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A COMPARATIVE STUDY OF DIFFERENT NUTRIENT MEDIA ON THE *IN VITRO* ASYMBIOTIC SEED GERMINATION OF TWO THREATENED WILD ORCHIDS

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Abstract

The present study was conducted to develop a protocol for an efficient *in vitro* mass propagation of two floriculturally important and threatened wild orchids namely *Arundina graminifolia* and *Cymbidium aloifolium*. Seeds were cultured on six different nutrient media, full and half strength MS (Murashige and Skoog, 1962) both in semi solid and liquid form, VW (Vacin and Went, 1949) semi solid medium, and KC (Knudson, 1946) semi solid medium. Maximum germination (89.75%) and early protocorm development (83.00%; 26.80 days) of *C. aloifolium* were recorded in half-strength MS liquid medium. Maximum germination (89.87%) in *A. graminifolia* was noticed in half-strength MS semi-solid medium and early and maximum protocorm formation (86.25%; 23.83 days) was first observed in half-strength MS liquid medium.

Introduction

ORCHIDS BELONG to one of the largest families of flowering plants, the Orchidaceae. Their complex morphology and peculiar life cycle had been a field of marvel and scientific interests. The flowers of orchids being delicate, assorted with numerous rich colours and high vase life make them more valuable as ornamental plants. They possess one of the most saught after flowers in the trade by florists, for cosmetic, medicinal, and food purposes. However, most of the wild species are in danger of becoming extinct due to indiscriminate collections, over exploitation of their habitats, and human encroachment. This perilous position is due to their inherent nature that disables their seeds to germinate naturally without the association of suitable mycorrhizal fungus. Further, their natural populations are declining in nature due to their poor rate of regeneration capacity. Kaushik (1983) considered *Arundina graminifolia* (D.Don) Hochr. in list of threatened orchids along with some other orchids which also occur in Cambodia, Laos, and Vietman under *Endangered orchid*. Orchid species such as *Arundina graminifolia* (D.Don) Hochr. and *Cymbidium aloifolium* (L.) Sw. figure in the list of Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES, 2017), along with other orchid species.

A. graminifolia (D.Don) Hochr., a terrestrial orchid species with *Cattleya*-like flowers is amongst the largest and showiest of old world orchids. This tropical Asiatic genus is distributed from India, Nepal, Thailand, Malaysia, Singapore, and South China to Indonesia. Seed derived progenies are highly variable concerning the true-to-type colour of the flowers. Vegetative propagation through offshoots is cumbersome and time consuming. In addition, the rhizome of the species, which contains phenanthrene constituents (Liu *et al*., 2005) is important in several herbal drug preparations, and hence further such collections pose threats to the species. *Cymbidium aloifolium* (L.) Sw. is one of the highly valuable and threatened medicinal orchid of Nepal and NorthEast India. Kaushik (1983) included *Cymbidium aloifolium* (L.) Sw. under the chapter *Medicinal Value of the Orchid* in his monograph. The leaves of this species are extensively used for styptic properties in the treatments of boils and fevers. The roots are used to cure 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 (Das *et al*., 2008; Nongdam and Chongtham, 2011). Besides its medical importance, the species also attracts the floriculture market because of its long-lasting highly attractive beautiful flowers. Due to its various uses, plants are mercilessely uprooted from wild and hence its populations are getting threatened. *In vitro* asymbiotic seed germination technique provides a useful way to re-establish plants in the wild for germplasm preservation as well as for commercial propagation (Arditti *et al*., 1981; Bhowmik and Rahman, 2020; Gurudeva, 2019; Madhavi and Shankar, 2019; Mohanty and Salam, 2017; Pathak *et al.,* 2001; Thakur and Pathak, 2020).

The seeds of orchids are minute and contain undifferentiated embryos which lack enzymes to metabolize polysaccharides and lipids. As such, orchids need to form a symbiotic relationship with mycorrhizal

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fungus for their successful germination, in nature. Through the pioneer work of Knudson (1922), *in vitro* technique of asymbiotic seed germination and plantlet regeneration have been successfully used in a wide range of epiphytic and terrestrial orchid species (Arditti *et al*., 1981, 1982; Oliva and Arditti, 1984; Pathak *et al.,* 2001; Thakur and Pathak, 2020). Development of efficient protocols for orchid seed germination and the subsequent growth and development of protocorms into fully formed seedlings is a must for successful application of tissue culture techniques. Tissue culture and micropropagation techniques in various laboratories have also demonstrated that *in vitro* techniques allow production of a large number of plants in a given time which cannot be achieved by normal methods of propagation (Anuprabha and Pathak, 2019; Bhowmik and Rahman, 2020; Paek *et al.,* 1993; Sembi *et al*., 2020; Vasundhra *et al*., 2019). According to Martin and Madassery (2006), propagation through *in vitro* strategy enables rapid production of sufficient number of plants of clonal nature within a limited time and has much pertinence in horticultural as well as agricultural crops, especially in those plants, which show high variability and low conventional propagation rate.

Material and Methods

Explant Source

The capsules of *Arundina graminifolia* and *Cymbidium aloifolium* were collected from the forest of Aibawk village of Aizawl district, Mizoram. These were shifted to the tissue culture laboratory, Department of Horticulture, University of Agricultural Sciences, Bengaluru via air transport and stored in a clean glass jar and seeds procured from these were used for the present experiments.

Preparation of Explants

The healthy and disease free immature unripe capsules (pods) were selected as explants for the experiments. The immature capsules were first transferred to a sterile bottle containing 100-200 ml of distilled water with 1-2 drops of Tween 20. They were sterilised for 30 min with intermittent shaking. They were then washed 10 times with sterile distilled water so as to remove all the traces of tween 20 from the surface. They were sterilised with 0.5% Bavistin for half an hour followed by washing 10 times with sterile distilled water. After this, the immature capsules were transferred to sterile bottle containing freshly prepared 0.1% mercuric chloride and surface sterilized by soaking for 3-4 min with intermittent shaking. The solution was drained and the explants were washed 4-5 times with sterile distilled water to remove all the traces of sterilizing agent from the surface. The excess moisture remaining on the explant was removed with the help of sterile filter paper placed on the sterile petri dishes. Later, the capsules were quickly dipped in 70% alcohol and flamed for a few seconds.

Culture Media

A total of six media namely full and half strength MS (Murashige and Skoog, 1962) media both in semi solid and liquid form, VW (Vacin and Went, 1949) semi solid medium, and KC (Knudson, 1946) semi solid medium were used for the study. The stock solutions were prepared and stored in sterile bottles in refrigerator at 4°C. The glasswares were cleaned by scrubbing with

Table 1. Effect of different culture media on *in vitro* asymbiotic seed germination and protocorms formation in *Arundina graminifolia* and *Cymbidium aloifolium*.

Nutrient media	Per cent germination		Per cent protocorm formation	
	Cymbidium aloifolium	*Arundina graminifolia*	*Cymbidium aloifolium*	*Arundina graminifolia*
M1	64.62	73.75	53.87	67.12
M2	83.12	89.87	75.50	85.00
M3	66.50	81.37	59.87	72.00
M4	89.75	89.62	83.00	86.25
M5	0.87	53.87	0.37	41.75
M6	66.25	75.12	42.75	71.12
C.D.	3.59	3.54	5.72	3.12
SE(m)	1.25	1.23	1.99	1.09

Where, M1, MS medium-semi solid-Full strength; M2, MS medium-semi solid-Half strength; M3, MS medium-liquid-Full strength; M4, MS medium-liquid-Half strength; M5, KC semi solid medium; M6, VW semi solid medium.

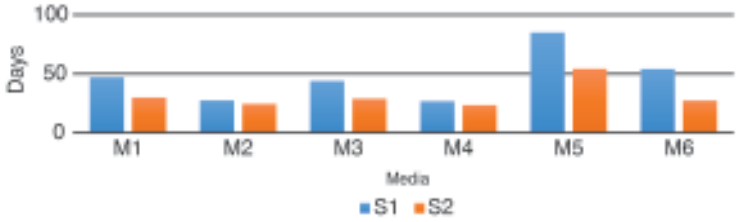

Fig. 1. Effect of different culture media on the number of days taken for formation of protocorms.

Where, M1, MS medium-semi solid-Full strength; M2, MS medium-semi solid-Half strength; M3, MS medium-liquid-Full strength; M4, MS medium-liquid-Half strength; M5, KC semi solid medium; M6, VW semi solid medium; DAC, Days after culturing; S1, *C. aloifolium*; S2, *A. graminifolia*.

liquid detergent and tap water. They were then rinsed with double distilled water and kept for autoclaving. Specific quantities of stock solution were drawn in required proportion using a measuring cylinder and mixed for different strengths of nutrient media. Sucrose (30 gl^{-1}) was dissolved with warm distilled water. The sucrose solution was added to the nutrient solution and the volume was made up to the required level. The pH was adjusted to 5.7 by addition of 1N HCl or 1N NaOH as required. After the pH has been adjusted, the nutrient medium is then heated. Liquid medium was devoid of agar, whereas, for solid medium, agar (6 gl^{-1}) was added. After bringing the nutrient media to a boil, it was dispensed to a culture bottle (30 ml per culture bottle). The bottles with media were properly labelled with sticky papers and then sterilised in the autoclave for 15 min at 121°C temperature and 15 psi pressure. Subsequently, it was allowed to solidify through cooling.

Inoculation

The inoculation was carried out under aseptic conditions inside a laminar airflow cabinet. The bottles, petri dishes, and instruments (blades, forceps *etc.*) required for inoculation were thoroughly washed, rinsed with double distilled water, covered airtight with autoclavable plastic covers, and were autoclaved at 121°C temperature for 45 min. The floor of the laminar air flow chamber were swabbed with 70% alcohol. Ultra-violet light was switched on for 15 min. The tools and hands were disinfected using 70% alcohol. Surface sterilized capsules were dried with sterilized blotting paper and were taken on a sterilized petri dish. They were further flame sterilised just before inoculation using spirit lamp inside the laminar airflow cabinet. The capsules were then incised longitudinally with a sharp, sterile surgical blade. Seeds, thus exposed were inoculated aseptically and carefully into culture bottles containing nutrient medium. A small and uniform quantity of inoculum had been used for initiation of germination.

Culture Conditions

The cultures were maintained at a regulated temperature of 25±2°C under 16 hrs photoperiod from cool white lights giving 2000 lux at culture level. Observations were made at regular intervals. Morphological observations of the seeds prior to and during germination were made.

Results and Discussion

Presently immature seeds from green capsules successfully responded to asymbiotic seed germination. *In vitro* production of seedlings from orchid seeds involved three sequential stages, namely, germination of seeds, formation of protocorms, and their subsequent development into seedlings. The first sign of germination of the cultured seeds was recorded as swelling of embryos after 2 wks of culture; protocorms were obtained after 3 wks.

The strength of the nutrient media played an important role in the seed germination and formation of protocorms (Table 1). The seeds of presently investigated species successfully germinated and subsequently developed into protocorms in all the nutrient media used except seeds of *Cymbidium*

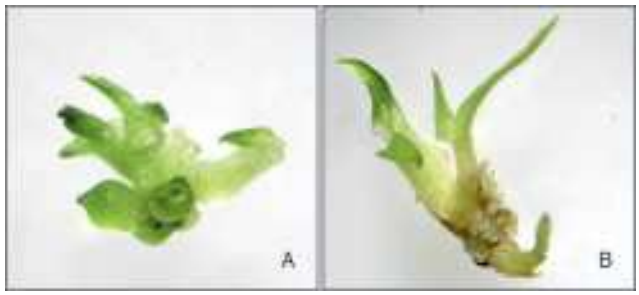

Fig. 2. A-B. Differentiation of leaf primordia and subsequent leaf formation at the upper part of the protocorm.

aloifolium which almost remained recalcitrant on KC semi solid medium. Amongst MS media both in semi solid and liquid form, the latter was observed to be more effective for germination and protocorm formation. The germinating entities on both liquid and semi solid

Fig. 3. A-B. *Arundina graminifolia* seeds cultures: A, The geminating entities on half strength MS liquid medium at 30 days after culturing (DAC); B, Cultures on half strength MS semi solid medium at 30 days after culturing (DAC).

formulations of full strength MS medium took longer as compared to half strength formulation. The half strength MS medium proved beneficial for inducing early germination and formation of protocorms (Fig. 1). High per cent seed germination and prolific protocorm formation was observed in half strength MS liquid and semi solid media in both *Cymbidium aloifolium* and *Arundina graminifolia*. The full strength MS liquid medium showed 81.37% and 66.5% seed germination while semi solid medium induced 73.75% and 64.62% germination in *A. graminifolia* and *C. aloifolium* respectively in 90 days. The half strength MS liquid medium showed 89.62% and 89.75% germination while 89.87% and 83.12% seeds germinated on semi solid medium in *A. graminifolia* and *C. aloifolium* respectively. The earliest formation of protocorms in *A. graminifolia* (23.5 days) and *C. aloifolium* (26.5 days) was observed in half strength MS liquid medium. The present investigation showed that half strength in comparison with full strength had augured well. Similar observations were also reported earlier by Naing *et al*. (2011) and Rani (2002). Further, it was observed that liquid culture medium proved to be better than semi solid medium for both the presently investigated orchid species. This is in agreement with the observations of Kanjilal *et al*. (1999) in *Dendrobium moschatum*, where liquid culture method proved to be highly efficient for cultures.

KC semi solid medium did not proved beneficial for inducing seed germination (0.87%) and protocorm formation (0.37%) in *C. aloifolium*. The seeds of *A. graminifolia* showed 53.87% germination response and 41.75% protocorm formation in KC semi solid medium. Germination was, however, delayed in *A. graminifolia* seeds. The low seed germination response of *C. aloifolium* on KC semi solid medium may be attributed to a high nitrate concentration and the inability of the protocorms to utilize nitrates during early growth and development as described by Raghavan and Torrey (1964) in *Cattleya*. VW semi solid medium was found to induce moderate germination response and protocorm formation in both the presently studied species. The germination response (75.12% and 66.25%) was recorded on VW semi solid medium for *A. graminifolia* and *C. aloifolium* respectively. In KC and VW semi solid media, formation of protocorms in *C. aloifolium* was observed in 85 days and 54.12 days respectively (Fig. 1). The difference in germination percentage may also be due to difference in the composition of nutrient medium in terms of the chemical constituents. These two media differ in the additional presence of $Ca_3(PO_4)_2$ (200 mgl^{-1}) and KNO_3 (525 mgl^{-1}) in modified Vacin and Went medium and $Ca(NO_3)_2$ (1000 mgl^{-1}) in modified Knudson C medium (Kanjilal *et al*., 1999; Mitra *et al*., 1976).

A perusal of data presented in Table 1 reveals a differential response of the two orchid species, namely, *A. graminifolia* and *C. aloifolium* cultured in different media regarding seed germination and formation of protocorms. At the initial stage of protocorm development, leaf primordium appeared at the upper part of the protocorm (Fig. 2). As the protocorm grows further, an opening developed at the closed ridge, first leaf primordium comes out and complete seedling was produced (Batygina *et al*., 2003). Considering the previous reports on the effectiveness of various basal nutrient media for asymbiotic seed culture of orchids, MS medium has been the universally accepted for the culturing of plant species. The strength of media has been an interest of study amongst scientists and asymbiotic germination response in some of the orchid species has been successfully tested on half strength MS medium. Of the six nutrient media tested during the present study, 90% germination was achieved on half strength MS medium both in liquid and semi solid form after 90 days of culture initiation (Fig. 3). Various changes associated with the development of protocorms and development of seedlings during seed culture has been studied to a certain degree in the two orchid species. Morphogenetic changes were similar in *A. graminifolia* and *C. aloifolium* where the seeds after germination produced protocorms which directly differentiated into seedlings.

Amongst the two species studied, *A. graminifolia* gave the earliest response (23.5 days after initiation of culture) in half strength MS liquid medium. In comparison, the seed culture took more than 85 days for formation of protocorms in *C. aloifolium* on KC semi solid medium. In the present study, though the response for protocorms initiation was delayed, it was possible to establish formation of protocorms with prolonged culture. As observed in seed germination, the liquid media showed outstanding response in the formation of protocorms. This is in accordance with the observations made by Pradhan *et al*. (2014) on *C. aloifolium.* The protocorms cultured on both full and half strength MS liquid media showed large sized healthy protocorms, which further differentiated faster as compared to other media. This could be due to the fact that the liquid media covers the surface of the explant like a thin film aiding in better absorption of the nutrients.

Conclusion

The results of the present study indicated that successful asymbiotic seed germination was achieved *in vitro* without the aid of symbiotic fungus using a suitable nutrient medium within a short period of time. Such studies may be extended to some other related orchid taxa as well.

References

Anuprabha and Promila Pathak. 2019. *In vitro* asymbiotic seed germination and seedling development in *Coelogyne fimbriata* Lindl. *J. Orchid Soc. India,* **33**: 83-89.

Arditti, J., J. D. Michaud, and A. P. Oliva. 1981. Seed germination of North American orchids. I. native California and related species of *Calypso, Epipactis, Goodyera, Piperia,* and *Platanthera*. *Bot. Gaz.,* **142**: 442-53.

Batygina, T. B., E. A. Bragina, and V. E. Vasilyeva. 2003. The reproductive system and germination in orchids. *Acta Biol. Cracov. Ser. Bot.,* **45**: 21-34.

Bhowmik, Tapash Kumar and Md. Mahbubur Rahman. 2020. *In vitro* seed germination and micropropagation of *Dendrobium chrysotoxum* Lindl. (Golden Bow): A highly fragrant orchid species of Bangladesh. *J. Orchid Soc. India,* **34**: 69-77.

CITES. 2017. *Convention on International Trade in Endangered Species of Wild Fauna and Flora, Appendices I and II.* http://www.cites.org/app/appendices.php.

Das, P. K., S. Sahoo, and S. Bal. 2008. Ethnobotanical studies on orchids of Niyamgiri Hill Ranges, Orissa, India. *Ethnobot. Leaflets,* **12**: 70-78.

Gurudeva, M. R. 2019. *In vitro* seed germination and developmental morphology of seedlings in *Dendrobium ovatum* (L.) Kraenzl. *J. Orchid Soc. India*, **33**: 31-41.

Kanjilal, B., D. D. E. Sarker, J. Mitra, and K. B. Datta. 1999. Stem disc culture: Development of a rapid mass propagation method for *Dendrobium moschatum* (Buch-Ham) Swartz., an endangered orchid. *Curr. Sci*., **77**(4): 497-500.

Kaushik, P. 1983. *Ecological and Anatomical Marvels of the Himalayan Orchids*. Today and Tomorrow's Printers & Publishers, New Delhi, India.

Knudson, L. 1922. Nonsymbiotic germination of orchid seed. *Bot. Gaz.,* **73**: 1-25.

Knudson, L. 1946. A new nutrient solution for germination of orchid seed. *Am. Orchid Soc. Bull*., **15**: 214-17.

Liu, M. F., Y. Ding, and D. M. Zhang. 2005. Phenanthrene constituents from rhizome of *Arundina graminifolia*. *Zhongguo Zhongyao Zazhi*, **30**: 353-56.

Madhavi, M. and P. C. Shankar. 2019. Effects of different growth additives on seed germination of *Vanda tessellata* (Roxb.) Hook. ex G. Don- A medicinal orchid. *J. Orchid Soc. India,* **33**: 105-12.

Martin, K. P. and J. Madassery. 2006. Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. *Sci. Hortic*., **108**: 95-99.

Mitra, G. C., R. N. Prasad, and R. A. Choudhury. 1976. Inorganic salts and differentiation of protocorms in seed callus of orchid correlative changes in its free amino acid content. *Indian J. Exp. Biol.,* **14**: 350-51.

Mohanty, C. R. and P. Salam. 2017. *In vitro* seed culture studies in *Dendrobium* orchid *cv.* Banyat Pink. *J. Orchid Soc. India*, **31**: 93-96.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with Tobacco tissue culture. *Physiol. Plant*, **15**: 472-97.

Naing, A. H., J. D. Chung, and K. B. Lim. 2011. Plant regeneration through indirect somatic embryogenesis in *Coelogyne cristata* orchid. *Am. J. Plant Sci*., **2**(2): 262.

Nongdam, P. and N. Chongtham. 2011. *In vitro* rapid propagation of *Cymbidium aloifolium* (L.) Sw.: A medicinally important orchid *via* seed culture. *J. Biol. Sci*., **11**(3): 254-60.

Oliva, Allison P. and J. Arditti. 1984. Seed germination of North American Orchids. II. Native California and Related Species of *Aplectrum*, *Cypripedium*, and *Spiranthes*. *Bot. Gaz*., **145**: 495-501.

Paek, K. Y., J. D. Park, and G. B. Shim. 1993. Growth response of several terrestrial orchids to Fe-EDTA level *in vitro*. *Korean J. Hortic. Sci. Technol.,* **34**(4): 308-14.

Pathak, Promila, K. C. Mahant, and A. Gupta. 2001. *In vitro* propagation as an aid to conservation and commercialization of Indian orchids: seed culture. *In: Orchids: Science and Commerce* (eds. Promila Pathak, R. N. Sehgal, N. Shekhar, M. Sharma, and A. Sood) pp. 319-62. Bishen Singh Mahendra Pal Singh, Dehradun, India.

Pradhan, S., B. Tiruwa, B. R. Subedee, and B. Pant. 2014. *In vitro* germination and propagation of a threatened medicinal orchid, *Cymbidium aloifolium* (L.) Sw. through artificial seeds. *Asian Pac. J. Trop. Biomed*., **4**(12): 971-76.

Raghavan, V. and J. G. Torrey. 1964. Inorganic nitrogen nutrition of the seedling of the orchid, *Cattleya*. *Am. J. Bot.,* **51**: 264-74.

Rani, L. C. 2002. *Intra and Interspecific Hybridization in Dendrobium spp*. Ph.D. Thesis, Kerala Agricultural University, Thrissur, Kerala, India.

Sembi, Jaspreet K., Promila Pathak, and Jagdeep Verma. 2020. Regeneration competence of leaf explants in *Cymbidium eburneum* Lindl. (Orchidaceae). *J. Orchid Soc. India,* **34**: 17-21.

Thakur, Babita and Promila Pathak. 2020. *In vitro* propagation of *Herminium lanceum* (Thunb. ex Sw.) Vuijk (Orchidaceae), through asymbiotic seed germination: A therapeutically important and endangered orchid from NorthWestern Himalayas. *J. Orchid Soc. India,* **34**: 61-67.

Vacin, E. F. and F. W. Went. 1949. Some pH changes in nutrient solutions. *Bot. Gaz*., **110**: 605-13

Vasundhra, Promila Pathak, and Ankush Prakash. 2019. *In vitro* shoot induction and regeneration potential of floral buds in *Crepidium acuminatum* (D.Don) Szlach., A medicinal ayurvedic plant from NorthWestern Himalayas. *J. Orchid Soc. India*, **33**: 43-48.