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Effect of different basal media and PGRs on *in vitro* seed germination and seedling development of medicinally important orchid *Cymbidium aloifolium* (L.) Sw.

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Abstract
A comparative study on *in vitro* seed germination and seedling development of *Cymbidium aloifolium* (L.) Sw., an epiphytic medicinal orchid, was carried out on three different conditions of KC, MS, PM & VW media *viz.* half strength, full strength and medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA. Varied response was found in terms of seed germination, protocorm like bodies formation and seedling development was observed on four different basal media. Medium supplemented with hormones favored optimum condition for the germination (approx. 95%) of seeds followed by full strength and half strength on KC, MS, PM and VW media. MS medium supplemented with 0.5mg/l BAP and 0.5mg/l NAA showed comparatively better response within 6 weeks of culture than other conditions of MS medium as well as KC, PM and VW media. Based upon the results, it was found that MS medium was more effective than KC, PM and VW media for germination, PLBs and plantlet formation. The present study has provided useful information that the high concentration of nutrient compounds and vitamins supplemented with PGRs are required for earlier *in vitro* germination, plantlet developed from immature seeds of *C. aloifolium*. It could be an important protocol to conserve this medicinally important orchid species by establishing an efficient *in vitro* regeneration system using immature seed culture.

Keywords: *Cymbidium aloifolium*, medicinal orchid, PGRs, PLBs

1. Introduction
The orchidaceae represents a peak in the evolution of monocots and is one of the most successful family of flowering plants, as is clear from its wide distribution and numerous species [1]. Orchids are one of the most striking decorative plants all over the world. They are highly priced in the national and international markets because of their enduring and beautiful flowers. They account for 7% of total flowering plant species which represent one of the most expensive ornamental known today and dominate the international cut flower trade [2]. Besides their high ornamental values, orchids are of considerable importance in medicines as well. They have rich contents of alkaloids, glycosides and other useful phytochemicals [3]. *Cymbidium* comprises more than 70 natural species and hundreds of manmade hybrids. This rich variety has contributed significantly to the development of the international trade in orchid cut flowers [4]. *Cymbidium aloifolium* (L.) Sw. is one of the horticulturally as well as highly medicinally important epiphytic orchid of Bangladesh. The plant is reported to have emetic and purgative properties [5]. People use its pseudobulb and leaves for various medicinal purposes. 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, fever and other inflammatory conditions. The whole plant can also be used as tonic and in the treatment of vertigo, weakness of eyes, burns and sores [6, 7, 8]. This orchid is highly demanded in floriculture market because of its exquisite highly intricate beautiful flowers. Indiscriminate collections by orchid lovers, habitat destruction and over exploitation for medicinal purposes are the main factors that have threatened the survival of this species. Therefore conservation of this orchid is now a matter of universal concern. Tissue culture technique has been widely used for the *in vitro* mass propagation of several commercially important orchids [9]. Orchid's seeds are very minute, non-endospermic and need mycorrhizal association for germination in nature and require up to 8-10 years for their *in vivo* growth before reaching reproductive maturity. This problem can be overcome by tissue culture technique. *In vitro* culture of orchid seed does not require any fungal association and asymbiotic seed germination can be successfully performed leading to plant regeneration. Asymbiotic seed germination of orchids is greatly influenced by several factors such as developmental stage of embryos, age of green pod and

different nutrient media with adjuvant and plant growth regulators [10]. Modification of traditional tissue culture technique by adding specific plant growth regulators, activated charcoal, peptone and changing culture condition are reported to enhance germination percentage and subsequent development of protocroms in many orchids [6]. *Cymbidium aloifolium* being highly medicinal orchids, its propagation and domestication is an urgent need. The present research highlighted the best medium and concentration for *in vitro* seed germination, protocorm like bodies (PLBs) and seedling development of *C. aloifolium*. It has generated standardized protocol for propagation and their conservation.

2. Methodology

Immature pods of *C. aloifolium* collected from natural habitat from Madhob Kundo Eco Park, Moulvibazar, Bangladesh.

2.1 Surface sterilization of explants

Immature green pods of *C. aloifolium* were used as explants for the present investigation. Young green pods were first cleaned with detergent and rubbed with savlon soaked cotton and finally washed in running tap for 30 minutes till all the detergent was washed off clearly. After that, green pods were surface sterilized sequentially with 70% ethyl alcohol for 1 minute, 0.1% $HgCl_2$ solution for 10 minutes and finally rinsed thoroughly three times with sterile distilled water. Chemical surface sterilization process was carried out on laminar airflow cabinet.

2.2 Culture medium and incubation

In the present investigation, half strength, full strength and plant growth regulators (PGRs) *viz.* BAP (0.5 mg/l) and NAA (0.5 mg/l) supplemented media of KC [11], MS [12], PM [13] and VW [14] were used for *in vitro* seed germination and seedling development. Basal medium were fortified with 30g/l sucrose for MS and 20g/l sucrose for KC, PM, VW and with or without different plant growth regulators like BAP & NAA (Table 1). Agar (0.8% w/v) was used as a gelling agent for all tested media. pH of the media was adjusted at 5.8 in case of MS and 5.4 in KC, PM and VW by using 0.1N NaOH or HCl. Agar was dissolved by boiling the mixture and about 50 ml of media was dispensed into 100 ml each culture vessel and autoclaved at 121 ^{0}C for 20 minutes at 15 lb/cm^2 pressure. All cultures were maintained at 25±2 ^{0}C under 350-500 lux illumination for 14h photoperiod using white fluorescent tubes and 10h dark.

2.3 Seed culture

Surface sterilized immature green pods were kept on sterilized petri dish containing sterilized filter paper for drying. They were cut longitudinally with the help of sharp sterilized surgical blade. The immature seeds were scooped out with the help of sterilized spatula and transferred to and spread over the surface of different strength of KC, MS, PM and VW medium supplemented with or without combination of NAA and BAP. Sub-culturing was carried out every six weeks into fresh medium and five replicates were used for each treatment. The initiation and rate of seed germination was recorded regularly. The entire experiment was performed in aseptic condition under laminar air flow hood to prevent contamination.

3. Results and Discussion

Half strength, full strength and PGRs (0.5 mg/l BAP and 0.5 mg/l NAA) supplemented full strength of KC, MS, PM and VW media were used for *in vitro* seed germination and seedling development of *C. aloifolium*. With varied response and more than 80% seeds were germinated in all cultured conditions (Table 1). In MS basal medium, the first visible sign of germination was observed as the swollen yellowish green spherule like protocorm within 9 weeks of primary culture. First leaf primordium was developed in 16 weeks of culture while Shreeti Pradhan *et al.* [15] found after 19 weeks. In 24 weeks of culture, complete seedlings were obtained. Though MS basal medium favored seed germination but the rate of seed germination was less in comparison to MS medium supplemented with BAP (0.5mg/l) and NAA (0.5mg/l) where the initiation of seed germination was observed within 6 weeks of primary culture and around 95% seeds were successfully germinated. In this condition, after 8 weeks of culture, protocorms were developed which underwent further differentiation to form complete seedlings. First leaf primordia emerged out in 12 weeks of culture which finally developed into embryonic photosynthetic leaves and root developed in 18 weeks of culture. Within 24 weeks of culture, complete seedlings were obtained. Hence, in the present study, half strength of KC, MS, PM and VW media were found to be efficient for the germination of immature seeds only up to the protocorm development but failure to seedling development. Whereas with or without PGRs supplemented full strength KC, MS, PM and VW media was found to be the most suitable culture condition for immature seed germination of *C. aloifolium* up to seedling development. Here also noted that PGRs supplemented media took lesser time for germination, protocorm proliferation and further differentiation of leaf primordia than full strength of PGRs free medium. Therefore the present study showed that full strength KC, MS, PM and VW media supplemented with PGRs was the best and followed without PGRs free full and half strength KC, MS, PM and VW media respectively.

Immature seeds of *C. aloifolium* undergo various developmental stages during germination. Germination of orchid seeds is different from other seeds. Orchid seeds are produced in large numbers inside a pod. The seeds are very minute and contain undifferentiated embryos and lacks endosperm. In certain orchids self pollination is not possible and even if it is possible as in the case of *Vanda*, one has to wait for 4-6 months for pod development [16]. In the present investigation, immature green pods were taken for *in vitro* culture. Due to non-endospermic nature of seed, the germination in nature is a unique phenomenon and requires specific mycorrhizal fungal infection. Germination is much more successful in *in vitro* condition. Cells of immature seeds first turned into smooth walled globular structures called spherules with sticky hair after six weeks of seed culture due to the swelling of embryos. This indicates the first visible sign of germination of cultured seeds. Light yellowish cultured seeds first changed into light green and finally to green in color during development of protocorms. Protocorms develop further to give rise to shoot and root without undergoing callus formation in *C. aloifolium*. All the testing conditions showed seed germination but full strength MS media supplemented with BAP (0.5mg/l) and NAA (0.5mg/l) was the most effective condition for enhancing seed germination and seedling development.

Four different basal media employed in present study were different from one another in their chemical composition. MS medium is highly enriched with macro and micro nutrients

with different vitamins whereas PM, VW and KC medium contained comparatively low amount of macro and micro nutriments and with or without vitamins [6] respectively. Due to this, maximum percentage of seed germination was observed in different conditions of MS, PM and VW medium rather than Knudson medium as they lack vitamins. There are several reports explaining enhancement of germination and seedling growth and development by vitamins on different orchids. Addition of various vitamins into the medium was reported to be promotive for seed germination and seedling growth of *Cymbidium elegans* and *Coelogyne punctulata* [17]. Mariat [18] reported that vitamin B favored germination and differentiation in *Cattleya* seedlings. He showed that thiamine, nicotinic acid and biotin were most effective in *Cattleya* hybrids production. In one another study, Pyridoxine was shown to be essential for chlorophyll synthesis and combination of nicotinic acid and biotin favored better germination of *Orchis laxiflora* seeds [19]. In present investigation, half strength of KC, MS, PM and VW medium were not effective for plantlet formation as they have very low amount of macro and micro nutrients. All of the basal media of full strength gave satisfactory result but the time taken for germination and seedling development was quite longer than PGRs supplemented medium. In *in vitro* culture, cytokinins like BAP, Picloram and Kinetin are generally known to induce both axillary and adventitious shoots formation and auxins like NAA, IAA and IBA for root induction [20]. PGR like BAP was known to enhance the germination frequency of *Cypripedium candidum* [21]; protocorm multiplication and shoot formation in *Cymbidium pendulum* [22]. The synergistic effect of cytokinin and auxin in germination and plantlet development as found in present study has also been reported in *Phaius tancarvilleae* [3], *Cymbidium elegans* [23] and *C. iridiodes* [24]. Protocorms produced in present investigation were globular, hairy and chlorophyllous in all testing conditions of MS and PM medium whereas on KC and VW medium, they were light yellowish in color.

The number of protocorms was also lesser on Knudson medium than MS medium but size of protocorms was superior on Knudson medium. Low amount of macro and micro elements of Knudson medium could have been effective for enlargement of protocorms due to nutritional stress but not for increasing their number. Production of larger size of protocorms indicates that culture seeds require sufficient amount of nutrients. Thus, the nutrient regime for orchid culture is species specific and no single culture medium is universally applicable for all the orchid species. From above result, it was concluded that MS medium enriched with high concentration of nutritional compounds was suitable for earlier germination, large number of protocorm formation and seedling development rather than KC, PM and VW media. Similar findings were reported in *Malaxix khasiana* [25], *Cleisostoma racemifefum* [26], *Coelogyne suaveolens* [27] where MS medium was shown to be the most suitable medium over other nutrient media. In the present investigation, a successful attempt was made to compare the *in vitro* seed germination and their subsequent differentiation of *C. aloifolium* on four media *viz.* KC, MS, PM and VW. This protocol might be useful for selection of best condition for mass propagation and *ex situ* conservation of this valuable orchid species.

Table 1: Effects of different strength of medium and plant growth regulators on seed germination of *Cymbidium aloifolium* (L.) Sw.

Medium	Culture condition	Time taken in weeks					Remarks
		Initiation of germination	Development of protocorm	Differentiation of			
				1st leaf primodia	1st root primodia	Seedling	
KC	*	17	20	-	-	-	+
	**	12	16	21	28	35	+
	***	10	12	17	23	29	+
MS	*	12	15	-	-	-	+
	**	9	12	16	23	29	+
	***	6	8	12	18	24	++
PM	*	14	17	-	-	-	+
	**	10	12	17	24	31	+
	***	8	10	14	21	27	+
VW	*	15	19	-	-	-	+
	**	10	13	18	25	32	+
	***	9	11	15	21	27	+

*Half, **Full, ***PGRs (BAP 0.5mg/l + NAA 0.5mg/l), + = Germination favoured, ++ = Germination (Best)

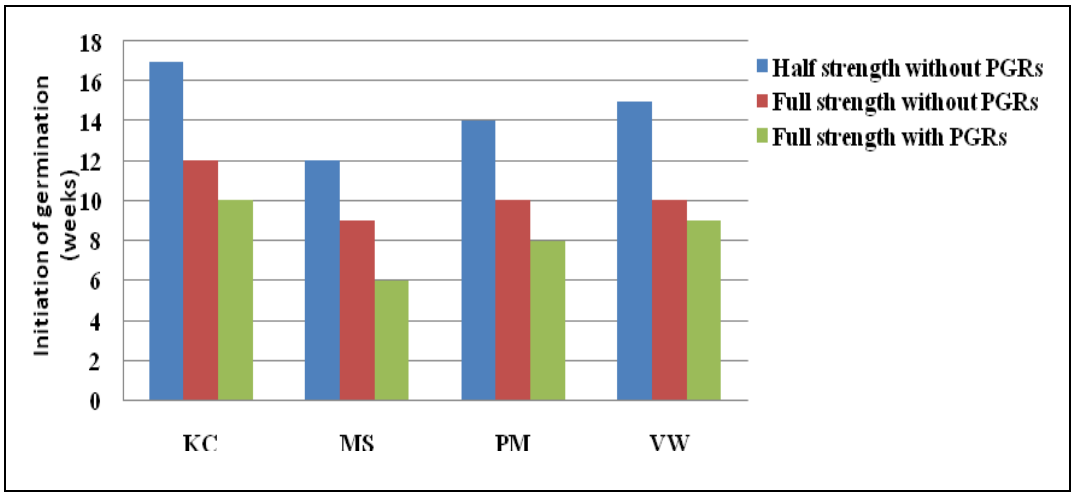


Fig 1: Initiation of germination pattern of *Cymbidium aloifolium* (L.) Sw.

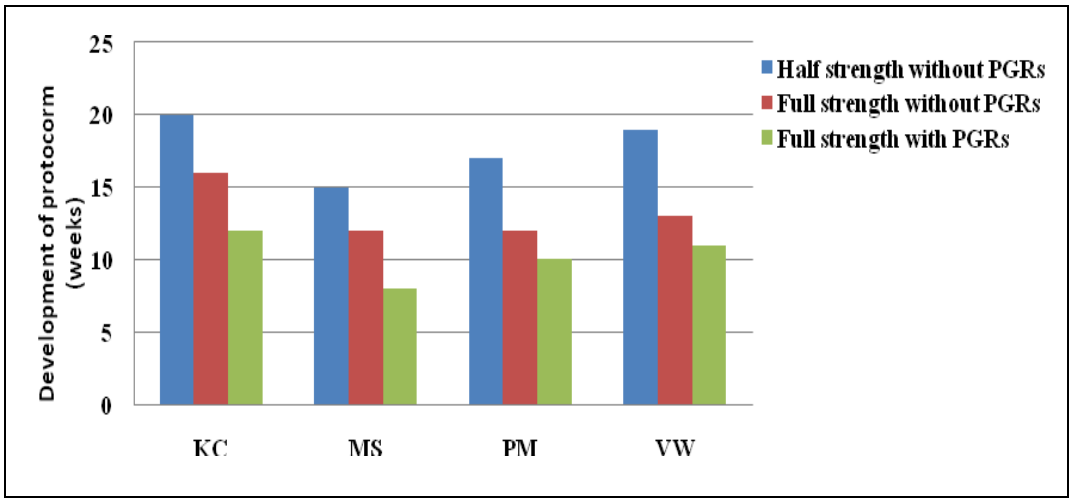
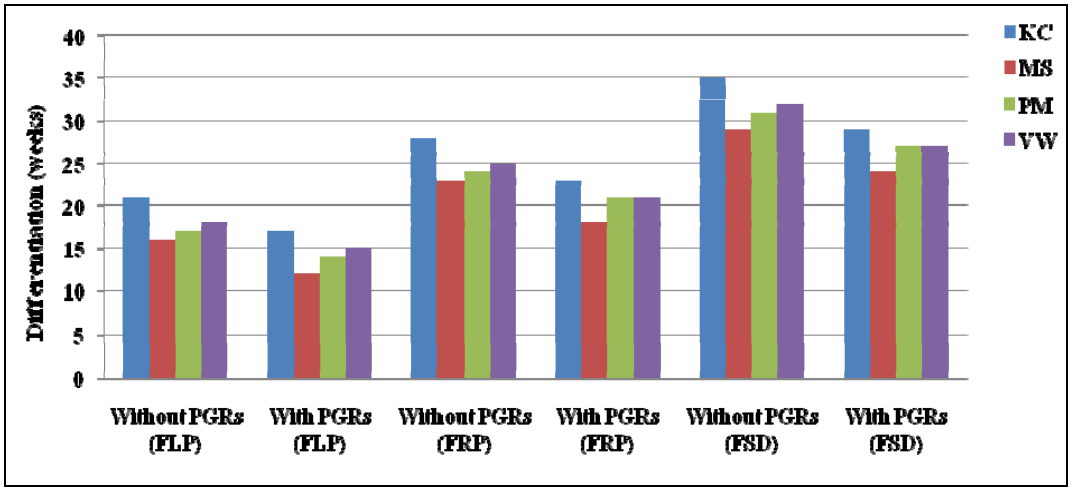


Fig 2: Development of protocorm pattern of *Cymbidium aloifolium* (L.) Sw.



FLP = First Leaf Primodia, FRP = First Root Primodia, FSD = First Seedling Development

Fig 3: Differentiation pattern of *Cymbidium aloifolium* (L.) Sw.

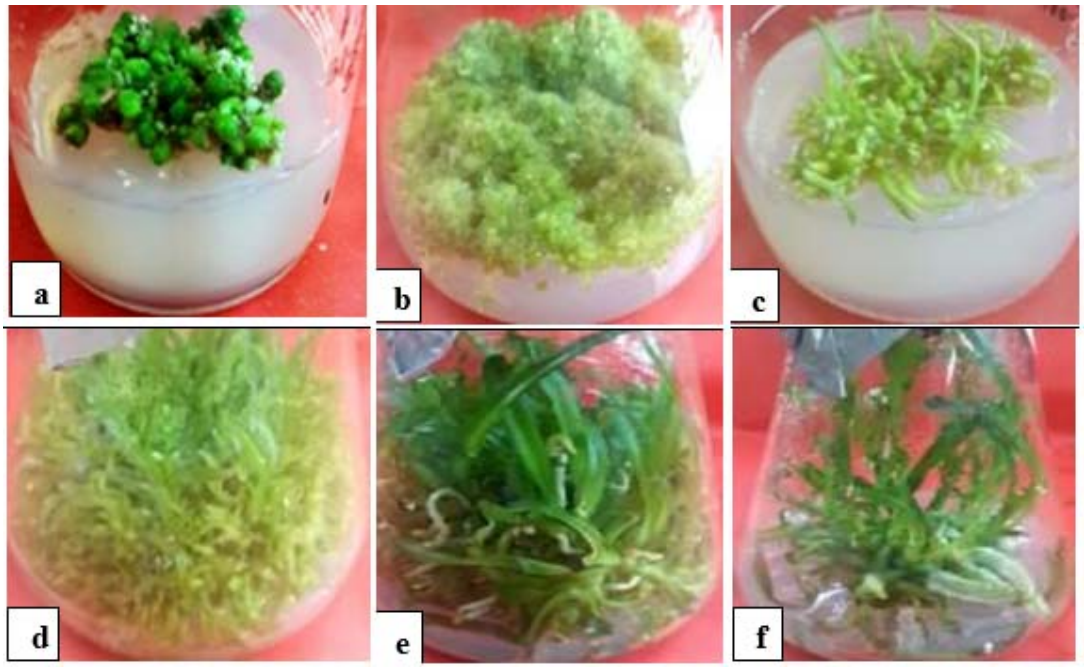


Fig 4: Different stages of *in vitro* seed germination and seedling development of *Cymbidium aloifolium*: a. Immature seeds turned into larger spherules on ½ KC medium; b. Immature seeds turned into small spherules on MS medium; c. PLB's with small shoot developed on full strength of VW medium; d. Development of small shoot on full strength PM medium; e. Development of small root on full strength MS medium; f. Multiple plantlets formed on MS + 0.5mg/l BAP+ 0.5mg/l NAA.

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