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Full Length Research Paper

Propagation and conservation of *Dactylorhiza hatagirea* (D. Don) Soo, an endangered alpine orchid

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***Dactylorhiza hatagirea* (D. Don) Soo; a source of high quality 'salep' used as nervine tonic, has been categorized as critically endangered due to over-exploitation from the wild. This report deals with the propagation of this species using both *in vitro* and conventional methods. For green pod culture, four nutrient media, Knudson C (KC), Murashige and Skoog (MS), Vacin and Went (VW) and Vejsadova (VJ) were tested by adding different growth additives. MS medium supplemented with peptone (P) (1.0 g/L), morphoinoethane sulphonic acid (MES) (1.0 g/L) and activated charcoal (AC) (0.1%) was found to be the most effective medium for the development of protocorm like bodies (PLBs), development of chlorophyll and for the plantlet formation. To improve vegetative multiplication, tubers were treated with α- naphthalene acetic acid (NAA), indole-3- butyric acid (IBA) and indole acetic acid (IAA) before planting. Rooting was observed in only apical segments. Maximum rooting (38.88%) was induced with 50.0 µM IBA. This study is helpful in the propagation of this endangered orchid at lower elevation which could be a successful effort of conservation of this endangered orchid at lower elevation and this could also reduce pressure on its population in the wild.**

Key words: *In vitro* propagation, protocorm like bodies (PLBs), vegetative propagation, conservation.

INTRODUCTION

Generally, orchids are propagated by vegetative means as well as through seeds. The rate of vegetative propagation is very slow and seed germination in nature is also very poor that is, 0.2 to 0.3% (Vij, 2002). A single orchid capsule contains millions of seeds, which lack metabolic machinery and do not have any endosperm. In spite of a very large number of seeds produced, only few seeds germinate in nature (Kalimuthu et al., 2007). Terrestrial species have more stringent requirements for germination but little is known about their specific requirements (De Pauw et al., 1995). Many researchers have reported difficulties in germinating seeds of terrestrial orchids (Arditti et al., 1981; Rasmussen, 1992; Zettler et al., 2001). Terrestrial orchids might be more dependent on a mycorrhizal fungus than epiphytic species (Stoutamire, 1974), making *in vitro* asymbiotic seed germination difficult. *In vitro* germination of orchid seeds is an important part of the conservation and multiplication programme, as the 'dust seeds' are tiny and contain few food reserves.

Vegetative propagation is an established method of preserving the unique characteristics of a plant in the subsequent progeny. Plant growth regulators (PGRs) are known to affect adventitious root formation, and the role of auxins, cytokinins, gibberellins, abscisic acid and the polyamines in rooting has been examined (Hartman et al., 2002).

Limited reports exist on the vegetative propagation of Himalayan alpine herbs of medicinal value. These include

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Abbreviations: KC, Knudson C; **MS,** Murashige and Skoog; **VW,** Vacin and Went; **VJ,** Vejsadova; **PLBs,** protocorm like bodies; **NAA,** α- naphthalene acetic acid; **IBA,** indole-3- butyric acid; **IAA,** indole acetic acid; **P,** peptone; **AC,** activated charcoal; **MES,** morphoinoethane sulphonic acid.

species like *Picrorhiza kurrooa* (Nautiyal et al., 2001), *Aconitum balfourii* syn *A. atro x* (Rawat et al., 1992; Pandey, 2002), *Heracleum candicans*, *Angelica archangelica* (Joshi, 2002).

Dactylorhiza hatagirea (D.Don) Soo (Family Orchidaceae) is a high altitude monocotyledonous, perennial, terrestrial orchid. Its natural distribution extends from Europe to North-Africa and Central, West and South-East Asia (Vij et al., 1992). Bhatt et al. (2005) reported it's presence in temperate to alpine regions (2500 to 5000 m) in India, Pakistan and Nepal. The tubers of this species, commonly sold as 'Salampanja,' are known to yield a high quality 'Salep' which is extensively used in local medicine as nervine tonic for its astringent and aphrodisiac properties (Vij et al., 1992).

It has been categorized as critically endangered (Conservation Assessment and Management Plan; CAMP status), critically rare (International Union for Conservation of Nature and Natural resources; IUCN status) and listed under appendix II of Convention on International Trade in Endangered Species (CITES) (Bhatt et al., 2005). On the basis of distribution pattern, population status and level of anthropogenic pressures, *D. hatagirea* was placed under the category of 'restricted distribution and heavy pressure' (Uniyal et al., 2002). It is a habitat specific and inherently slow growing species in nature (Vij et al., 1992; Agarwal et al., 2008) and poorly regenerating species because of pollinator specificity and requirement for mycorrhizal association for seed germination. It was also observed that *D. hatagirea* had localized distribution in their habitat (Semwal et al., 2007). As the annual consumption of the 'Salep' obtained from this species in India is about 7.38 tones (valued at about Rs.50.0 lakhs), most of it imported from the other countries (Vij et al. 1992).

Since the plant is palatable and has high medicinal value, various levels of disturbances, like grazing pressure, overexploitation and unawareness of collection and propagation etc. have resulted in the decline of this species from its natural habitat (Giri et al., 2008).

One of the most appropriate actions for safeguarding overexploited species is to improve propagation techniques and to encourage cultivation. Therefore, this present study was carried out to propagate and conserve this endangered alpine orchid at lower elevation by using *in vitro* and conventional methods. On the basis of concerned literature, there is no any previous report on vegetative propagation of *D. hatagirea* by using tuber cuttings at lower elevation.

MATERIALS AND METHODS

Green pod culture

Green pods of *D. hatagirea* were collected from different alpine meadows of Tungnath (30° 30' N -79° 15' E, 3300- 4200 m), Garhwal Himalaya during the month of July. After washing under running tap water with few drops of labolene, these pods were treated with a systematic fungicide (bavistin, 1.0%, w/v, 10 min), washed with distilled water to remove any traces of fungicide and kept inside the laminar air flow cabinet. Surface disinfection was carried out with aqueous solution of mercuric chloride ($HgCl_2$, 0.1%, w/v, 3 min) and washed with sterilized double distilled water (x 4). These surface disinfected pods were cut into two halves, seeds were carefully dissected and inoculated on different media that is Knudson (KC, 1946), Murashige and Skoog (MS, 1962), Vejsadova (VJ, 2002) and Vacin and Went (VW, 1949) medium supplemented with different growth additives (Table 1). Five flasks were used in each treatment and one pod per flask.

Shoot bud culture

Daughter tubers (having shoot buds) of *D. hatagirea* were collected from different alpine meadows of Tungnath (30° 30' N _ 79° 15' E, 3300 to 4200 m), Garhwal Himalaya during the month of July and brought to the laboratory. After washing under running tap water with few drops of labolene, these tubers were treated with a systematic fungicides (bavistin, 1.0%, w/v, 20 min) followed by washing (x 5) with double distilled water and kept inside the laminar air flow cabinet.

Surface disinfection was done with aqueous solution of $HgCl_2$ (0.1% w/v, 7 min) followed by washing with sterile double distilled water. These daughter tubers with shoot buds were inoculated on MS medium (Murashige and Skoog, 1962). Sucrose (3.0%, w/v) was added as a carbon source and medium was gelled with agar (0.8% w/v). The basal medium was supplemented with different concentration of 6-benzyladenine (BA) and thidiazuron (TDZ) in different trials (Table 2).

Tuber segment culture

After shoot emergence in nature, young tubers (Photo plate 1, E) of *D. hatagirea* were collected from different alpine meadows of Tungnath in the month of July to August. After washing under running tap water with few drops of labolene, these tubers were treated with a systematic fungicide (bavistin 1.0%, w/v, 30 min), washed with double distilled water (x 3) to remove any traces of fungicide and kept inside the laminar air flow cabinet. Surface disinfection was done with aqueous solution of $HgCl_2$ (0.1%, w/v, 5 min) followed by washing with sterilized double distilled water (x 4) to remove any traces of surface disinfectant. These sterilized tubers were cut into small segments and inoculated on MS medium supplemented with different PGRs that is α- naphthalene acetic acid (NAA), BA (0.5, 2.5, 5.0, 10.0 and 20 µM each) and with combination of BA + NAA (2.5 + 5.0; 2.5 + 10.0; 2.5 + 15.0; 2.5 + 20.0). Activated charcoal (0.1%) was also added to the medium. Sucrose (3.0%, w/v) was added as a carbon source and medium was gelled with agar (0.8% w/v).

Leaf segment culture

Leaves of *D. hatagirea* were taken from the non-flowering plants growing inside the poly house of the department. After washing carefully, surface disinfection was done by using aqueous solution of $HgCl_2$ (0.1%, w/v, 3 min). These leaves were cut into small segments and inoculated on MS and VW basal media supplemented with different concentration of PGRs that is MS + NAA (2.5, 5.0,10.0 and 20.0 µM), MS+ 2, 4-dichlorophenoxy acetic acid (2,4-D) (2.5, 5.0,10.0 and 20.0 µM), VW+ 2,4-D (2.5, 5.0,10.0 and 20.0 µM), BA+ NAA (2.5 + 5.0; 2.5 + 10.0; 2.5 + 20.0 ; 2.5 + 25.0 µM). Sucrose (3.0%, w/v) was added as a carbon source and medium was gelled with agar (0.8% w/v).

Culture condition

The pH of the medium was adjusted to 5.8 before autoclaving (1.05 kg cm^{-2}, 121°C, 20 min). All the chemicals were obtained from Hi Media Laboratory Pvt. Ltd, Mumbai, India. All cultures were placed inside the culture room at 25 ± 1°C in 16 h light (42 µ mol m^{-2} s^{-1}; cool fluorescent lights, Philips 40 W) and 8 h dark cycle. The observations were made regularly and subcultured as and when required.

Vegetative propagation

Tubers of *D. hatagirea* were collected from different alpine meadows of Tungnath (30° 30' N - 79° 15' E, 3300~ 4200 m) Garhwal Himalaya during the month of October (Photo plate 1,E). These tubers were immediately sown in earthen pots containing alpine soil, till they were used for experimental work. After 12 weeks (last week of January 2009) all the tubers were carefully dugout from the pots, washed under running tap water to remove adhering soil, rinsed with distilled water (x3) and then allowed to air-dried at room temperature.

Tubers were placed in aqueous solutions of various PGRs for 24 h followed by air drying (at room temperature, 25 ± 2°C) and then again treated with freshly prepared solution of used PGRs for a further period of 24 h followed by air drying. The used PGRs were α- naphthalene acetic acid (NAA), indole-3- butyric acid (IBA) and indole acetic acid (IAA). The concentrations of each used PGRs were 10.0, 50.0 and 100.0 µM (Table 3). Following treatments, each tuber was cut into small segments (apical, middle and basal). Only the apical segment had a dormant shoot bud while the remaining two segments, that is, middle and basal segments had no bud (at the time of planting). These tubers were planted in polythene bags (at 1" depth) containing forest soil, during last week of January 2009. Eighteen tuber cuttings (in triplet; six cutting in each replicate) were used in each treatment and poly bags were kept inside the poly house. They were watered at regular intervals. After 16 weeks of planting, all cuttings were uprooted and observations were made on percent rooting, root number and root length.

Data analysis

Standard Error (SE) and analysis of variance (ANOVA) were calculated following the method of Snedecor and Cochran (1967).

RESULTS

Green pod culture

Green pods of *D. hatagirea* showed varied responses on the various tested nutrient media (Table 1). The seeds when inoculated on these media, showed their first response by swelling (Photo plate 1A). Then the seeds became round and the embryos came out of the seeds by rupturing the wall but this process was very slow and tedious, took very long time (up to 08 to 17 weeks) (Table 1). These embryos were later on (after 20 weeks of seed germination) converted into white round structures called protocorm like bodies (PLBs) but only in few treatments (Table 1; Photo plate 1B).

Good seed germination (+++) was achieved in half strength MS medium supplemented with peptone; P (1.0g/L), morphoinoethane sulphonic acid; MES (1.0 g/L) and activated charcoal (AC) (0.1%), within 9 weeks whereas average seed germination (++) occurred in KC medium supplemented with kinetin (KN) (1.0 mg/L) and AC (0.1%). It took 15 weeks time for their germination. Poor seed germination was shown by all the media but in few treatments (Table 1).

PLBs were formed only in four treatments (Table 1). Out of these, the maximum number of PLBs (11.0) was formed in ½ strength MS medium supplemented with P (1.0 g/L), MES (1.0 g/L) and AC (0.1%) and it took 20 weeks time from seed germination to PLBs formation. Photo plate 1C shows the enlarge view of protocorm like body with clear shoot apex and rhizoids region after 22 weeks of seed germination. In KC medium, only 5 PLBs were formed in the presence of KN (1.0 mg/L) and AC (0.1%) after 26 weeks of germination. Although less time was required for the formation of PLBs in VW (supplemented with YE (1.0 g/L) and AC, 0.1%) and VJ (supplemented with KN, 1.0 mg/L and AC, 0.1%) media (16 weeks and 13 weeks, respectively) but the number of PLBs was very less (only 2 PLBs) in both media. Out of these four treatments, which were able to form PLBs, only MS ½ strength medium supplemented with P (1.0 g/L), MES (1.0 g/L) and AC (0.1%) was effective for the development of chlorophyll in formed PLBs, however, it was again a very lengthy process as it required 41 weeks time for the development of chlorophyll. Out of the eleven PLBs, chlorophyll was developed in only five PLBs. This treatment was very effective as the subculturing of these green PLBs in the same medium favoured the plantlet formation after 91 weeks of seed inoculation (Photo plate 1D).

Shoot bud culture

For shoot bud culture, daughter tubers having shoot buds **(**Photo plate 1F) were used as explants. Initially only 40. 50% of explants were found to be free from fungal as well as bacterial contamination. Sprouting started in shoot buds within four weeks of inoculation. Out of different used treatments, MS medium supplemented with TDZ (10.0 µM) showed positive response towards sprouting (11.10%) in shoot buds **(**Table 2). The rest of the used treatments shoot buds did not sprout and became brown and died after four weeks of sprouting. Later on, shoots developed from these sprouted buds and attained an average length of 2.72 cm (Photo plate 1G and H). When these shoots were transferred to the shoot multiplication medium, they could not survive and died (data not shown).

Tuber and leaf segment culture

No response was observed in any trials even after 24

Table 1. *In vitro* study of *D. hatagirea* by using green pod culture.

Medium	Growth additive	Germination response	Average germination time(weeks)	Development of PLBs		Development of chlorophyll		Development of plantlet
				Number of PLBs	Average time(weeks)	Number of PLBs	Average time(weeks)	
K\C	Basal	-	-	-	-	-	-	-
	KN (1mg/l) + AC (0.1%)	++	15	05	26	-	-	-
	P (1g/l) + AC (0.1%)	+	13	-	-	-	-	-
	CH (1g/l) + AC (0.1%)	+	14	-	-	-	-	-
	YE (1g/l) + AC (0.1%)	+	17	-	-	-	-	-
	(½ strength) + BA (1mg/l) + CW (10%).	-	-	-	-	-	-	-
	(½ strength) + P (1g/l) + MES (1g/l) + AC (0.1%)	-	-	-	-	-	-	-
MS	Basal	-	-	-	-	-	-	-
	KN (1mg/l) + AC (0.1%)	+	13	-	-	-	-	-
	P (1g/l) + AC (0.1%)	-	-	-	-	-	-	-
	CH (1g/l) + AC (0.1%)	+	12	-	-	-	-	-
	YE (1g/l) + AC (0.1%)	-	-	-	-	-	-	-
	(½ strength) + BA (1mg/l) + CW (10%).	-	-	-	-	-	-	-
	(½ strength) + P (1g/l) + MES (1g/l) + AC (0.1%)	+++	09	11	20	05	41	Favoured
VW	Basal	-	-	-	-	-	-	-
	KN (1mg/l) + AC (0.1%)	+	16	-	-	-	-	-
	P (1g/l) + AC (0.1%)	+	11	-	-	-	-	-
	CH (1g/l) + AC (0.1%)	+	15	-	-	-	-	-
	YE (1g/l) + AC (0.1%)	+	14	02	16			
	(½ strength) + BA (1mg/l) + CW (10%).	-	-	-	-	-	-	-
	(½ strength) + P (1g/l) + MES (1g/l) + AC (0.1%)	-	-	-	-	-	-	-
VJ	Basal	-	-	-	-	-	-	-
	KN (1mg/l) + AC (0.1%)	+	08	02	13	-	-	-
	P (1g/l) + AC (0.1%)	-	-	-	-	-	-	-
	CH (1g/l) + AC (0.1%)	-	-	-	-	-	-	-
	YE (1g/l) + AC (0.1%)	-	-	-	-	-	-	-
	(½ strength) + BA (1mg/l) + CW (10%).	-	-	-	-	-	-	-
	(½ strength) + P (1g/l) + MES (1g/l) + AC (0.1%)	-	-	-	-	-	-	-

KC (1946) = Knudson C medium, VW (1949) = Vacin and Went medium, MS (1962) = Murashige and Skoog medium, Vejsadova (VJ, 2002), AC= Activated charcoal, YE = Yeast extract, P = Peptone, KN = Kinetin, BA = Benzylaminopurine, NAA = α- Naphthalene acetic acid, µM= Micro-mole, PLBs = Protocorm -like bodies, CW= Coconut water; PGRs= Plant growth regulators; MES= 2 Morphoinoethane sulphonic acid.

Table 2. Effect of MS medium supplemented with BA and TDZ on shoot formation in shoot buds of *D. hatagirea*

Treatment	Concentration (µM)	% sprouting	Average shoot length (cm)
Control	(0.0)	00.00 ± 0.00	00.00 ± 0.00
BA	(2.5)	00.00 ± 0.00	00.00 ± 0.00
	(5.0)	00.00 ± 0.00	00.00 ± 0.00
	(10.0)	00.00 ± 0.00	00.00 ± 0.00
TDZ	(2.5)	00.00 ± 0.00	00.00 ± 0.00
	(5.0)	00.00 ± 0.00	00.00 ± 0.00
	(10.0)	11.10 ± 5.55	02.72 ± 0.97

Values are mean ± standard error (SE), n = 9, 3 replicates, 3 explants per flasks. MS: Murashige and Skoog (1962) medium.

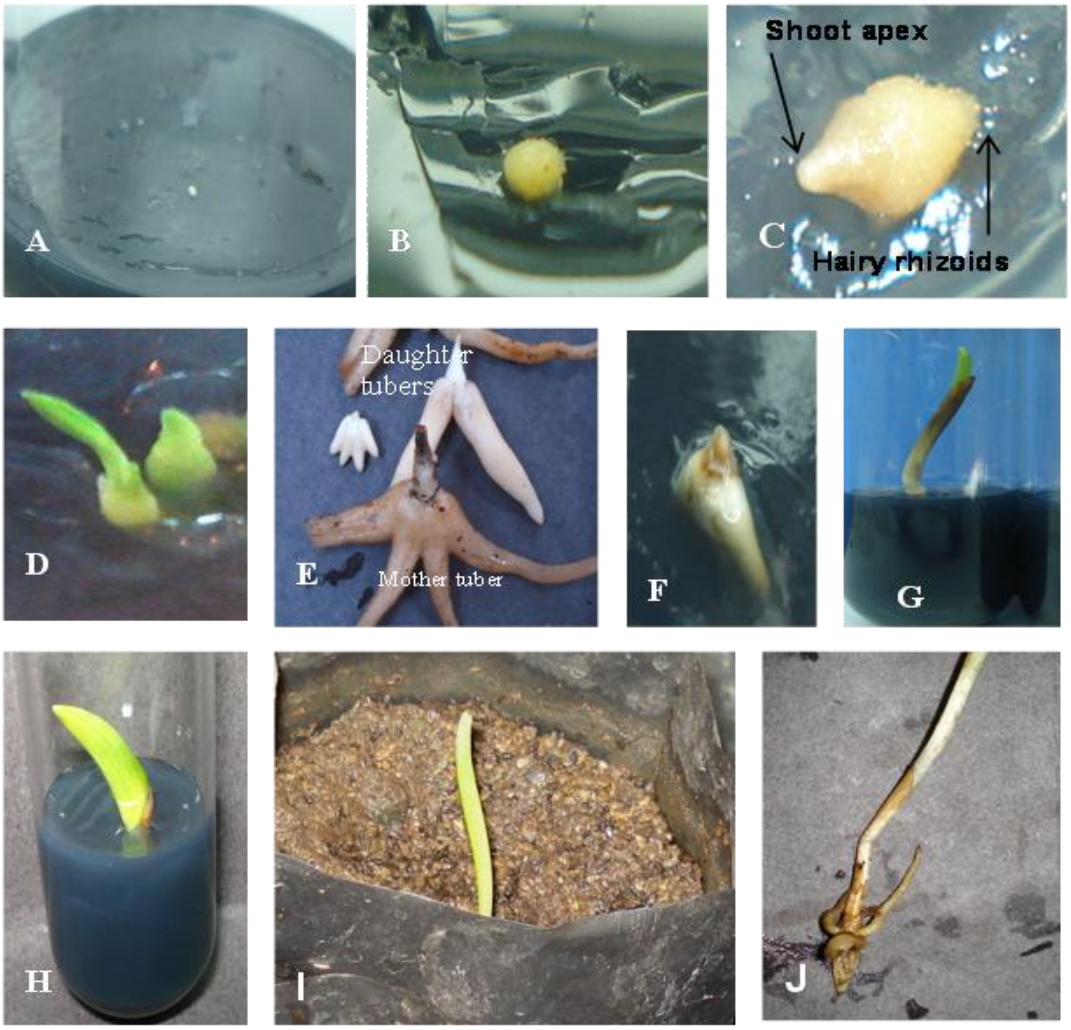

Photo plate 1. Propagation study of *D. hatagirea.* A. Swelling of seeds after 09 weeks of inoculation, B. Development of protocorm after 20 weeks of seed germination, C. An enlarge view of protocorm showing distinct shoot apex and rhizoids after 22 weeks of seed germination**,** D. *In-vitro* raised plantlets after 91 weeks of seed inoculation on MS medium, E. Photo plate showing mother tuber and daughter tubers, F. Daughter tubers having shoot buds used as an explants. G and H. Shoots developed from the sprouted buds and attained an average length of 2.72 cm on MS medium supplemented with TDZ (10.0 µM) and AC (0.1%, I. Sprouted plantlet inside the poly house after 12 weeks of planting**,** J. Root emergence in apical segments of tuber after 16 weeks of planting.

Table 3. Effect of different PGRs on rooting of tuber cuttings of *D. hatagirea.*

Treatment	Concentration (µM)	Sprouting (%)	Rooting (%)	Average root number	Average root length (cm)
Control	0.0	22.21 ± 5.55	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
	10.0	16.66 ± 0.00	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
NAA	50.0	38.88 ± 5.55	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
	100.0	61.10 ± 5.55	22.21 ± 5.55	0.59 ± 0.27	0.96 ± 0.20
	10.0	22.21 ± 5.55	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
IBA	50.0	33.33 ± 0.00	38.88 ± 5.55	1.51 ± 0.40	1.06 ± 0.26
	100.0	44.44 ± 5.55	22.21 ± 5.55	1.50 ± 0.63	2.10 ± 0.30
	10.0	22.22 ± 5.55	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
IAA	50.0	27.77 ± 5.55	00.00 ± 0.00	00.00 ± 0.00	00.00 ± 0.00
	100.0	44.44 ± 5.55	11.10 ± 5.55	0.74 ± 0.37	1.03 ± 0.60

Values are mean ± standard error (SE), six tuber cuttings in each treatment (in triplet). Data were recorded after 16 weeks of planting.

weeks (in leaf segment) and 48 weeks (in tuber cultures) of inoculation.

Vegetative propagation

Data for the effect of different PGRs on rooting of tuber cuttings of *D. hatagirea* is given in Table 3. In this present study, only apical segment of cuttings exhibited sprouting and rooting (Photo plate 1I and J). Middle and basal portion did not respond at all. Out of the ten treatments, only NAA (100.0 µM), IBA (50.0, 100.0 µM) and IAA (100.0 µM) were able to induce rooting; however, sprouting occurred in all the treatments. Values for percent sprouting ranged from 16.66 to 61.10% (Table 3). Among these auxin treatments, IBA (50.0 µM) was most effective as the maximum rooting percentage (38.88%) was observed in this treatment in comparison to the control (0.0% rooting). The average root number was also maximum (1.51 roots/cutting) in this treatment whereas maximum root length (2.10 cm) was observed in higher concentration of IBA (100.0 µM). Higher concentrations of both NAA and IBA (100.0 µM) were able to induce only 22.21% rooting in the apical segment of *D. hatagirea,* whereas the higher concentration of IAA (100.0 µM) showed minimum percent rooting (11.10%). Analysis of variance (ANOVA- one way) showed a significant variation (at 1 and 5%) in % sprouting, % rooting, average root number and average root length (cm) in both, IBA and NAA (Table 4).

DISCUSSION

This present study concluded that in this terrestrial orchid, *D. hatagirea*, the *in-vitro* seed germination by using green pods is very poor, slow, tedious and difficult. Out of 28 used treatments, only one treatment was responded well from seed germination to plantlets formation. Yam and Arditti (1990) also reported that many terrestrial genera of orchids are difficult to germinate and these difficulties have often been attributed to the development of inhibiting factor in their embryos (Stoutamire, 1974) and/or the persistent nature of the inner integument of their seeds which impedes hydration (Veyret et al., 1969). In this present study, activated charcoal (AC) was used in all the treatments as the AC in the medium enhances the plant regeneration. The promontory effect of AC on plant regeneration, mass multiplication and rhizome enlargement were reported by several researches (Sungkumlong and Deb, 2008). One possible explanation of the effects of charcoal is that it improves aeration (Ernst, 1974).

A second possibility is that the charcoal absorbs ethylene (Ernst, 1975) which can inhibit growth and proliferation. Terrestrial orchids have minute seeds with presumably several types of dormancy (Vander Kinderen, 1987). Their germination is difficult (Arditti, 1979), having specific requirements for nutrients (Arditti et al., 1982) and environmental conditions (Oliva and Arditti, 1985; Van Waes and Debergh, 1986a). Hence, *in-vitro* germination has not been very successful and has only been achieved with a limited number of species (Arditti et al., 1990). After the formation of non-chlorophyllous PLBs, in this present study, the chlorophyll development and multiplication of PLBs was a major problem in most of the cultures. Chlorophyll was developed in only few PLBs after 41 weeks of seed germination in MS medium. There was no multiplication of PLBs in any culture (data not shown). Stoutamire (1974) indicated that PLBs in the ground growing taxa of open grass land and well drained or seasonally dry soils are non-chlorophyllous. The protocorms of certain orchid species require more than a year for further development. In *Dactylorhiza maculata*,

Table 4. ANOVA summary of vegetative propagation of *D. hatagirea*.

Auxin	Source of variance[#]	% Sprouting			% Rooting			Average root number			Average root length (cm)		
		DF	MS	F	DF	MS	F	DF	MS	F	DF	MS	F
NAA	I	3	1196.10	17.22*	3	370.18	15.98*	3	0.26	4.75**	3	0.69	22.44*
	II	8	0069.44		8	023.15		8	0.05		8	0.03	
IBA	I	3	0339.64	04.88**	3	1072.3	23.15*	3	2.27	5.72**	3	3.03	25.55*
	II	8	0069.47		8	046.31		8	0.39		8	0.11	
IAA	I	3	0331.90	03.58 ^{NS}	3	092.51	04.00**	3	0.41	3.99**	3	0.79	02.88^{NS}
	II	8	0092.62		8	023.12		8	0.10		8	0.27	

[#]Source of variance: I; Between Group, II; Within Groups.
*Level of significant at 0.01 (at 1%), **level of significant at 0.05 (5%) and NS not significant. DF, Degree of freedom; MS, mean of square.

one year after dissemination the protocorm possesses a fully formed apical bud, from which the shoot develops in the next year (Vinogradova, 1999). Stoutamire (1974) reported that after development of protocorms their subsequent growth remains arrested unless these were transferred to a carbohydrates regime.

In this present study, MS medium was found to be best for seed germination, PLBs formation as well as for plantlets formation and KC medium did not respond well after PLBs development. Similar results were reported by Sungkumlong and Deb (2008) in *Coelogyne suaveolens* orchid. They found that the MS medium favoured formation of healthy PLBs and subsequently formation of healthy plantlets whereas in Knudson (KC) medium the PLBs formation was arrested right after nodular swelling of the seeds. Stoutamire (1964) also reported that some species of terrestrial orchids did not germinate on KC medium. The duration of the period in which the protocorm is modified into juvenile plants is taxon-specific. In *D. hatagirea,* in this present study, the whole process that is, seed germination to plantlets formation was also very lengthy.

After more than one year (91 weeks after seed inoculation), we got a very few numbers of *in vitro* raised plantlets using green pod culture in MS medium. These plantlets were inoculated in different multiplication media (data not shown) but the response was null and we could not achieve a multiple number of plantlets. These *in vitro* raised plantlets were transferred to soil for hardening but they could not survive and thus died soon. The major cause of death of these *in vitro* raised plantlets during hardening may be poor root development and environmental factors. However, we also tried leaf segment, tuber segment culture and shoot bud culture for regeneration of this plant besides, green pod culture but we could not get any positive response from these cultures. However, sprouting occurred in shoot bud cultures in MS medium supplemented with TDZ (10.0 µM) and shoots of 2.72 cm height were obtained but after 8 weeks the growth was arrested and they could not multiply and became brown and died later. Sheelavantmath et al. (2000) successfully propagated the endangered terrestrial orchid *Geodorum densiflorum* through rhizome section culture from *in vitro* seed derived rhizome but when we used the tuber segments of *ex vitro* raised plants we did not get any response.

This can be concluded from this present study that the *in vitro* propagation of this orchid species is very difficult and slow. Agarwal et al. (2008) also reported that the complete *in vitro* process that is, from seed germination to plantlets formation takes about one year. The *in vitro* seed germination is slow not only in *D. hatagirea* but also in the other species of *Dactylorhiza.* In *D. ruthei,* and *D. praetermissa* seeds started germinating after four months of culture and only 20.0 to 25.0% germination was achieved in Norstog medium after eight month of inoculation (Vaasa and Rosenberg, 2004). Vij et al. (1995) also reported that the *in vitro* propagation using green pod culture was time taken and slow. Research on *in vitro* germination of terrestrial orchid seeds is often a long, slow process due to the considerable periods of time required for germination to occur. Conclusively this study indicate that this species is slow growing and require

more than one year for their complete process that is, seed germination to plantlet formation under *in vitro* condition. After these difficulties, we got a little success in relation to *in vitro* propagation of this endangered species which could be successful efforts towards *ex situ* conservation strategy of this species.

In this study propagation through vegetative means (tuber segments) was found to be successful (38.88% rooting). This is a general agreement and reported in several plant species such as, *Acacia concinna* (Reddy et al., 1998), *Pongamia pinnata* (Negi and Tewari, 1984), etc. The multiplication of plants through vegetative means offers cost-effective mass production of pathogen free uniform plants of elite genotype and could be useful in selection, genetic improvement programmes and conservation efforts of genetic diversity within a species (Mesen et al., 2001). The advantage of vegetative propagation is also that the desirable traits are perfectly preserved (Kesari et al., 2010). Raising the crop through tuber segments is an additional method for obtaining propagules and is advantageous because it eliminates difficulties associated with seed germination and seedling survival and being a clonal method of propagation avoids variation found in the seedling populations.

This present study emphasizes that by using different auxins treatments, we could be able to obtain this high value endangered alpine orchid species at lower elevation. However, this present study also indicates that the rate of vegetative propagation by using tuber cuttings of this species is not so high at lower elevation but it could be enhanced by standardizing more concentration regimes of auxins and suitable environmental conditions at lower elevation. Only apical segments of tuber cuttings were sprouted and induced rooting. However, this study is helpful in propagation and conservation of this endangered orchid at lower elevation and rooting was obtained in some treatments which could be helpful in propagation of this species.

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