International Journal of Pharmaceutical Science and Research
www.pharmacyjournal.net
ISSN: 2455-4685
Received: 20-01-2023, Accepted: 05-02-2023, Published: 22-02-2023
Volume 8, Issue 1, 2023, Page No. 5-12

An effective *ex situ* regeneration of critically endangered medicinal orchid *Dactylorhiza hatagirea* (D. Don) Soő

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Abstract
Orchids are unique and highly evolved group of flowering plants with a remarkable diversity and exotic beauty. They are rich in alkaloids and other phytochemicals thereby are used in indigenous medicines to cure a variety of ailments. *Dactylorhiza hatagirea* (D. Don) Soő tubers are used to prepare 'salep' a local drug having aphrodisiac properties. It is a medicinally important orchid with threatened status and used as cut flower in many countries including India, so in the present investigation micropopagation techniques have been employed. The technique of asymbiotic seed germination has added new domains to orchid propagation for cultivation and conservation. The *ex-situ* conservation strategies involved propagation using different explants like inflorescence, petioles, apical tips, stems, leaves, tubers and seeds. Green and mature pods containing seeds germinated on medium supplemented with cytokinin and auxin leading to seedling regeneration via PLB's regeneration. In another treatment auxins particularly IBA have been found to induce germination and plantlet regeneration in tuber sections. Therefore an efficient method of propagation was developed so that this critically endangered medicinal orchid can be easily propagated to achieve its mass multiplication.

Keywords: immature seed, mass multiplication, mature seeds, micropopagation, PLB's, tubers

Introduction

The use of orchids in herbal medicines has a very long history. There is not a single ailment for which, therapeutic and curative elements are not found in one or the other orchid species. Orchids hold great repute as medicine with strengthening and demulcent qualities in Ayurveda. Orchids are grown as cash crop in several countries including Thailand, Malaysia, Singapore, South Korea and Sri Lanka [1]. Our research team conducted surveys in the north, south and central Kashmir Himalaya harbouring a rich diversity of valuable orchids in order to enumerate the species number and to develop the medicinal plants sector. It was found that majority of the species are terrestrial growing in sub-alpines. These inhabit three types of habitats *viz*: marshy lands, shady coniferous forests of sub-alpines and open moist land of alpines. The maximum representation of orchid species occurs between altitudinal gradient of 2000-3000m asl because the zone provides suitable condition for the growth and development of the orchids [2]. Some orchids prefer the places with high moisture, shade and humus rich soils [3]. All the orchids of temperate region are highly puzzling and peculiar. Our earlier studies regarding the conservation of temperate terrestrial orchids include Padder *et al.* 2013, 2015 [4, 5] are worth mentioning for those studying medicinal orchids along the globe. *Dactylorhiza hatagirea* (D. Don) Soő *syn. Orchis latifolia* var. Indica (Lindl.) plants are perennial short-lived, terrestrial, autotrophic herbs with tubers or rootstocks containing mycorrhizal fungi in roots. It is the principal cut flower among the temperate orchids of Jammu and Kashmir including cold desert Ladakh and is marketed both as potted plant and as cut flower. It has been identified as critically endangered (CAMP status), critically rare (IUCN status) and is listed under Appendix II of CITES (according to which its trade is banned internationally) [6, 7, 8]. It has aphrodisiacal properties due to which the species has great demand in national and international markets [9, 10]. The species is used in various system of Indian (Ayurveda, Siddha, Unani) and traditional medicines to cure dysentery, diarrhoea, chronic fever, cough, stomachache, wounds, cuts, burns, fractures, general weakness particularly in debilitated women after delivery and for increasing regenerative fluid [11, 12]. The fleshy tubers are rich in starch, mucilage, sugar, phosphate, chloride and glucoside- loroglossin [11]. Seeds germinate very poorly in nature because they require an appropriate fungus for the purpose.

Conservation and *in vitro* propagation of medicinal as well as ornamental orchid species is one of the biggest challenges as the demand for them is increasing day by day. Large scale production of medicinal plants has been achieved successfully using tissue culture techniques. Several workers have used various explants, culture media and introduced tissue culture methods for regeneration of orchids. Propagation of orchids through the production of protocorms (PLB's) has been achieved in a number of orchids namely; *Oncidium* sps. [13], *Cypripedium candidum* [14], *Cypripedium acaule* [15], *Satyrium nepalense* [16]. Seed germination of temperate terrestrial orchids is difficult [17-20]. Information relating to the factors that complicate seed germination of these species remains fragmentary and obscure. In most orchids, higher frequencies of germination have been achieved by culturing immature seeds than by culturing mature seeds [21-25, 18, 26]. Similarly Padder *et al.* [27] has reported immature seed germination leading to protocorm formation in *Cephalanthera longifolia* L. Fritsch species on MS (1962) medium in supplementation of AC. It was confirmed that dormancy is induced by some undefined changes during seed development and maturation, accumulation of some inhibitory substances such as phenolics in *Cymbidium goeringii* [28] and Abscisic acid in *Dactylorhiza maculata* and *Epipactis helleborine* (L.)

Crantz [29], induction of a physiologically dormant state in embryos [22] or by increasing impermeability of the embryos during seed maturation [30]. *Dactylorhiza hatagirea* (D. Don) Soő is a temperate orchid species of Kashmir Himalaya and Ladakh region valued for its beautiful flowers, inflorescence and medicinal properties. Its seeds are minute and dust-like. Multiplication of the species in nature is through seeds and only 0.3% of seeds germinate in the presence of suitable mycorrhiza [16, 31]. *Dactylorhiza hatagirea* (D. Don) Soő is rapidly dwindling in nature and in coming few years can be declared critically rare species which is alarming need for its conservation. A little work has been done so far on conservation of *Dactylorhiza hatagirea* (D. Don) Soő species using tissues other than seeds and protocorms. It is in this context that the present studies were carried out for the standardization of an effective and low cost reproducible protocol for the conservation and commercialization of this critically endangered species.

Materials and Methods

In vitro studies of seeds

Explants were obtained from the plants established at KUBG (Kashmir University Botanical Garden) grown plants. These explants include leaf, shoot apex, petioles, inflorescence, flower stalk, mature and green pods. Juvenile leaves selected after sterilization were cut with sharp surgical blade to get 1 cm^2 long leaf explants for regeneration. Similarly 0.5 cm long shoot tip, flower stalk and inflorescence were used as explants. All these explants had similar sterilization and inoculation procedure with differences in case of pod explants. Explants without green pods were washed under running water for 30 min. and then immersed in an aqueous solution of 5% liquid detergent (Labolene) for 5–6 minutes and rinsed five to six times with distilled water. These were then sterilized in 2% NaOCl solution for 5 min. and rinsed three times with sterile double distilled water. Green pod was washed gently under running tap water. After this, the pod was immersed in 70% ethanol solution for 1 min followed by immersion in 2% NaOCl solution for 10 min with 5 drops of (Tween-20) and constant shaking for 5 min under laminar air flow. Finally, the pod was gently rinsed 3 times with sterile double distilled water. The sterilized capsule was cut with sterilized surgical blade and the seeds were carefully scooped out and cultured aseptically on MS 1962 medium [32] Knudson C 1946 medium [33], Knudson C Morel modification 1976 medium [34] supplemented with cytokinins, auxins and gibberellins in varied concentrations. The pH of the media was adjusted to 5.8 with 1M KOH or HCl prior to autoclaving for 15 min at 121ºC after the addition of the growth regulators. Nearly 10 ml of medium was dispensed into 35 ml culture vial and only one explant of leaf, shoot tip, flower stalk or inflorescence were inoculated per culture vial during inoculation. In case of mature/ green pods many seeds 80±20 in number were inoculated per culture vial. Ten number of culture vials were taken as a unit of replication with twelve replicates per treatment. The cultures were maintained under cool white fluorescent lights at 25±2ºC with 16 hour photoperiod.

In vitro studies of tubers

Field trips were organized to collect fresh plants from the natural habitat. Tuber explants were washed under running water for 30 min. and then immersed in an aqueous solution of 5% (1:1) liquid detergent (Labolene) for 5–6 min followed by rinsing five to six times with distilled water. The tubers were cut longitudinally with sharp surgical blade into smaller sections where each section was having at least one bud. These tubers sections were sterilized with an aqueous solution of 0.1% mercuric chloride solution or 3% NaOCI solution for 5 minutes and rinsed five to six times with sterile double distilled water. After this the tuber sections were subjected to pretreatment with various hormones in different concentrations. They were then kept in petridishes containing aqueous solutions of cytokinins, auxins, gibberellins (GA_3) of varied ppm concentrations. Nearly 5ml of aqueous solution was dispensed into sterilized petri dishes and thereafter sealed with parafilm. Ten number of petri dishes with one rhizome section each was taken as a unit of replication with three replicates per treatment. Initial observations were recorded after 72 hours of pretreatment of the tuber sections.

Direct plantation of Propagules

Fresh plants were collected from the natural habitat. Tubers used as propagules were washed under running water for 30 minutes and then immersed in an aqueous solution of 5% (1:1) liquid detergent (Labolene) for 5–6 min followed by rinsing five to six times with distilled water. These were sown in pots and soil beds with different soil mixtures. 20 propagules in pots (one each) and 16 in soil beds were taken as a unit of replication with three replicates.

Results and Discussion

During the present investigation leaves, petioles, apical tips, tuber sections and seeds (mature as well as immature) were used as explants. Mature as well as immature seeds were inoculated on MS 1962 medium, Knudson C Orchid 1946 medium and Knudson C Orchid medium (Morel modification, 1976). A contamination rate of 50% was observed in shoot apex explants used at vegetative phase whereas no contamination in inflorescence explants was observed. Shoot apexes and leaf explants showed 50% and 15% survival, respectively after 8 weeks of culture. The survival rate of floral explants was significantly high and the browning rate was low as compared with other explants. However a significant 90% of survival percentage was achieved in case of immature seeds (Table 1).

Table 1: Effect of sterilization on different explants in *Dactylorhiza hatagirea* (D. Don) Soő

Explant	Contamination (%)	Browning (%)	Survival (%)
Shoot apex	25	20	50
Leaf	7	80	15
Flower Stalk	33	67	0
Inflorescence	0	7	93
Mature seed	30	5	80
Immature seed	20	10	90

In vitro response of mature seeds

Mature seeds from ripe pods inoculated on MS medium responded only to media supplemented with varied concentrations of BAP and NAA added together along with AC $5gl^{-1}$. BAP and NAA in concentration range of 2.0-3.0 mgl^{-1} and 0.5-2.0mgl^{-1} induced germination of seeds leading to PLB’s formation. BAP alone has no effect on the germination of seeds and PLB’s formation. BAP when added together with NAA induced germination of seeds.

Maximum frequency of PLB's development was observed on MS medium supplemented with BAP 3.0 mgl^{-1} + NAA 1.5 mgl^{-1}+ Activated Charcoal 5gl^{-1} with 70% of culture response after 12±1 weeks of inoculation (Table 2; Fig1. a, b, c, d, e). These PLB's remained quiescent for three months but were unable to form shoots.

Table 2: *In vitro* mature seed germination and PLB's development on MS medium (1962) supplemented with varied concentrations of BAP and NAA.

Treatment	**Percentage frequency of seed germination and PLB's development**	**Initiation of response**
MS medium + BAP +NAA (mgl^{-1}) + AC 5gl^{-1}		
MS basal (control)	-	12±1weeks
0.0 + 0.5	-	
1.0 + 0.5	-	
2.0 + 0.5	20	
2.0 + 1.0	40	
2.5 + 1.0	40	
3.0 + 1.5	70	
3.5 + 1.5	20	
4.0 + 2.0	-	

-No Response
12 replicates per treatment

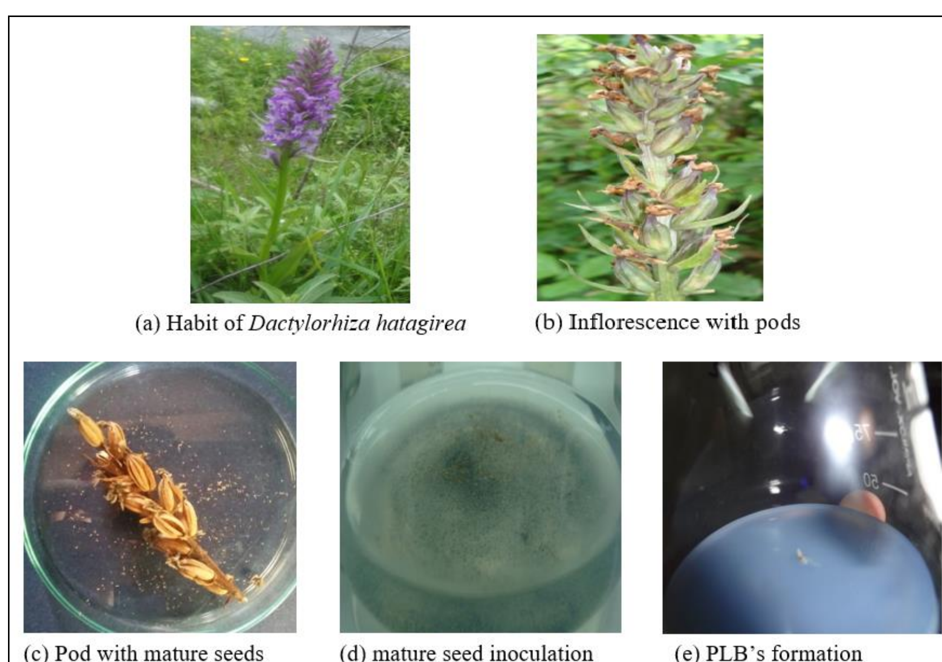

Fig 1: *In vitro* mature seed germination and PLB's formation

In vitro response of immature seed

Immature seeds from green pods responded well on Knudson C Orchid (Morel modification) medium supplemented with varied concentrations of BAP and NAA. BAP and NAA alone did not induce any response. KCM medium when supplemented with BAP and NAA together in range of 2.0-4.0 mgl^{-1} and 1.0-2.0 mgl^{-1} resulted in development of PLB's. The maximum frequency of seed germination i.e.; PLB'S formation was achieved on BAP 2.5 mgl^{-1} + NAA 1.5 mgl^{-1} when added together after 9±1 weeks with 80% culture response. Seed germination on average began after 9 weeks of culture. The colour of protocorms was milk white and three to four epidermal hairs were produced at this stage. Single protocorm was inoculated per culture vial during sub culturing. These PLB's showed little activity of elongation which resulted into development of seedling on KCM medium supplemented with BAP 4.0 mgl^{-1} + NAA 2.0 mgl^{-1} added together with maximum shoot length of 2.8±0.4 cm on BAP 4.0 mgl^{-1} + NAA 2.0 mgl^{-1} treatment after 7±1 weeks with 90% of culture response. A shoot length of 1.2±0.1 and 0.8±0.06 cm was achieved on BAP 4.5 + NAA 2.5 mgl^{-1} and BAP 3.5 mgl^{-1} + NAA 1.5 mgl^{-1} with little and moderate culture response respectively (Table 3, 4; Fig 2. a, b, c, d, e).

Table 3: *In vitro* immature seed germination and PLB's development on Knudson C Orchid medium (Morel modification, 1976) supplemented with varied concentrations of BAP and NAA

Treatment	**Percentage frequency of seed germination and PLB's development**	**Initiation of response**
Knudson C Orchid medium (Morel modification, 1976) + BAP + NAA mgl^{-1} + AC 5gl^{-1}		
Knudson C medium (control)	-	9±1weeks
1.0 + 0.5	-	

2.0 + 0.5	-	
2.0 + 1.0	20	
2.5 + 1.5	80	
3.5 + 1.5	40	
3.5 + 2.0	20	
4.0 + 2.0	20	

-No Response
12 replicates per treatment

Table 4: *In vitro* seedling development from PLB's subculture on Knudson C Orchid medium (Morel modification, 1976) supplemented with varied concentrations of BAP and NAA

Treatment Knudson C Orchid medium (Morel modification, 1976) + BAP + NAA mgl^{-1}+ AC 5gl^{-1}	Percentage frequency of seedling development	Length of shoot (cm)	Initiation of response
2.0 + 0.5	-	-	7±1weeks
2.5 + 1.0	-	-	
3.0 + 1.5	30	0.4 ± 0.03	
3.5 + 1.5	60	0.8 ± 0.06	
4.0 + 2.0	90	2.8 ± 0.4	
4.5 + 2.5	40	1.2 ± 0.1	

-No Response
12 replicates per treatment

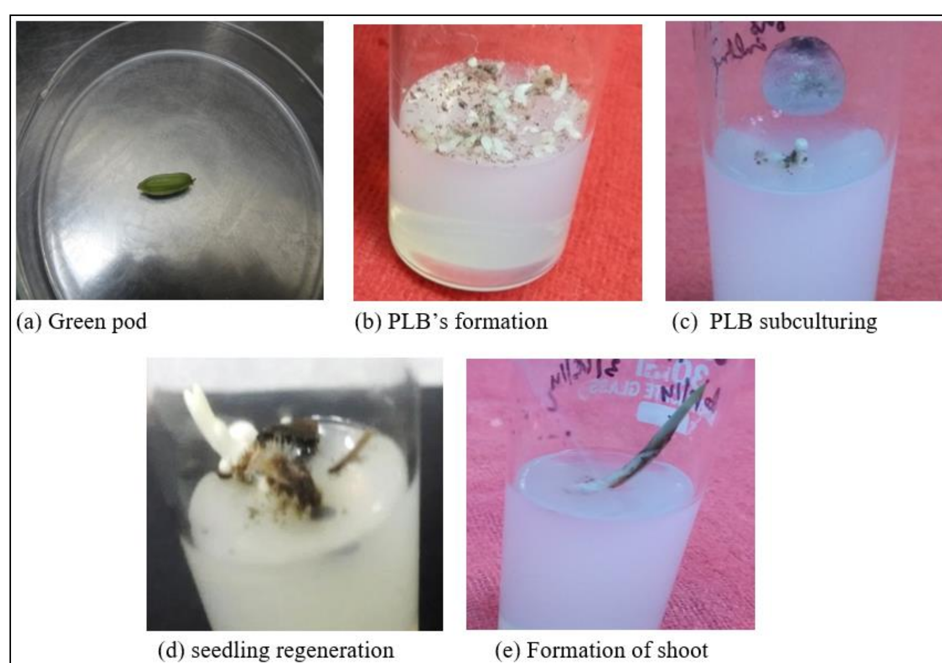
(a) Green pod (b) PLB's formation (c) PLB subculturing
(d) seedling regeneration (e) Formation of shoot

Fig 2: *In vitro* immature seed germination leading to seedling development

As the terrestrial orchids are adapted to variable habitats, optimal *in vitro* culture conditions suitable for one species may differ for other species [35]. Protocorms were developed by culturing immature seeds from unripe fruits on KCM medium supplemented with BAP 2.5 mgl^{-1} + NAA 1.5 mgl^{-1} when added together after 9±1 weeks with 80% culture response. However, when these protocorms were sub cultured, complete plantlet was achieved on KCM medium supplemented with BAP 4.0 mgl^{-1} + NAA 2.0 mgl^{-1} after 7±1 weeks with maximum shoot length of 2.8±0.4 cm. These findings are supported by those reported by Kalimuthu *et al.* [13], Mitsukuri *et al.* [36], R Eszeki [37] and Zenga *et al.* [38]. Sungkumlong and Deb [39] confirmed that the development stage of the seeds and nutrient media significantly influences their germination frequency.

The number of orchid species is rapidly and steadily declining because of their low rate of propagation in nature and the ongoing collection from nature. Careless collection of these species has led to serious genetic and ecological erosion; many have already been listed as endangered species [40, 41]. Therefore, *in vitro* propagation could be useful for the mass propagation of orchids for commercial purposes. Tissue culture techniques have been widely used for the *in vitro* mass propagation of several commercially important orchids over the past few decades [42, 43]. Bud production on the roots has been observed in many temperate terrestrials, which would make propagation by division successful [18]. In some temperate terrestrial orchid species a root tip meristem can even transform directly into a shoot meristem [44, 18]. So tuber sections were used to regenerate complete plants by inducing buds with pretreatment of different hormones.

Pretreatment

Among the various treatments given NAA, 2,4-D and GA_3 at 200, 300 and 500 ppm induced little germination. BAP

200 and 300 ppm was effective in regenerating plantlets from 50% of tuber sections with shoot length of 0.8 cm and 0.6 cm. However, IBA at 200 ppm was highly effective in inducing germination and development of shoot with maximum shoot length of 4.0 cm where 100% of tuber sections regenerated shoots. After successful development of plantlets, they were transferred to pots (Table 5; Fig.3. a-i). In three months the plants got well established. After three months the initiation of flowering took place with a short flowering duration of ten days. Hence a fourfold multiplication rate of the species was achieved at 200 ppm IBA being the optimum hormonal treatment. These results are in accordance with those of Sheelavanmath *et al.* [45] who standardized an *in vitro* regeneration protocol in *Geodorum densiflorum* through rhizome section culture on MS media supplemented with growth regulators and 0.1% activated charcoal.

Table 5: Effects of different hormonal treatments on tuber germination and plantlet development

Treatment	Hormone Concentration (ppm)	% age of tuber sections regenerated plantlets	Length of shoot (cm)
NAA	200	50	-
	300	50	-
	500	50	-
IBA	200	100	4.0±0.03
	300	75	1.8±0.02
	500	75	1.6±0.004
2,4-D	200	50	-
	300	50	-
	500	50	-
BAP	200	50	0.8±0.002
	300	50	0.6±0.001
	500	50	-
GA_3	200	50	-
	300	50	-
	500	50	-
Distilled Water (Control)	-	-	-

No. of replicates=3
Data obtained after 72 hours of culture

Fig 3: Pretreatment of Propagules

Direct plantation of propagules

Here the propagation was done in pots and soil beds. Propagules were subjected to establishment in earthen pots (radii 10 cm) and propagation beds (4x6 feet) at KUBG. The soil mixture containing soil charcoal, moss (sphagnum) and pieces of earthen pots (3:1:2:1) was filled in pots. In soil beds the propagules were spaced uniformly. The pots were watered at alternate intervals and monitored regularly for

three consecutive years. The plant propagules 16 in number in two rows (8 in each) were planted in soil bed. Similarly propagules were planted in 20 pots with one propagules in each pot (Table 6; Fig.4. a-e). The charcoal used helped to develop pathogen free conditions during growth and development period. The pieces of earthen pots resulted in proper drainage within pots so as to prevent root-rot of rhizomes due to excess of water during watering of the pots. The growth was excellently well with flowering after a growth period of 12 ± 2 weeks. During the transplanting phase it is necessary to compile a soil mix which is suited to the needs of the temperate species. Soil from deciduous woods or orchid habitats is ideal as a part of substrate for terrestrial orchid cultivation.

Table 6: Survival percentage in propagation beds and pots at KUBG

Propagation Type	No. of Propagules	Survival percentage		
		First year	Second year	Third year
Pot	20	20, 100%	16, 80%	12, 75%
Soil bed	16	16, 100%	13, 81%	10, 77%

(a) *Dactylorhiza* tuber (b) Sprouting of tuber in soil bed (c) Flowering in soil bed (d) Sprouting of tuber in pot (e) Sprouting of tuber in pot

Fig 4: Direct plantation of Propagules in soil beds and pots

Conclusion

An effective and successful regeneration protocol in three different ways was standardized for the mass multiplication of *Dactylorhiza hatagirea* (D. Don) Soő. The micropopagation from mature seeds with 70% frequency of PLB's formation after 12±1 weeks, micropopagation from immature seed from unripe fruits with 80% frequency of PLB's formation after 9±1 weeks of inoculation proved comparatively better to propagation from mature seeds. Germination of immature seed from unripe fruits in *Dactylorhiza hatagirea* (D. Don) Soő with higher percentage of PLB's induction than the mature seed is a major breakthrough in the studies of temperate terrestrial orchids because several workers have reported it too difficult to raise temperate terrestrial orchids from seeds. Also it has been observed that inflorescence explants proved as virus free explants thus can prove best for regeneration studies. Propagation from tuber sections proved very helpful. A fourfold multiplication rate of the species was achieved. Using tubers as propagules direct plantation of the species yielded better results as already described. Therefore the present protocol could be employed for the mass multiplication of this endangered medicinally important orchid growing in Kashmir Himalaya.

Acknowledgements

The authors are highly thankful to the Head Department of Botany University of Kashmir Hazratbal Srinagar 190006 for providing necessary laboratory facilities in Tissue Culture Research Laboratory of the Department. Also the author Burhan M. Padder is thankful to UGC New Delhi, India for providing financial assistance under UGC-BSR fellowship programme.

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