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In vitro germination and propagation of a threatened medicinal orchid, *Cymbidium aloifolium* (L.) Sw. through artificial seed

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ABSTRACT

Objective: To study the *in vitro* germination and plantlet regeneration from artificial seeds of *Cymbidium aloifolium* (*C. aloifolium*), a highly threatened medicinal orchid of Nepal.

Methods: Artificial seeds were produced *in vitro* by encapsulation of protocorms with 4% sodium alginate and 0.2 mol/L calcium chloride solution. *In vitro* germination and plantlet regeneration of the artificial seeds were tested by culturing them on different strength of Murashige and Skoog (MS) liquid media (0.25, 0.5 and 1.0) and MS liquid medium supplemented with 0.5 mg/L benzyl amino purine and 0.5 mg/L naphthalene acetic acid. Freshly produced artificial seeds were stored up to 28 d at 4 ℃. In order to check the viability, storage artificial seeds were treated with five different sterilization techniques (T_1, T_2, T_3, T_4, T_5) and inoculated on full strength (1.0) of MS liquid medium after each 7 d of interval upto 28th days.

Results: The highest percentage of germination (100%) of artificial seed was obtained on quarter (0.25), half (0.5) and full (1.0) strength of MS liquid medium. Experimentally, full strength of MS liquid medium was more effective for earlier seedling development of *C. aloifolium*. Artificial seeds were successfully stored at 4 ℃ till 28th days. Treatments T_1 and T_2 showed 97.5% viability of storage artificial seeds and hence considered as the most effective sterilization techniques to recover the plant from storage artificial seeds. Plantlets developed from artificial seeds were successfully acclimatized in potting mixture containing cocopeat, litter and sphagnum moss with 85% survival rate.

Conclusions: The present study revealed that artificial seeds are the good alternative explants for *in vitro* mass propagation and short term conservation of *C. aloifolium*.

1. Introduction

Orchids represent the most evolved and one of the largest groups among the angiosperm. They are of immense horticultural as well as medicinal importance which fetches a very high price in the international market. They also play a very useful role to balance the forest ecosystems. Demand for high quality of orchids has been increasing day by day due to their popularity in horticulture industry[1]. A single orchid capsule contains millions of seeds, which lack any metabolic machinery and do not have any endosperm. In spite of a very large number of seeds produced, only few seeds germinate in nature as they require specific fungus[2]. Currently, wild orchid population decreases rapidly due to biodiversity loss, illegal trade and consumption by local people. Therefore, the development of an artificial means of propagation is needed to reduce collection pressures on wild population. Nowadays, encapsulation technique for producing artificial seeds has become an important asset in micropropagation[3].

Cymbidium aloifolium L. Sw (*C. aloifolium*) is one of the highly valuable and threatened medicinal orchids of Nepal. The leaves of this species are extensively used for styptic properties in the treatments of boils and fevers. The roots are used to cure

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paralysis and chronic illness. The whole plant can also be used as tonic and in the treatment of vertigo, weakness of eyes, burns and sores[4-7]. Besides its medical importance, this orchid also attracts the floriculture market because of its long-lasting highly attractive beautiful flowers. Due to its various uses, people uprooted this plant from wild and hence reach to the threatened category[8]. Thus, an attempt was made to produce artificial seeds of *C. aloifolium*, their short term storage and mass propagation within a short span of time by using tissue culture technique. Asymbiotic germination of artificial seed through protocorms provides a useful way to re-establish plants in the wild for germplasm preservation as well as for commercial propagation.

2. Materials and methods

2.1. Source of explants

Protocorms derived from *in vitro* culture of seeds of *C. aloifolium* were used as explants in present study. The undehisced capsules of *C. aloifolium* were harvested from nature at an elevation of 500 m from tropical region of Central Nepal[8]. Capsule contains a large number of orchid seeds. The freshly collected capsules were first thoroughly washed under running tap water for at least 30 min to remove the external particles attached to it. They were then washed with detergent Tween 20 (0.1%) for 15 min and again washed with tap water until all detergent washed clearly. After that capsules were surface sterilized by dipping it in 70% alcohol for 2 min followed by 1% sodium hypochlorite solution for 15 min and were subsequently rinsed in sterile water for at least three times. The sterilized capsules were dried on Whatman 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 Murashige and Skoog (MS) basal medium under aseptic condition. Protocorms were started to develop after 10 weeks of culture of seeds and 21-days old protocorms were selected as primary explants for present study.

2.2. Germination medium

In vitro germination and subsequent development of artificial seed was assessed on different strength of liquid MS medium[9], *i.e.* full (1.0), half (0.5) and quarter (0.25) and full-strength MS liquid media supplemented with different concentration of plant growth regulators *viz.* 0.5 mg/L benzyl amino purine (BAP) and 0.5 mg/L naphthalene acetic acid (NAA) (Table 1). MS medium was fortified with 3% sucrose as carbon source without solidified with agar. The pH of MS media was adjusted to 5.8 before autoclaving. About 16-20 mL of media was dispensed into each culture tube (150×25 mm, Borosil) and autoclaved at pressure 15 psi and temperature of 121 ˚C for 20 min. All cultures were maintained at (25±2) ˚C under 500-1 000 lux illuminance for 16/8 h. (light/dark) photoperiod using cool white light (Philips, India).

2.3. Gel matrix and encapsulation

Protocorms were used as primary explants to produce artificial seeds. For encapsulation of protocorms, sodium alginate was used with calcium chloride dihydrate for complexation. Sodium alginate of 4% and 0.2 mol/L of calcium chloride dihydrate ($CaCl_2.2H_2O$) solution were prepared separately by dissolved in sterile water. Individual protocorms were separated and mixed in 4% sodium alginate matrix. Alginate matrix containing single protocorm were taken up with the help of sterile micropipette and then gently dropped into 0.2 mol/L $CaCl_2.2H_2O$ solution. The drops each containing single protocorm were left in $CaCl_2.2H_2O$ solution for at least 30 min to harden the alginate beads. After that, the alginate beads were washed with sterile water for three times and blot dried. The alginate beads were then called as artificial seeds or synthetic seeds or encapsulated seeds. Artificial seeds [(3±1) mm diameters] were placed on sterile filter paper containing sterile glass petriplates (30 beads per plate), sealed with parafilm and stored at 4 ˚C or used immediately. Freshly prepared artificial seeds were inoculated on different strength of MS liquid medium *i.e.* 0.25 MS, 0.5 MS, 1.0 MS, and 1.0 MS liquid 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 h photoperiod from cool-white-light in culture room.

2.4. Sterilization and viability test of storage artificial seed

Artificial seeds stored at 4 ˚C were regularly taken out at regular intervals of 7 d till 28th d and were inoculated on culture tubes

Table 1
In-vitro germination and seedling development of freshly inoculated artificial seeds containing protocorms of *C. aloifolium* (L.) Sw.

MS Media	Plant growth hormone concentrations (mg/L)	Observation (weeks)					Percentage of germination (%)
		Germination (mean)	Initiation of shoot	Initiation of leaf	Initiation of root	Seedling	
0.25	-	7.00	8.00±0.89	9.50±1.22	14.75±3.59	16.16±0.98	100.00
0.5	-	7.00	8.16±0.75	12.16±3.43	17.20±4.81	18.16±1.17	100.00
1.0	-	7.00	8.00±0.89	14.60±5.68	15.75±6.39	16.00±0.89	100.00
1.0	0.5 BAP+ 0.5 NAA	7.00	8.33±1.03	17.50±1.73	13.00±5.65	18.50±1.37	83.33

Data of initiation of shoot, leaf, root and seedling is expressed as mean±SD, 6 replicates were used in each condition.