OPTIMIZATION OF LIQUID MEDIUM FOR INCREASING THE BIOMASS OF *DACTYLORHIZA HATAGIREA* (D.DON)

Enrollment Number - 123819

Name of student - Devanshi Popli

Name of supervisor- Dr. Hemant Sood

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY, WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitled "**Optimization of liquid medium for increasing the biomass of *Dactylorhiza hatagirea*"** pursued by **DEVANSHI POPLI** (123819) in partial fulfillment for the award of degree Bachelor of Technology in Biotechnology from Jaypee University Of Information And Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other university or institute for the award of any degree or appreciation.

Dr. Hemant Sood

Assistant Professor (Senior Grade)

Department Of Biotechnology And Bioinformatics

Jaypee University Of Information And Technology, Waknaghat

Dist- Solan, H.P.- 173232

Email: hemant.sood@juit.ac.in

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I express my thanks to Dr. Rajinder Chauhan , Head, Department of Biotechnology and Bioinformatics, Jaypee University Of Information and technology. And of course Mrs. Mamta Mishra and other staff members for their constant help and support.

DEVANSHI POPLI

DATE:

SUMMARY

The orchid *Dactylorhiza Hatagirea*, a critically endangered species, is a medicinal orchid used to cure various diseases including dysentery; diarrhea; chronic fever; cough; stomach ache; wounds; fractures; cuts; burns and general weakness. It is indigenous to the Himalayas and exclusively found in the Ladakh (altitude -3000 meters) region of Jammu and Kashmir, India. They are of great value to floriculture industry as cut flowers and potted plants. The field grown microshoots were taken and cultured on both liquid and solid MS media, and maximum biomass (6.29 ± 0.20) was obtained in plantlets cultured in liquid media containing BAP (3mg/l) and IBA (4mg/l). Mass multiplication of shoots and roots was observed. The in- vitro grown plantlets were again sub-cultured on fresh media for further mass multiplication. Plantlets containing 2-3 shoots were transferred to potting mixture containing coco peat, vermiculite and perlite (1:1:1), for acclimatization to field conditions and further multiplication. One hundred percent survival was obtained after one month of the transplantation in the green house. In present study the culturing conditions of *Dactylorhiza hatagirea* has been successfully optimized. This study holds the robust potential in large scale production of the plant and secondary metabolite production.

DEVANSHI POPLI

DATE:

DR. HEMANT SOOD

DATE:

LIST OF ABBREVIATIONS

Abbreviations	Full forms
KN	Kinetin
IBA	Indole-3-butyric acid
GA_3	Gibberellic acid
BAP	6- Benzylaminopurine
NAA	Napthalene acetic acid
2,4-D	2,4 dichlorphenoxyacetic acid
IAA	3- indoleacetic acid
MS	Murashige and skoog

CHAPTER -1

INTRODUCTION

CHAPTER-1

1.1 INTRODUCTION

Dactylorhiza hategirea (D. Don) ***Soo*** is a monocotyledonous species of family Orchidaceae. It is also known as *Panch aunle, Hatajadi* (Nepali), *Aralu, Salap* (Sanskrit), *Ongu lakpa* (Sherpa) and *Lob.*

1.1.1Distribution:

It is a Himalayan endemic medicinal orchid which is found in Hindu Kush Himalaya range. Its occurrence is sub-alpine and alpine zones from 2800-4200 m above from sea level. Other than Nepal Himalayas, it occurs in the same altitudinal ranges of India, Pakistan, Bhutan and China also. In India, it is reported from Jammu and Kashmir, Sikkim, Arunachal Pradesh, Uttarakhand and Himachal Pradesh .

1.1.2 Morphological characteristics:

It is a terrestrial, erect herb, up to 60 cm high, with palmately divided tuberoids.

LEAVES: Leaves are broadly lanceolate or oblong-lingulate or elliptic.

FLOWERS: Flowers are 1.7 to 1.9 cm long with curved spur, purplish-lilac, rose or rarely white and in many-flowered plant, cylindrical inflorescence is observed. The inflorescence consist of a compact raceme with 25 to 50 flowers developed from axillary buds.

ROOTS: The plant store a large amount of water in their tuberous roots to survive under arid conditions.

SEEDS: It is propagated by seed and rhizomes. Seeds are very small (dusty) and contain few food reserves. Seed germination in nature is very poor, i.e. 0.2% to 0.3%. 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. Seeds require symbiosis with mycorrhiza for germination .

The flowering Period of this plant is June-July and the fruiting Period is August-September.

1.1.3 Indigenous uses:

It is a high value medicinal orchid used in Indian system of medicine, particularly ayurveda, siddha, and unani medicine. Tubers are sweet, cooling, emollient, astringent, demulcent, nervine,

The classification of *D. hatagirea* is as follows:

Kingdom: Plantae

Division: Angiosperms

Class: Monocots

Order: Asparagales

Family: Orchidaceae

Subfamily: Orchidoideae

Tribe: Orchideae

Sub tribe: Orchidinae

Genus: *Dactylorhiza*

Species: *hatagirea*

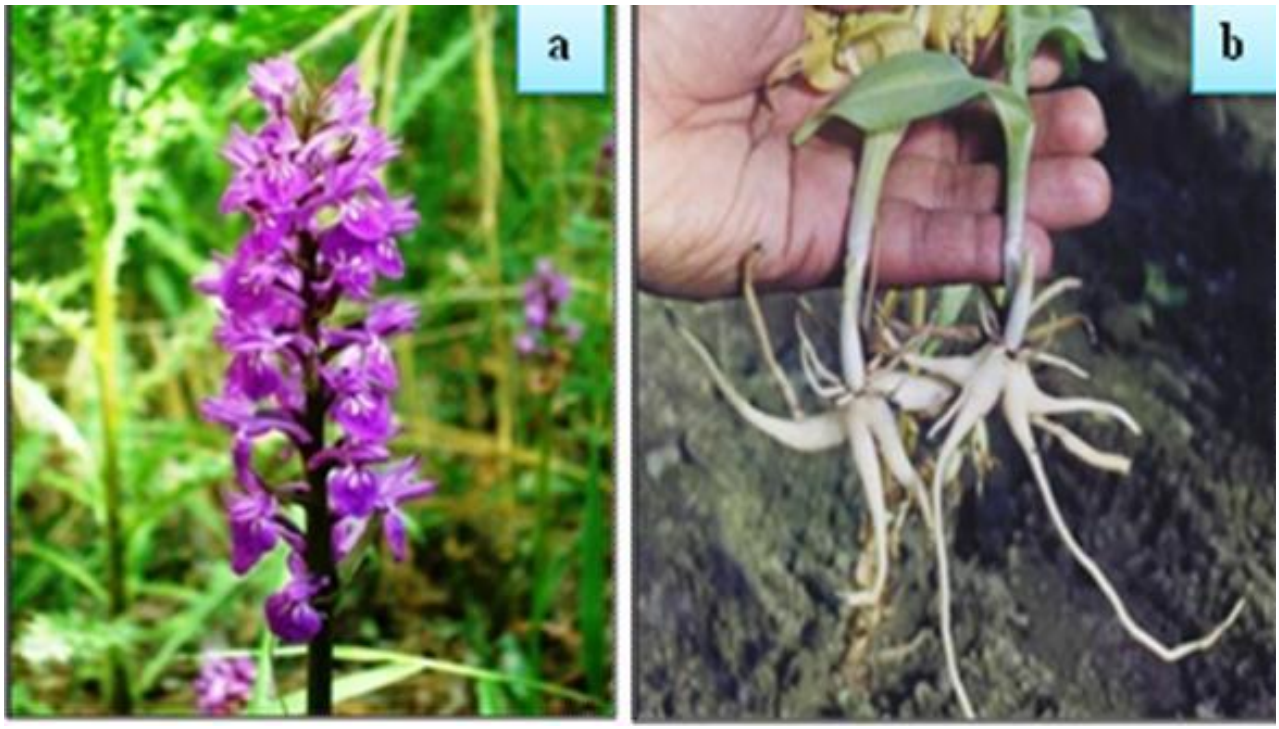

Figure 1.1 Plant morphology of *D. hatagirea* a. Inflorescence b. Tuber (Ashish warghat et al, 2014)

and rejuvenating tonic. They are useful in diabetes, hemiplegia, dysentery, phthisis, chronic diarrhoea, seminal weakness, neurasthenia, cerebropathy, emaciation and general debility. A decoction of tuber is given in colic pain. Powder is used to relieve fever; it is sprinkled over wounds to check bleeding. Root is also used in urinary troubles; also used as farinaceous food. The bulbous roots of Dactylorhiza hatagirea which are synonymous to the tubers of Orchis macula (Orchidaceae) and serve as source of **Salep**, are used traditionally in Indian subcontinent specially in the Northern region and Nepal as aphrodisiac and sexual stimulant. It is considered as a nutritive and restorative tonic and also as an alternative source of Salep used very commonly in Europe.

1.1.4 Chemical composition:

Tubers contain a glucoside-loroglossin, a bitter substance, starch, mucilage (45%), albumen, phosphate(2.7%), chloride, a trace of volatile oil and ash. Chemically, dactylorhins A - E, dactyloses A and B and lipids etc. are found as major constituents.

1.1.5 Pharmacological activity:

It has been found that rhizomatous part of *D. hatagirea* has shown resistanceagainst all Gram positive and Gram negative bacteria, but its aerial part has shown limitedresistance against some bacteria. Zonation of inhibitions (ZOIs) between two parts of *D. hatagirea* indicates that the rhizome part is more effective than the aerial part against all tested organisms, except *E. coli*. Further, it is interesting to note that *E. coli*, one of the very resistant bacteria to synthetic drugs, was found to be very susceptible to the extract of this plant. This finding is distinctive from the folkloric uses of *D. hatagirea*. Hence, this plant can be a potential source for evolving newer antimicrobial compounds for treating dysentery caused by *E. coli*. *D. hatagirea* showed effectiveness in improving and preventing functionality of sexual organ and may be helpful in improving the sexual behavior and performance also. The results also corroborate the hype that the plant is capable of being nominated as herbal cure for sexual dysfunction. A study gave evidence that plant increased testosterone level in adult male rats. Clinical data on testosterone also suggested that slightly increased level of testosterone in adult male's results in increased sexual desire and arousability.

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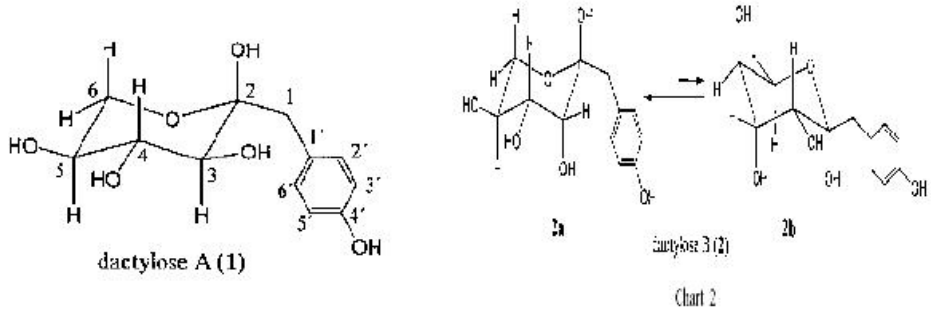

Figure 1.2 Structure of dactylose(Ashish warghat et al, 2014)

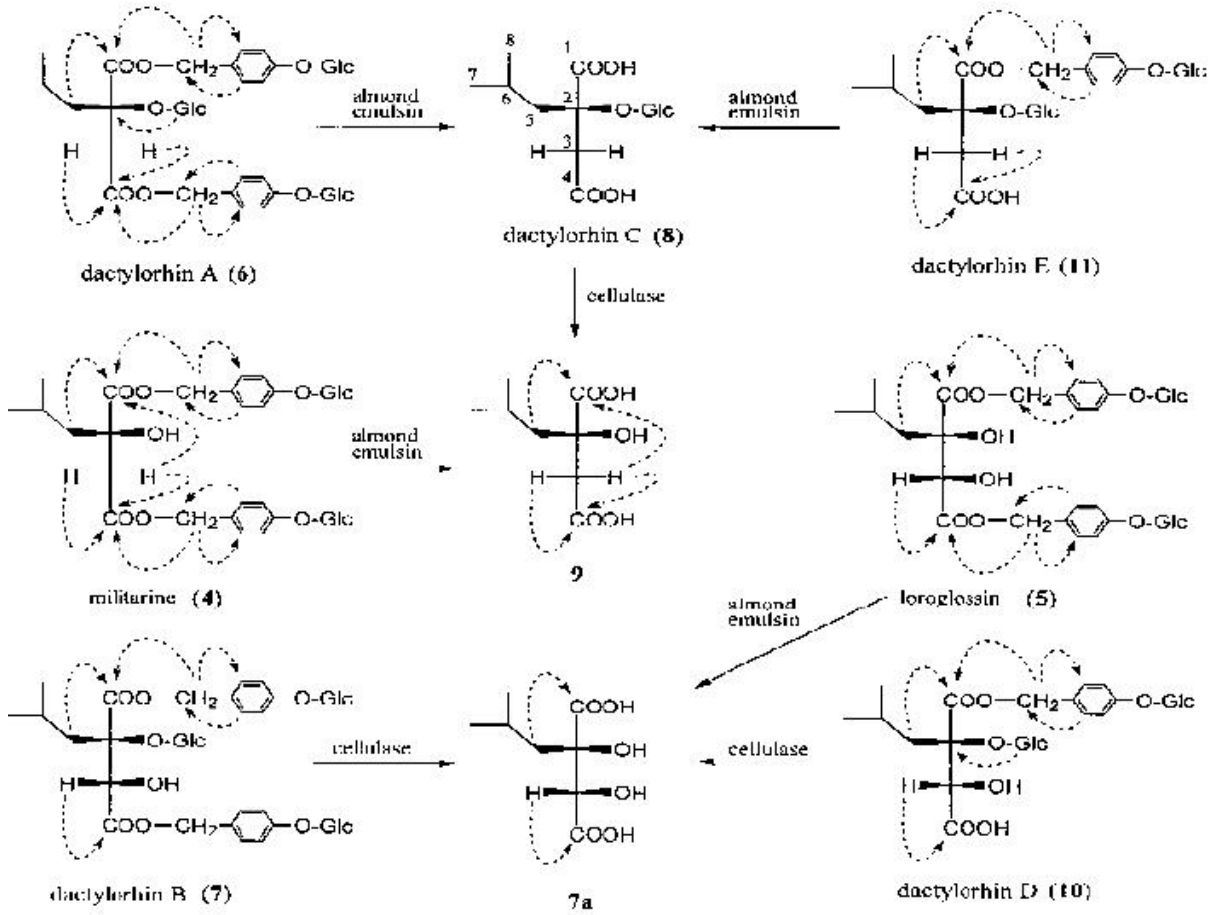

Figure 1.3 Structure of Dactylorhin (A to E) (Ashish warghat et al, 2014)

1.1.6 Conservation Status:

According Forest Act 1993, and Forest Regulation 1995, the rhizome of *D. hatagirea* is banned to collect, trade and process. If the collection is done with the government authority, the government royalty is Rs. 500 per piece according to Forest Regulation 1995 and its amendment 2005. MFSC, Department of Plant Resources, Kathmandu has listed the plant under national priority species of medicinal herbs for cultivation and conservation. Convention on International Trade in Endangered Species (CITIES) and Conservation Assessment and Management Plan (CMAP) have listed the plant under endangered and vulnerable species respectively.

1.1.6.1 Ex-situ Conservation:

It is propagated by seed and rhizomes. A gentle slope, open moist areas, humus soil, well drained sandy soil with high organic matters is suitable. The light humid, moist climate of alpine and subalpine is suitable. Collected seed are shown in nursery bed during April- May at the spacing of 40-60 cm for each seedling. The dried yield in natural condition is found to be 250-300 kg from a hectare. 5 kg of seed is required for one hectare of land for cultivation. 15-20 tonnes/ ha compost fertilizer is required for manure. During cultivation 3 to 4 hoeing and weeding is necessary. The germination percentage is 85-90%.

1.1.6.2 In-situ Conservation:

For sustainable harvesting, collection of rhizome is done only after flowering of plants. Collection of mother plant takes place by leaving l immature tubers by filling with layer of soil with the help of sharp *kuto* (a small spade like hand tool). Harvesting period is September to November after seed ripening and fall. Proper care of the surrounding vegetation should be taken while rooting out the tubers of the *D. hatagirea*. Collection of plant should be done by applying rotating system. The rotation of the plant is 4-5 years for harvesting. Sustainable harvestable amount is 80%. Both ex-situ and in-situ approaches are important for the protection of rare and endangered orchid species. The threat to the species is due to some anthropogenic activities , low rate of propagation , poor seed germination and as well as due to habitat destruction.

Tissue culture is one of the most important measures in ex-situ conservation of terrestrial orchid. Knowledge of the physiochemical and morphological aspects of the germination and development of a particular orchid species is of critical importance for the establishment of tissue culture.

The current study reports for the optimization of liquid MS media for increasing the biomass of endangered *Dactylorhiza hatagirea*. This holds a robust potential for large scale propagation and secondary metabolite production which is required to meet the demand of national and international market. According to a report, the annual demand of this species is approximately 5000 tons. This leads to over-exploitation of the species from wild habitat. Local inhabitants collect this high value medicinal plant for illegal trading. The local inhabitants could collect Rs. 100 to 200 per kg of dried roots of *D. hatagirea.* For 1 kg of dried roots, 90 to 100 mature plants are exploited. As a result, so many areas are there where *D. hatagirea* is present in abundance, but now, a few individuals of this species are seen. This indicates that if the casual factors continue to operate, this species may become extinct within a few years. During the survey, it was also observed that local inhabitants carry their livestock in the higher regions of the valley for grazing. This is another level of disturbance, because due to the grazing and trampling, the under-ground part of the *D. hatagirea* get exposed or removed. These levels of disturbances, like grazing pressure, over exploitation and unawareness of proper procedure of collection and propagation, etc., are the other major factors for declining of this species from its natural habitats.

CHAPTER-2

REVIEW OF LITRATURE

2.1 **Population status of *Dactylorhiza***

Rinchen et al [2012] studied population census of *D. hatagirea* in Suru valley of Jammu and Kashmir, India. He found 11 associated species across the study sites and out of forty eight sites, only sixteen sites showed its presence. They found least density of *D. hatagirea* (8.00 and 6.1 ind/m2) among other associates. The maximum density was found in *Equisetum* sp. (3.45 to 17.2 ind/m2) followed by *Polygonum* sp. (2.6 to 4.35 ind/m2), *S. chrysanthemoides* (2.05 to 5.75 ind/m2), *M. lupalina* (1.75 to 5.6 ind/m2), *R. hirtellus* (1.5 to 4.55 ind/m2) and *P. hexandrum* (1.95 to 3.65 ind/m2).

Giri et al [2008] studied analysis of associated vegetation and present status of *D. hatagirea* in Garhwal Himalaya, India. He found 24 herb species across the study sites and out of six study sites, only two sites showed its presence. The maximum density was found in *P. alpinum* L. (141.52-201.28 ind/m2) followed by *G. trichophylla* Royle, (14.2-75.0 ind/m2), *D. cachyemyriana* Jaub. and Spach, (8.32-40.32 ind/m2), *P. depressa* Willd.(15.0-58.4 ind/m2) and *A. aptera* DC (2.80- 32.2 ind/m2) and dominant herbs in all study sites. *D. hatagirea* showed minimum density (0.70- 1.8 ind/m2) in all study sites.

D. hatagirea is facing extremely high risk of extinction due to overexploitation of tubers for medicinal purposes. Therefore, it is listed into the Indian Red Data Book. Near about all the species of *Dactylorhiza* spread world-wide are listed in red data book. Therefore, there is urgent need of conservation. *In situ* conservations including complete ban of its collection from its natural habitat must be implemented at an early date. The standardization of agro-technologies and cultivation in new areas will give further boost to its conservation. The orchids are propagated through vegetative means as well as seeds. However, the rate of vegetative propagation of *D. hatagirea* is very slow and seed germination in nature is very poor, i.e. 0.2% [Vij, 2002]. Therefore, *in vitro* germination of seeds and micro propagation technology are an important part in orchid multiplication and conservation programmes.

2.2 Tissue culture of *Dactylorhiza*

Znaniecka and lojkowska [2004] used mature seeds for establishment of *in vitro* collection of endangered European *D. majalis*. *D. majalis* seeds started to germinate after 6 weeks of culture on Fast medium.

Vaasa and Rosenberg [2004] used semi ripened seeds for preservation of *D. ruthei and D. praetermissa*. *D. ruthei* and *D. praetermissa* seeds started to germinate after four months of culture on Norstog medium.

Rajasekaran et al [2009] studied multiplication and conservation of *Dactylorhiza hatagirea* and found satisfactory results.

Aggarwal and Zettler [2010] used green capsule for reintroduction of *D. hatagirea* symbiotically. They found 100% germination within 10 days of sowing and seedling after 3 months.

Giri and Tamta [2012] used green pod culture for conservation of *D. hatagirea*. They have tried Knudson C (KC), Murashige and Skoog (MS), Vacin and Went (VW) and Vejsadova (VJ) media for seed germination and found better response in MS medium supplemented with peptone (P) (1.0 g/L), morphoinoethane sulphonic acid (MES) (1.0 g/L) and activated charcoal (AC) (0.1%).

Warghat et al [2014] used seeds for in vitro protocorm development and mass multiplication of an endangered orchid, *D. hatagirea.* The surface sterilized immature seeds were cultured on germination medium. The germinated seeds further differentiated into protocorm like bodies on the same medium. The protocorm like bodies were maintained on optimal basal germination medium for further differentiation to form leaf primordia. The protocorm like bodies with leaf primordia were then cultured on regeneration medium containing MS and BM-2 media supplemented with combinations of plant growth regulators (IBA-3mg/l and KN-3mg/l. The resulting microshoots were separated from regeneration medium for mass multiplication. Maximum root number, maximum root length, maximum shoot length and maximum shoot number was observed. Plantlets with 2-3 shoots were transferred to potting mixture of cocopeat, vermiculite, and perlite (1:1:1) for acclimatization , which produced 75 shoots after 1 month of transplantation in greenhouse.

The present study is carried out with the objective of optimizing the liquid MS medium for increasing the biomass of *Dactylorhiza hatagirea*.

CHAPTER- 3

MATERIALS AND METHODS

3.1 MATERIALS AND METHODS

3.1.1 Plant Source:

The young micro-shoots were collected from the field grown plants of *Dactylorhiza* which were kept in glass house of Department Of Biotechnology, JUIT, Waknaghat and maintained under natural conditions.

3.1.2 Selection Of Explants And Culture Conditions:

Shoot apex, micro-shoot and regenerated shoot from the plant can be used as explants. We have used developed micro shoot from the field, as explants which were further surface sterilized and cultured on tested liquid and solid medium for their micro propagation under culture conditions 25±2° C and 60%-70% humidity.

3.1.3 Nutrient media:

Six MS media, solid and liquid both, with different concentration of plant growth hormone were prepared for in-vitro mass multiplication of micro shoots. Plant growth hormones used were IBA, BAP and KN, but the concentrations were different as shown in table - 3.1. Sucrose and agar (for static media) was added to MS media containing plant growth hormone and volume was raised tad lesser than the required volume. pH was set between 5.6- 5.7 using 0.1N HCl and 0.1N NaOH and volume was raised to the required scale. Now, 30- 40ml of the suspension media and 45- 50ml of static medium (without agar) was dispersed into the culturing jars and autoclaved for 20 minutes at 121°C and 1.05kg /cm² pressure.

3.1.4 Surface sterilization of micro shoots and inoculation:

Micro shoots were taken from the field and washed with laboline followed by tap-water followed by rinsing in distilled water (2-3 times). After that, micro shoots were taken to well set laminar air flow where further sterilization was done using 0.5% bewestin and 0.1% mercuric chloride. Micro shoots were kept in bewestin for 3-4 min and washed with distilled water 2-3 times. After that, they were kept in mercuric chloride for 30- 60 seconds and again washed with distilled water 2-3 times.

These surface sterilized micro shoots were then cultured on different MS media with agar and without agar, supplemented with different concentrations of plant growth hormones and cultures were incubated in a growth chamber maintained at 25±1°C under a 16/8-h photoperiod with illumination of 3000 lx intensity of white light.

3.1.5 Micro propagation and mass multiplication:

The plantlets were successfully developed from cultured micro shoots. For multiplication of plantlets, the liquid MS media supplemented with combination of plant growth regulators BAP (2-5mg/L) and IBA (1-5mg/L) and static MS media containing agar supplemented with IBA (3mg/L) and KN(1mg/L) was tested. The resulting in- vitro shoots were separated from respective media for sub culturing, for further mass multiplication. During mass multiplication, data were recorded for shoot length; shoot number; root length root number and number of days required for shoot and root formation after 28 days. The well developed plants were maintained on the same medium before transferring for hardening.

3.1.6 Acclimatization and in-vivo multiplication of plantlets:

Fully grown plantlets were taken from the medium and washed with tap water. Plantlets with 20-25cm shoot length, with 10-15 roots, were successfully transplanted to controlled greenhouse conditions into the potting mixture containing cocopeat, vermiculite and perlite in 1:1:1 ratio. Initially, for 15-20 days the plants were covered with the glass jars to provide them the sufficient humid environment and avoid desiccation until the plantlets show new growth. During the hardening process, the glass jars were taken off every day for 1-2 hour so as to acclimatize the plantlets to the external environment and data was recorded for percent survival, number of shoots and roots, and length of shoots and roots.

3.1.7 Data analysis:

Statistical procedures were used to analyze the data. Mean and standard deviation was calculated.

CHAPTER-4

RESULTS

4.1 Results and Discussions:

4.1.1 Shoot formation and multiplication:

The micro shoots developed into plantlets with multiple shoots and roots within 14 to 18 days of incubation in media. Growth and development of plantlets with maximum number of shoots (43.50±0.04), shoot length (31.06±0.63), number of roots (15.00±0.52),maximum root length (14.20±0.24) and maximum biomass (6.29 ± 0.20) occurred on MS medium supplemented with IBA (4 mg/L) + BAP (3 mg/L) within 25 to 32 days of incubation. Whereas liquid MS medium containing different concentration of plant growth hormone and MS media containing agar showed relatively less number of shoots and other respective parameters of growth and development until 35 to 42 days of culturing, as shown in table- 4.1 and 4.2.

Table 4.1: Effect of MS media for shoot growth and development in *Dactylorhiza hatagirea.*

MEDIA	NO. OF SHOOTS	SHOOT LENGTH	NO. OF ROOTS	ROOT LENGTH	NO. OF DAYS
MS + 5mg/L IBA+ 4mg/L BAP	10.00 ± 0.63	12.67±0.16	5.45±0.66	4.78±0.34	38-43
MS + 4mg/L IBA + 5mg/L BAP	12.67±0.73	16.00±0.28	5.96±1.06	5.09±1.04	38-46
MS + 1mg/L IBA + 2mg/L BAP	18.34±0.61	17.00±1.39	6.46±1.36	6.92±0.81	35-45
MS + 2mg/L IBA + 3mg/L BAP	24.40±0.50	20.10±0.59	6.89±0.90	6.30.±0.45	32-36
MS + 3mg/L IBA + 4mg/L BAP	30.34±0.06	26.00±0.09	10.09±1.09	7.99±0.07	30-35
MS + 4mg/L IBA + 3mg/L BAP	43.50±0.04	31.06±0.63	15.00±0.52	14.20±0.24	28-32

*Data shown are the mean of 3 replicates ± S.E.

Figure 4.1 A) Cultured micro shoot in liquid MS media on first day. B) Excessive shooting in liquid MS media on 19th day of culturing.

Figure 4.2 A) Cultured seedling on solid MS media on first day. B) Complete plantlet formation on 31st day of culturing.

Figure 4.3: A) and B) shows in- vitro plantlet formation on 28th day of culturing on liquid MS media having roots and shoots.

According to a report there is an efficient and reproducible procedure outlined for rapid in vitro multiplication of two commercially important orchids *Cymbidium aloifolium* and *Dendrobium nobile* through high frequency shoot proliferation from thin cross sections (TCSs) of protocorm-like bodies (PLBs). The TCS were cultured on Murashige and Skoog's medium supplemented with any of the three cytokinins such as zeatin riboside (ZR), N^6-benzyladenine (BA) or kinetin (Kn). PLB development from TCS explants in both the species was enhanced by the use of suspension culture.

Therefore, liquid media gives a booming response as compared to the solid media and thus liquid media are far better than the solid media.

4.1.2 Mass multiplication and in-vivo transplantation

In-vitro grown plantlets from liquid as well as from solid control MS media were collected and were transferred to a potting mixture containing vermiculite, perlite and cocopeat in 1:1:1 ratio for their acclimatization to field conditions and for further growth and multiplication. 100% plant survival was found with high shoot number, high root number, high shoot length and high root length, after 28 days of transplantation as shown in figure.

Table-4.2 Comparative analysis of biomass generated from liquid medium and solid medium.

Parameters	**Liquid medium (MS media without agar)**	**Solid medium (MS media with agar)**
Number of shoots	43.50±0.04	16.09 ± 0.40
Shoot length	31.06±0.63	13.00 ± 0.34
Number of roots	15.00±0.52	5.67 ± 0.89
Root length	14.20±0.24	7.88 ± 0.31
Plant biomass	6.29 ± 0.20	1.76 ± 0.01

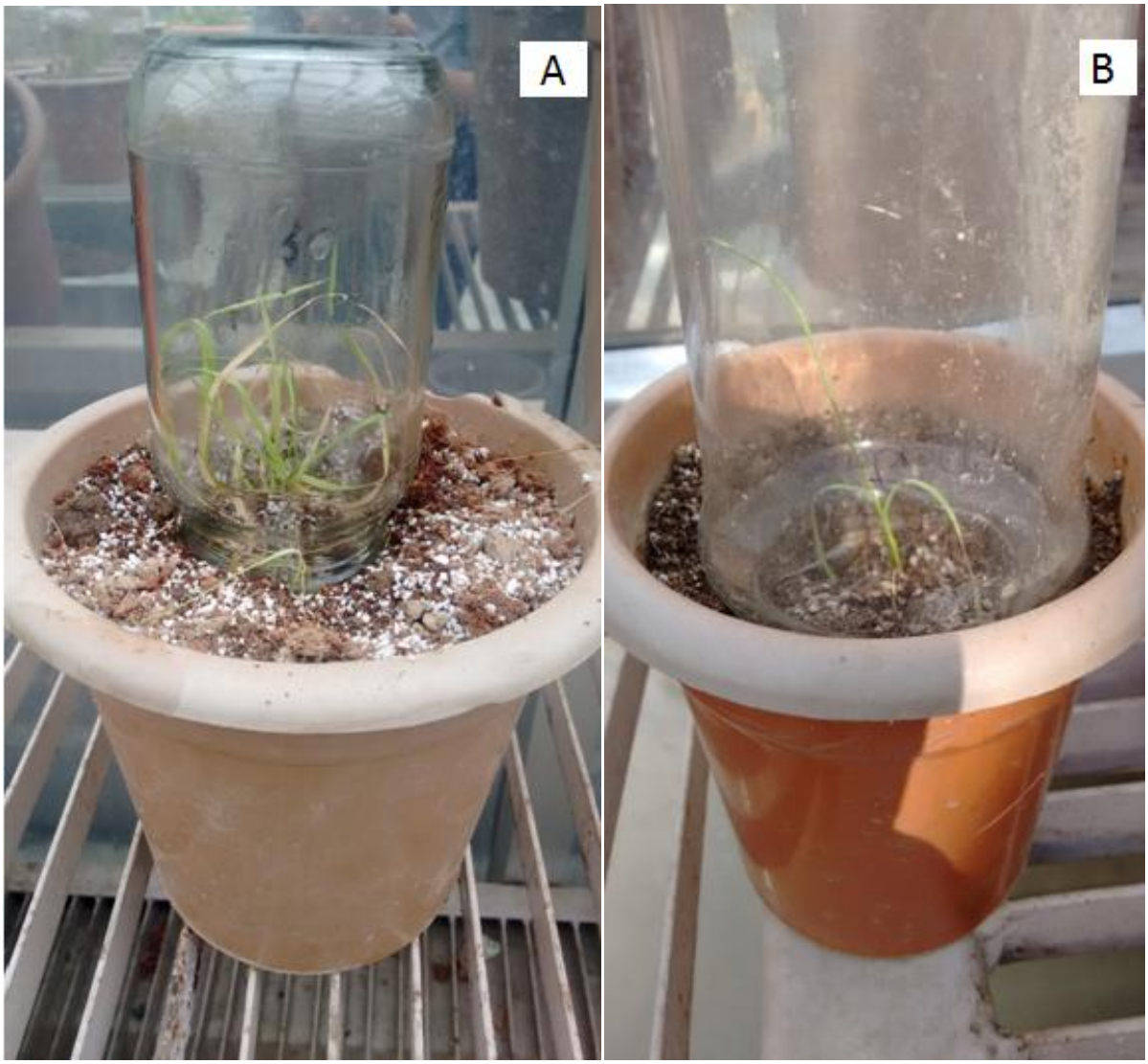

Figure 4.4: A) Transplantation of plantlet obtained from liquid medium, kept in green house for acclimatization. B) Transplantation of plantlet obtained from solid medium, kept in green house for acclimatization.

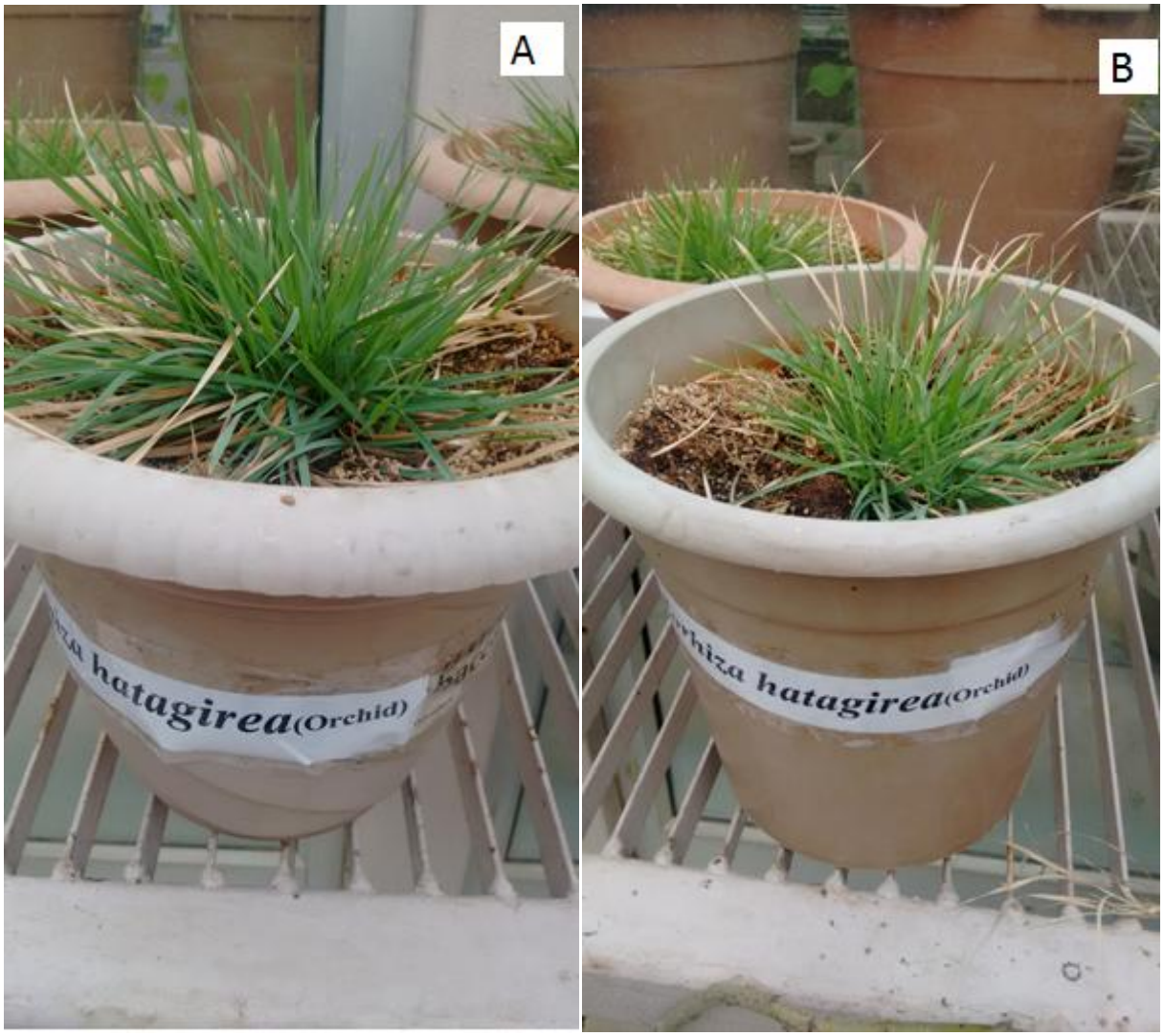

Figure 4.5: A) and B) show mass multiplied *Dactylorhiza hatagirea* ready for transfer to field conditions.

4.2 Conclusion:

Dactylorhiza hatagirea is a plant from Hindu Kush Himalayan region and is critically endangered species. Thus, very less population of this plant is available, only in the some pockets of Hindu Kush Himalayan region. Therefore, the plant is required to be propagated so that its population can be increased and conserved. As the plant is having low seed germination rate (0.2%) and low seed viability (0.1%-0.3%, as reported by warghat et al, 2014), therefore, alternative technologies are needed to be developed so that plants can be multiplied and conserved. This study describes the rapid in-vitro mass-production protocol of *Dactylorhiza hatagirea* through micro propagation. Under this study, I have optimized that liquid media which gives us four times increased fold of biomass, as compared to solid media, which could be used as a platform for its conservation and its mass propagation. This technique holds a robust potential in mass multiplication and propagation of *Dactylorhiza hatagirea* and secondary metabolite production as this plant contains glucoside-loroglossin, a bitter substance, starch, mucilage (45%), albumen, phosphate(2.7%), chloride, a trace of volatile oil, ash and chemically, dactylorhins A - E, dactyloses A and B and lipids, therefore, this technique can be utilized by pharmaceutical industries. This technology will help not only in multiplying the plantlets but can also play a major role in the conservation of this endangered orchid species.

PUBLICATIONS:

Devanshi Popli and Dr. Hemant Sood, 2016. Optimization of liquid media for increasing the biomass of *Dactylorhiza hatagirea*. 3rd prize in IEEE project presentation. 14th may 2016. JUIT, Waknaghat, Solan

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ANNEXURE

ANNEXURE

Composition of Murashige and Skoog basal medium (MS MEDIUM)

STOCKS	CHEMICALS	ORIGINAL STRENGTH(mg/l)	STOCK(g/l)	FINAL VOLUME
A-10X	KNO_3	1900	19	
	$MgSO_4.7H_20$	370	3.7	100 ml/l
	KH_2PO_4	170	1.70	
B-20X	NH_4NO_3	1650	33	150 ml/l
C-100X	$CaCl_2.2H_2O$	440	44	10 ml/l
D-100X	Na_2EDTA	37.26	3.72	10 ml/l
	$FeSO_4.7H_2O$	27.85	2.28	
E-100X	KI	0.83	0.083	100 ml/l
F-100X	H_3BO_3	6.2	0.62	
	$CoCl_2.6H_2O$	0.025	0.0025	
	$ZnSO_4.7H_2O$	8.6	0.86	10 ml/l
	$CuSO_4.5H_2O$	0.025	0.0025	
	$MnSO_4.4H_2O$	22.3	2.23	
	$Na_2MbO_4.2H_2O$	0.25	0.025	
G-100X	m-INOSITOL	100	10	10 ml/l
	GLYCINE	2	0.2	
H-100X	PYRIDOXINE-HCL	0.5	0.05	
	NICOTINE ACID	0.5	0.05	10 ml/l
	THIAMINE	0.1	0.01	