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# *IN VITRO* PROPAGATION OF *HERMINIUM LANCEUM* (THUNB. EX SW.) VUIJK (ORCHIDACEAE), THROUGH ASYMBIOTIC SEED GERMINATION: A THERAPEUTICALLY IMPORTANT AND ENDANGERED ORCHID FROM NORTHWESTERN HIMALAYAS

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**Abstract**

An efficient and reproducible protocol for asymbiotic seed germination of an endangered and therapeutically important terrestrial orchid, *Herminium lanceum*, commonly known as *Jalya*, has been established. Its leaves and stem are mainly used for curing fever, diabetes, and urinal disorders. With a view to enlarging its population base, an attempt was presently made to culture its mature seeds procured from dehisced capsules on modified Knudson C (KC, 1946) medium fortified with different concentrations of auxins (IAA, IBA, NAA; 1 $mgl^{-1}$ each) and cytokinins (KN, BAP; 1$mgl^{-1}$ each) in different combinations. The aim of present investigation was to study the effect of different combinations of growth regulators on seed germination and their subsequent development into seedlings. Seeds showed initial signs of germination within 29±0.88 days of inoculation. The first visible sign of germination was the swelling of embryos followed by apical rupturing of seed coat so as to release the spherules. Within 95±3.05 days of inoculation, the spherules developed into oval, green protocorms with absorbing hair. The complete seedlings were obtained within 149±1.15 days of inoculation. The highest germination percentage (83.02%) and early development of healthy seedlings were observed on medium augmented with combination of IBA and KN.

## Introduction

THE ORCHIDS belong to the family Orchidaceae which is an assemblage of superlatives having the most varied, utmost beautiful, and highly exotic flowers; there are reports of 28,237 accepted species spanning 736 genera (Christenhusz and Byng, 2016; Willis, 2017). In India, orchids are represented by 1256 species which belong to 155 genera (Singh *et al*., 2019). The orchids stand distinct from other flowering plants in having spectacular array of adaptations that are linked to several innovative features including zygomorphic flowers with well-developed gynostegium, elaborated perianth and resupinated ovary, presence of pollinia, specialized pollination mechanisms, production of microscopic and non-endospermic seeds with undifferentiated embryos, symbiotic association with mycorrhizal fungi, colonization of epiphytic habitats, velamenous roots, and crassulacean acid metabolism (Givnish *et al*., 2015; Kaushik, 2019; Manoharachary, 2019; Pal *et al.,* 2019; Pathak *et al.,* 2001; Silvera *et al*., 2009; Vij, 1995). Though most orchids have a long juvenile period, slow growth rate, and low photosynthetic capacity, yet they are of great value in ornamental, medical, conservation, and evolutionary research (Janakiram and Baskaran, 2018; Kumar *et al.,* 2018; Pathak *et al.,* 2010; Prakash and Pathak, 2019; Prakash *et al.,* 2018; Zhang *et al.,* 2018). Their survival in nature is ensured by a complex symbiotic association with soil mycorrhizal fungi, which are of particular importance during seed germination and early plant development (Manoharachary, 2019). Orchidaceae is one of the most threatened of any plant families due to over exploitation of orchids from the wild, habitat destruction, large scale deforestation, and more recently the threat of climate change (Backhouse, 2007; Barua *et al.,* 2019; Bhandari *et al.,* 2018; Devi *et al.,* 2018; Kumar *et al.,* 2017; Ninawe and Sapna, 2017; Prakash and Pathak, 2019; Seaton *et al*., 2010; Sharma *et al.,* 2017; Singh *et al.,* 2019). The entire family has been placed in the Appendix-II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), while a number of selected species have been placed in its Appendix-I and are totally banned for any international trade (CITES, 2017). The technique of asymbiotically culture orchid seeds *in vitro,* serves both functions to increase the availability of the plants to meet the increasing demand, and to serve as reservoirs for restocking the wild populations which are declining due to habitat destruction, fragmentation, and over harvesting (Anderson, 1990; Anuprabha *et al.,* 2017; Arora *et al.,* 2016; Bhatti *et al.,* 2017; Kaur *et al.,* 2017; Pathak *et al.,* 2017; Sibin and Gangaprasad, 2016; Singh *et al.,* 2006).

The genus *Herminium* L. was established in 1758 by Linnaeus in his *Opera Veria*. The genus is represented by 49 species, predominantly terrestrial in the subtribe Orchidinae and is widely distributed in Europe through most of continental Asia to Japan, the Malay

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Archipelago and New Guinea (Raskoti *et al*., 2017; Yonzone *et al*., 2012). *Herminium lanceum* (Thunb. ex Sw.) Vuijk, commonly known as *Jalya*, *Kath Jhakri* (Gairola *et al*., 2010; Malla *et al*., 2015), is characterized by having a globose or ellipsoid tuber, linear lanceolate leaves, and small greenish-white flowers. The orchid is found commonly in shaded, wet forests and rock shelters in montane, and alpine Himalayas within an altitudinal range of 1100-4200 m amsl. Flowering and fruiting takes place during the months of July and October. The whole plant is harvested in summer or autumn and used to treat diabetes, cold, fever, rheumatism, hernia, sores, eczema, snake bites, and reduce swelling and pain. The extract of the plant is also given to cure suppressed urination (Joshi *et al.,* 2009; Malla *et al.,* 2015; Pant, 2013). Consequently, its natural populations are succumbing to commercial collection pressures. The situation is further compounded by the destruction of its natural habitats due to rapid urbanization, deforestation, and increase of agricultural land. Since the species has become endangered in nature, conservation measures need to be taken up to work out suitable strategies for its propagation and to protect its survival in natural habitats. The aim of present work was to study the asymbiotic germination potential of its mature seeds procured from dehisced capsules with a view to developing an efficient mass propagation protocol for its conservation.

## Material and Methods

*Experimental Site*

During the present investigation, experiments were carried out in the Orchid laboratory, Department of Botany, Panjab University, Chandigarh.

*Sample Collection*

Mature seeds from dehisced capsules ('pods') of *Herminium lanceum* (Fig. 1A) were collected from Tara Devi Hills (Shimla, Himachal Pradesh), during last wk of August, 2017.

*Seed Viability Test*

Seed viability was determined by the ability to reduce 2,3,5-triphenyltetrazolium chloride (TTC) to the red coloured formazon (Brewer, 1949). In this experiment, the orchid embryo either turned red or stayed colourless after TTC staining. Therefore, seeds with TTC reduction ability (red coloured) were scored as viable.

*Culture Media and Culture Conditions*

The seeds were collected on sterilized filter paper and surface sterilized with 4% Sodium Hypochlorite solution with Teepol as the wetting agent for 10-15 min and then, thoroughly and repeatedly rinsed with autoclaved distilled water under a sterile laminar hood. Sterilized seeds were sown on modified Knudson C (KC, 1946) medium with 20 $gl^{-1}$ sucrose and 8 $gl^{-1}$ agar in test tubes, each containing 25 ml of medium. The pH of nutrient medium was adjusted to 5.6 prior to autoclaving at 121°C with 15 psi pressure for 20 min. The growth regulators [auxins (IAA, IBA, NAA) and cytokinins (KN, BAP)] in various combinations and concentrations were also used (Table 1). The cultures were maintained under a 12 hr photoperiod of 3500 lux light intensity and a temperature of 25±2°C, and observed regularly. The problem of phenolic exudates was overcome by frequent subculturing on fresh nutrient medium.

*Acclimatization*

Healthy plantlets with 2-3 well grown leaves and 1-2 roots were gradually hardened *in vitro,* by sequential elimination of growth additives, vitamins, sucrose, and minor salts from the nutrient medium at 15 days interval. The well rooted plantlets were taken out from culture vessel and thoroughly washed under running tap water for removal of agar attached to root surface and transferred to pots containing a potting mixture of sand, vermiculite, and bark in 1:1:1 ratio.

*Statistical Analysis*

The experiments were designed following complete randomize block design (CRD). The statistical analysis to determine the effects of auxin and cytokinin concentrations on germination percentage and seedlings development, experiments were performed using factorial analysis, with significant difference being accepted at the $p<0.05$ level. All the experimental manipulations were carried out under aseptic conditions and for each experiment, at least four replicates were used. The data was analyzed statistically using one-way analysis of variance (SPSS, 16.0 version), and the data means±standard error of the experiments were compared using Tukey's test.

## Results and Discussion

Orchids produce seeds in large quantity with tiny globular embryos with no functional endosperm. For the seed sample evaluated presently, the seed viability was determined by TTC (2,3,5-triphenyl tetrazolium chloride) test as the seeds have their ability to reduce TTC to red coloured formazon (Fig. 1B). Seeds were found to be viable, as determined by positive formazon staining, and morphologically most seeds appeared normal with viable embryos. Plant growth regulators

(PGRs) play important role in all aspects of plant growth and development. Auxins and cytokinins are the most frequently used growth regulators in the nutrient medium to initiate, increase, and regulate mitotic activities during germination of seeds (Emery and Atkin, 2006; Novak *et al.,* 2014). The percentage of seed germination in *Herminium lanceum* varied with different concentrations and combinations of auxins and cytokinins. The first sign of seed germination *i.e.*, swelling of embryo started after 29±0.88 days of inoculation (Fig. 1C). Swelling of embryos was followed by apical rupturing of seed coats (spherule stage; Fig. 1D). The protocorms formed after rupturing of seed coats are considered as unique structures with the primary goal to form shoot apical meristems. Cells at the apical end of protocorms divide rapidly, while those at the basal end enlarge and increase their ploidy level during asymbiotic germination (Chen *et al*., 2009). The early events in protocorm development are likely regulated by the pre-programmed long-lived mRNAs formed during seed maturation as indirectly demonstrated by Raghavan and Goh (1994). The

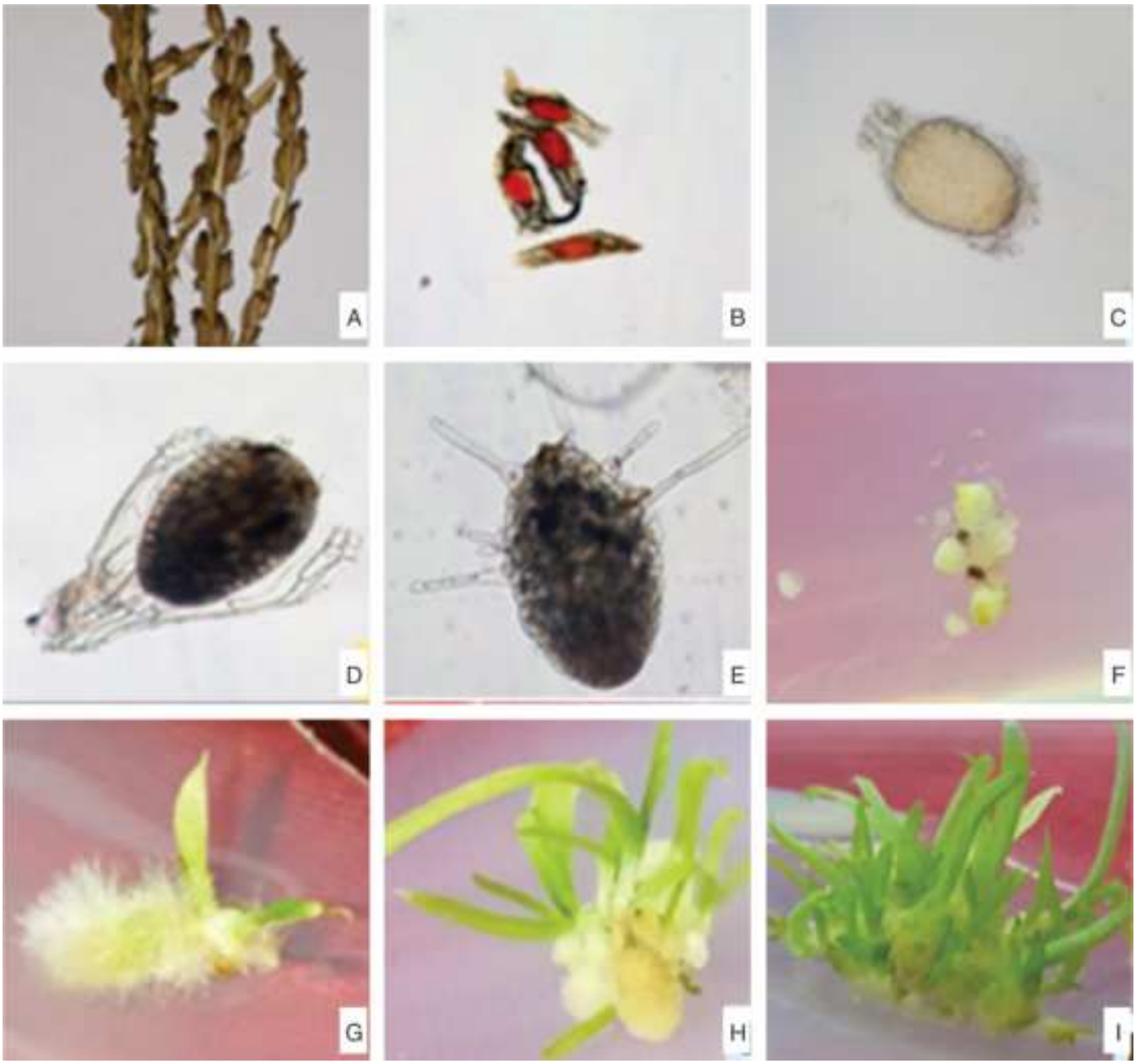


Fig. 1. A-I. *In vitro* asymbiotic seed germination and seedling development in *Herminium lanceum* on modified Knudson medium (KC): A, Part of the inflorescence with capsules; B, Seed viability testing by TTC method; C, Swelling of embryo; D-E, Spherules emerging out by rupturing of seed coats; F, Protocorm multiplication; G, Profuse growth of absorbing hair at the base of seedling; H, Tuberous leafy shoots; I, Luxuriant shoot growth.

Table 1. *In vitro* asymbiotic seed germination and seedling development in *Herminium lanceum* on modified Knudson medium (KC, 1946).

| Growth additives | Germination frequency (%) | Time taken in days for | | | | | | Remarks |
|---|---|---|---|---|---|---|---|---|
| | | Onset of germination | Spherule | Protocorms | 1st leaf primordium | 1st Root primordium | Seedlings | |
| Control | 82.88% | 32.66±0.88$^{ab}$ | 54.66±1.20$^{b}$ | 105.00±3.78$^{b}$ | 127.00±3.48$^{b}$ | 143.66±2.72$^{b}$ | 160.00±1.76$^{b}$ | Healthy seedlings |
| $IAA_1 + KN_1$ | 75% | 37.00±1.52$^{bc}$ | 59.33±0.88$^{c}$ | 120.00±2.08$^{d}$ | 141.00±0.88$^{c}$ | 148.66±1.20$^{b}$ | 163.66±1.20$^{b}$ | Healthy seedlings |
| $IBA_1 + KN_1$ | 83.02% | 29.00±0.88$^{a}$ | 37.33±0.88$^{a}$ | 95.00±3.05$^{b}$ | 112.00±1.45$^{a}$ | 123.00±1.15$^{a}$ | 149.00±1.15$^{a}$ | Protocorm multiplication; early seedling development |
| $NAA_1 + BAP_1$ | 38.35% | 41.00±0.88$^{c}$ | 0±0 | 0±0 | 0±0 | 0±0 | 0±0 | No further growth after swelling of embryos |
| $IAA_1 + BAP_1$ | 62.94% | 41.00±1.15$^{c}$ | 58.33±1.20$^{bc}$ | 130.66±2.02$^{a}$ | 146.00±2.96$^{c}$ | 161.00±1.85$^{c}$ | 171.00±1.55$^{c}$ | Healthy seedlings |
| $IBA_1 + BAP_1$ | 69.32% | 38.00±1.73$^{bc}$ | 61.00±1.15$^{c}$ | 124.66±2.33$^{a}$ | 138.00±2.96$^{c}$ | 158.00±1.45$^{c}$ | 178.00±1.85$^{c}$ | Healthy seedlings |

Entries in column numbers 3 to 8 are Mean±S.E.; same alphabetical letter in the superscript denotes that the corresponding means are in the same group using Tukey test at 5%.

protocorms became oval, green in colour with presence of marked absorbing hair (Fig. 1E-F) and in most of protocorms, shoot primordia developed within 112±1.45 days of inoculation. Observations as made during the present study have also been reported earlier by some workers (Decruse and Gangaprasad, 2018; Devi *et al.,* 2006; Pant *et al.,* 2008; Pathak, 1989; Pathak *et al.,* 1992) who reported that cytokinin alone or in combination with auxin, stimulated germination in orchid seeds. Species specific media for seed germination have been reported in orchids (cf. Arditti and Ernst, 1984; Pathak *et al.,* 2001). Establishment of shoot apical meristem indicates end of protocorm stage and the beginning of seedling stage (Fig. 1G-H). The complete seedlings were obtained after 149±1.15 days of inoculation (Fig. 1I). Maximum seed germination (83.02%) and early seedling development (123±1.15 days) was observed on KC medium augmented with IBA (1 $mgl^{-1}$) in combination with KN (1 $mgl^{-1}$). This treatment was followed by control and IAA (1 $mgl^{-1}$) in combination with KN (1 $mgl^{-1}$). IBA (1 $mgl^{-1}$) when used in combination with BAP (1 $mgl^{-1}$) induced germination in 38±1.73 days and plantlets were developed within 178±1.85 days. On the contrary, when the KC medium was supplemented with NAA and BAP (1 $mgl^{-1}$ each) in combination, the germinating entities failed to grow further (Table 1). The above results indicated that the difficulty in germinating *Herminium lanceum* seeds can be overcome partly by treatment of PGRs. The limited effect of some of combinations of hormones suggested the involvement of complex mechanism in seed germination and existence of great heterogeneity in seeds. Although orchid embryos are minute in size, these have been carefully used in developmental programmes ensuring successful asymbiotic seed germination. Some protocols are available which will aid to maintain and propagate important orchid species for further conservation purposes (Anuprabha and Pathak, 2019; Buyun *et al.,* 2004; Chen *et al.,* 2015; Gurudeva, 2019; Hernandez *et al.,* 2005; Kim *et al.,* 2019; Lekshmi and Decruse, 2018; Lin *et al.,* 2020; Madhavi and Shankar, 2019; Mohanty and Salam, 2017; Santos *et al.,* 2016; Vasudevan and Van Staden, 2010; Vasundhra *et al.,* 2019; Yamazaki and Miyoshi, 2006).

During the present investigation, *in vitro* raised seedlings were gradually hardened by sequentially removing the growth additives, vitamins, sucrose, and minor salts from the nutrient medium at regular intervals. After hardening *in vitro*, these plantlets were gently removed from the culture vessels with the help of a long forceps, washed thoroughly with lukewarm water in order to remove agar. The plants were then potted in 1:1:1 mix of sand, vermiculite, and bark pieces. The older the seedlings, the broader the tolerance range for various atmospheric conditions. The plantlets, thus raised have been reintroduced into the natural habitats and showed 60% survival rate.

## Conclusion

Orchids hold an unexplored reserve of various bioactive molecules which might prove beneficial to cure various disorders like Alzheimer's, asthma, diabetes, eczema, gastritis, gonorrhea, HIV/AIDS *etc.* (Bhattacharya and Staden, 2016; Malla *et al.*, 2015). There is not much work done on seed germination and micropropagation

of *Herminium lanceum* (Singh and Babbar, 2016; Verma, 2016) which is presently categorized as Endangered by IUCN. An efficient and reproducible protocol for asymbiotic seed germination of an endangered and therapeutically important terrestrial orchid, has been successfully established in *H. lanceum*. The present findings underline the stimulatory role played by combination of auxins and cytokinins in germination of its non-endospermic seeds on nutrient medium. The study also exhibits profuse multiplication of protocorms when the medium augmented with IBA in combination with KN (1 $mgl^{-1}$ each) which supports that *in vitro* propagation technique may help in mass propagation and conservation of this endangered species. A broad strategic approach which integrates biochemical analysis, metabolomics along with *in vitro* propagation will enable the scientific community to realize the tremendous medicinal potential within *H. lanceum* and will open new frontiers for the promulgation of sustainable conservation strategies for this therapeutically important orchid from NorthWestern Himalayas.

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