development of efficient plant regeneration from artificial seeds of *Cymbidium aloifolium* than other tested conditions of MS and Kn C medium. 30 days stored artificial seeds have showed highest percentage of viability *i.e.* 83.33% at 4°C on MS medium as compared to 60 and 90 days of storage. The development of protocol for direct recovery of plant from artificial seeds may have greater impact on tissue culture methods. Therefore, the present investigation concluded that artificial seeds have the excellent potential to be used as alternative planting materials for *in vitro* clonal mass multiplication, short to medium term storage, easy handling and transportation and *ex-situ* conservation of economically important species like *Cymbidium aloifolium*. Hence, the protocol established from present investigation can be employed for large scale propagation of *C. aloifolium* which may be not only useful for fulfilling the urgent local needs for effective conservation but also reliable for plant breeders and local orchid growers for storage of plant genetic resources, easy delivery and their commercial production by using minimum space with lower labour and maintenance cost.

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References

- [1] Murashige, T. (1977) Plant Cell and Organ Cultures as Horticultural Practices. *Symposium on Tissue Culture for Horticultural Purposes*, **78**, 17-30.
<http://dx.doi.org/10.17660/actahortic.1977.78.1>
- [2] Lambardi, M., Benelli, C., Ozudogru, E.A. and Ozden-Tokatli, Y. (2006) Synthetic Seed Technology in Ornamental Plants. *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues*, **2**, 347-354.
- [3] Asmah, H.N., Hasnida, H.N., Zaimah, N.N., Noraliza, A. and Salmi, N.N. (2011) Synthetic Seed Technology for Encapsulation and Regrowth of *In Vitro*-Derived Acacia Hybrid Shoot and Axillary Buds. *African Journal of Biotechnology*, **10**, 7820-7824.
<http://dx.doi.org/10.5897/AJB11.492>
- [4] Reddy, M.C., Murthy, K.S.R. and Pullaiah, T. (2012) Synthetic Seeds: A Review in Agriculture and Forestry. *African Journal of Biotechnology*, **11**, 14254-14275.
- [5] Datta, K.B., Kanjilal, B. and De Sarker, D. (1999) Artificial Seed Technology: Development of a Protocol in *Geodorum densiflorum* (Lam) Schltr.—An Endangered Orchid. *Current Science*, **76**, 1142-1145.
- [6] Bapat, V.A., Mhatre, M. and Rao, P.S. (1987) Propagation of *Morusindica* L. (Mulberry) by Encapsulated Shoot Buds. *Plant Cell Reports*, **6**, 393-395.
<http://dx.doi.org/10.1007/BF00269570>
- [7] Singh, F. (1991) Encapsulation of *Spathoglottis plicata* Protocorms. *Lindleyana*, **6**, 61-63.
- [8] Corrie, S. and Tandon, P. (1993) Propagation of *Cymbidium giganteum* Wall, through high Frequency Conversion of Encapsulated Protocorms under *In Vivo* and *In Vitro* Conditions. *Indian Journal of Experimental Biology*, **31**, 61-64.
- [9] Piccioni, E. and Standardi, A. (1995) Encapsulation of Micropropagated Buds of Six Woody Species. *Plant Cell, Tissue and Organ Culture*, **42**, 221-226.
<http://dx.doi.org/10.1007/BF00029990>
- [10] Chen, D.F., Chen, X.W. and Li, Z.D. (1996) Germination and Storage Characteristics of Ramie Artificial Seeds Made of Adventitious Buds. *China's Fibre Crops*, **2**, 1-5.
- [11] Yoshida, T. (1996) *In Vitro* Propagation of Hybrid Rice (*Oryzasativa* L.) 1. "Tissue Cultured" Shoot Primordia. *Japan Agricultural Research Quarterly*, **30**, 1-8.
- [12] Das, P.K., Sahoo, S. and Bal, S. (2008) Ethnobotanical Studies on Orchids of Niyamgiri Hill Ranges, Orissa, India. *Ethnobotanical Leaflets*, **12**, 70-78.
- [13] Pant, B. and Raskoti, B.B. (2013) Medicinal Orchids of Nepal. Himalayan Map House Pvt. Ltd., Nepal.
- [14] Chugh, S., Guha, S. and Rao, I.U. (2009) Micropropagation of Orchids: A Review on the Potential of Different Explants. *Scientia Horticulturae*, **122**, 507-520.
<http://dx.doi.org/10.1016/j.scienta.2009.07.016>
- [15] Mohanraj, R., Ananthan, R. and Bai, V.N. (2009) Production and Storage of Synthetic Seeds in *Coelogyne breviscapa* Lindl. *Asian Journal of Biotechnology*, **1**, 124-128.
<http://dx.doi.org/10.3923/ajbkr.2009.124.128>
- [16] Nayak, N.R., Rath, S.P. and Patnaik, S. (1997) *In Vitro* Propagation of Three Epiphytic Orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch-Ham) Sw. through Thidiazuron-Induced High Frequency Shoot Proliferation. *Scientia Horticulturae*, **71**, 243-250.
[http://dx.doi.org/10.1016/S0304-4238\(97\)00075-7](http://dx.doi.org/10.1016/S0304-4238(97)00075-7)
- [17] Hossain, M.M., Sharma, M. and Pathak, P. (2009) Cost Effective Protocol for *in Vitro* Mass

- Propagation of *Cymbidium aloifolium* (L.) Sw.—A Medicinally Important Orchid. *Engineering in Life Sciences*, **9**, 444-453. <http://dx.doi.org/10.1002/elsc.200900015>
- [18] Nongdam, P. and Chongtham, N. (2011) *In Vitro* Rapid Propagation of *Cymbidium aloifolium* (L.) Sw.: A Medicinally Important Orchid via Seed Culture. *Journal of Biological Sciences*, **11**, 254-260. <http://dx.doi.org/10.3923/jbs.2011.254.260>
- [19] Pradhan, S., Regmi, T., Parmar, G. and Pant, B. (2013) Effect of Different Media on *in Vitro* Seed Germination and Seedling Development of *Cymbidium aloifolium* (L.) Sw. *Nepal Journal of Science and Technology*, **14**, 51-56. <http://dx.doi.org/10.3126/njst.v14i1.8878>
- [20] Pradhan, S., Tiruwa, B., Subedee, B.R. and Pant, B. (2014) *In Vitro* Germination and Propagation of a Threatened Medicinal Orchid, *Cymbidium aloifolium* (L.) Sw. through Artificial Seed. *Asian Pacific Journal of Tropical Biomedicine*, **4**, 971-976. <http://dx.doi.org/10.12980/APJTB.4.2014APJTB-2014-0369>
- [21] Murashige, T. and Skoog, F. (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia plantarum*, **15**, 473-497. <http://dx.doi.org/10.1111/j.1399-3054.1962.tb08052.x>
- [22] Knudson, L. (1922) Non Symbiotic Germination of Orchid Seeds. *Botanical Gazette*, **73**, 1-25. <http://dx.doi.org/10.1086/332956>
- [23] Saiprasad, G.V.S. (2001) Artificial Seeds and Their Applications. *Resonance*, **6**, 39-47. <http://dx.doi.org/10.1007/BF02839082>
- [24] Latif, Z., Idrees, A., Nasir, A. and Riazuddin S. (2007) Indigenous Production of Synthetic Seeds in *Daucus carota*. *Pakistan Journal of Botany*, **39**, 849-855.
- [25] Gupta, A. (2016) Regeneration of *Renanthera imschootiana* Rolfe Using Synthetic Seeds. *International Advanced Research Journal in Science, Engineering and Technology*, **3**, 286-289.
- [26] Teixeira da Silva, J.A. (2012) Production of Synseed for Hybrid *Cymbidium* Using Protocorm-Like Bodies. *Journal of Fruit and Ornamental Plant Research*, **20**, 135-146. <http://dx.doi.org/10.2478/v10290-012-0023-7>
- [27] Nagananda, G.S., Satishchandra, N. and Rajath, S. (2011) Regeneration of Encapsulated Protocormlike Bodies of Medicinally Important Vulnerable Orchid *Flickingeria nodosa* (Dalz.) Seidenf. *International Journal of Botany*, **7**, 310-313. <http://dx.doi.org/10.3923/ijb.2011.310.313>
- [28] Sarmah, D.K., Borthakur, M. and Borua, P.K. (2010) Artificial Seed Production from Encapsulated PLBs Regenerated from Leaf Base of *Vanda coerulea* Griff. ex. Lindl.—An Endangered Orchid. *Current Science*, **98**, 686-690.
- [29] Zhang, Y., Yan, S. and Zhang, Y. (2011) Factors Affecting Germination and Propagators of Artificial Seeds of *Dendrobium candidum*. *International Conference on Agricultural and Biosystems Engineering*, Paris, 24-26 June 2011, 404-410.
- [30] Qin, Z., Zhao, T., Qiu, J., Lin, Y. and Cai, Y. (2008). Germination and Propagators of Artificial Seeds of *Dendrobium huoshanense*. *Chinese Journal of Biotechnology*, **24**, 803-809.
- [31] Saiprasad, G.V.S. and Polisetty, R. (2003) Propagation of Three Orchid Genera Using Encapsulated Protocorm-Like Bodies. *In Vitro Cellular & Developmental Biology-Plant*, **39**, 42-48. <http://dx.doi.org/10.1079/IVP2002360>
- [32] Mohanty, P. and Das, J. (2013) Synthetic Seed Technology for Short Term Conservation of Medicinal Orchid *Dendrobium densiflorum* Lindl. Ex Wall and Assessment of Genetic Fidelity of Regenerants. *Plant Growth Regulation*, **70**, 297-303. <http://dx.doi.org/10.1007/s10725-013-9801-z>
- [33] Danso, K.E. and Ford-Lloyd, B.V. (2003) Encapsulation of Nodal Cuttings and Shoot Tips for Storage and Exchange of Cassava Germplasm. *Plant Cell Reports*, **21**, 718-725.

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