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Optimization of Liquid MS Medium for Enriching Biomass of *Dactylorhiza Hatagirea*

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Abstract: *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 plants were taken and cultured on two different liquid MS media, one containing indole-3-butyric acid (IBA) (3 mg/l) and kinetin (KIN) (2 mg/l) and other containing 6-benzylaminopurine (BAP) (3 mg/l) and indole-3-butyric acid (IBA) (4 mg/l) and compared with the static media as control containing agar (8.5g/L). 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 indole-3-butyric acid (IBA) (4 mg/L) + 6-benzylaminopurine (BAP) (3 mg/L) within 25 to 32 days of incubation. 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 cocopeat, vermiculite and perlite (1:1:1), for acclimatization to field conditions and further multiplication. Successful survival was obtained after one month of the transplantation in the green house. In present study the culturing conditions for large scale multiplication of Dactylorhiza hatagirea has been successfully optimized. The current study possesses the robust potential in large scale propagation of this plant and its's secondary metabolite production.*

Keywords: *Dactylorhiza hategirea, MS media, indole-3-butyric acid , kinetin, 6-benzylaminopurine, indole-3-butyric acid , shoot length , shoot number, root length, root number, biomass.*

INTRODUCTION

Dactylorhiza hategirea (D. Don) *Soo* is a monocotyledonous species of family Orchidaceae. It is also known as Panch aunle, Aralu, Salap (Sanskrit), Lob, Hatajadi (Nepali) and Ongu lakpa (Sherpa). It is a Himalayan endemic medicinal orchid and occurs in sub-alpine and alpine zones from 2800-4200 m above from sea level. It can be commonly found in Jammu and Kashmir, Sikkim, Arunachal Pradesh, Uttarakhand and Himachal Pradesh of India and other countries like Pakistan, Bhutan and China [9]. It is a terrestrial, erect herb, up to 60 cm high. This plant has a high value as a medicinal orchid and is being used in Indian system of medicine, particularly ayurveda, siddha, and unani medicine [8]. Tubers are sweet, cooling, emollient, astringent, demulcent, nervine, and rejuvenating tonic.

They are useful in diabetes, dysentery, phthisis, chronic diarrhoea, seminal weakness, neurasthenia, cerebropathy, emaciation and general debility [11]. A decoction of tuber is helpful in colic pain. Its powder is used to relieve fever; it is sprinkled over wounds to check bleeding. Root is also used in urinary troubles and 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

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traditionally in Indian subcontinent specially in the Northern region and Nepal as aphrodisiac and sexual stimulant [2]. It is considered as a nutritive and restorative tonic. Tubers contain a glucoside-loroglossin, a bitter substance, starch, mucilage (45%), albumen, phosphate (2.7%), chloride, a trace of volatile oil and ash [3]. Chemically, dactylorhins A-E, dactyloses A and B and lipids etc. are found as major constituents [4]. 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 [10]. The risk of extinction to the species is due to some anthropogenic activities, low rate of propagation, poor seed germination and as well as due to habitat destruction [5]. The annual demand of this species is approximately 5000 tons [1]. This leads to over-exploitation of the species from wild habitat. Local inhabitants collect this high value medicinal plant for illegal trading. 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 plant has been listed as endangered and vulnerable species by Convention on International Trade in Endangered Species (CITIES) and Conservation Assessment and Management Plan (CMAP) respectively [12]. Both ex-situ and in-situ approaches are important for the conservation of this plant.

Tissue culture techniques can help in its conservation. 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 [7]. In-vitro protocorm development and mass multiplication of this plant has helped in conservation and increase in biomass of this plant, using solid MS media [11]. The current study aimed at optimization of liquid MS media for increasing the biomass, by much greater fold, of endangered *D. hatagirea*. This study possesses a robust potential for large scale propagation and secondary metabolite production of this plant which is required to meet the demand of national and international market. Therefore, the current study aimed at optimization of liquid MS media for increasing the biomass of endangered *D. hatagirea*.

MATERIALS AND METHODS

Plant Material

The young micro-shoots were collected from the field grown plants of *D. hatagirea* which were kept in glass house of Department Of Biotechnology, JUIT, Waknaghat and maintained under natural conditions. Micro shoots of *D. hatagirea* were used as explants. These shoots were surface sterilized using 0.5% bavistin and 0.1% mercuric chloride and cultured on different combinations of liquid and solid media.

Preparation of Media

Six Murashige and Skoog Media (MS media)[6], solid and liquid both, with different concentration of plant growth hormone were prepared for in-vitro mass multiplication of microshoots. Plant growth hormones used were IBA, BAP and KN, but the concentrations were different as shown in table-1. Sucrose was added to MS media containing plant growth hormone. pH was set between 5.6-5.7 using 0.1N HCl and 0.1N NaOH and volume was raised to the required scale. Agar was added to required media and media was heated until the clear solution was formed. 50 ml of the suspension media and static medium (without agar) was dispersed into the culturing jars and autoclaved for 20 minutes at 121°C and 1.05kg/cm² pressure. The 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, with relative humidity 60-70%.

Establishment of Axenic Cultures and Shoot Proliferation

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 and parallel control experiments were conducted where solid media with same composition was used for comparing growth. After forming well grown shoots, in-vitro rooting was induced to develop complete plantlets. 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.

Hardening and Acclimatization

Fully grown plantlets were taken from the medium and washed with tap water. Plantlets with 20-25 cm 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.

Data Analysis

All the experiments were done in triplicated and repeated thrice. Data was recorded as Mean ± standard deviation.

RESULTS AND DISCUSSIONS

Shoot Proliferation and Plantlet Development

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 1.

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

Table 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 + 5 mg/L IBA+ 4 mg/L BAP	10.00 ± 0.63	12.67 ± 0.16	5.45 ± 0.66	4.78 ± 0.34	38-43
MS + 4 mg/L IBA + 5 mg/L BAP	12.67 ± 0.73	16.00 ± 0.28	5.96 ± 1.06	5.09 ± 1.04	38-46
MS + 1 mg/L IBA + 2 mg/L BAP	18.34 ± 0.61	17.00 ± 1.39	6.46 ± 1.36	6.92 ± 0.81	35-45
MS + 2 mg/L IBA + 3 mg/L BAP	24.40 ± 0.50	20.10 ± 0.59	6.89 ± 0.90	6.30. ± 0.45	32-36
MS + 3 mg/L IBA + 4 mg/L BAP	30.34 ± 0.06	26.00 ± 0.09	10.09 ± 1.09	7.99 ± 0.07	30-35
MS + 4 mg/L IBA + 3 mg/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 triplicated (repeated thrice) ± S.E.

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*Data shown are the mean of triplicated (repeated thrice) ± S.E.

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 (N. Nayak; S. Sahoo; S. Patnaik; S.P. Rath, 2001). Biomass produced from solid media was much lesser than the biomass produced from liquid media as shown in Table 2.

Table 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

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

Plantlet Propagation in Glass House

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. These plants were kept in green house for their further growth

Figure 1: (A) Cultured micro shoot in liquid MS media containing IBA (4 mg/L) and BAP (3 mg/L) on first day. (B) Excessive shooting observed in liquid MS media on 24th day of culturing.

and propagation as shown in figure 4. 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 5.

CONCLUSION

The current study describes the rapid in-vitro mass-production protocol of *D. hatagirea* through micro propagation. Under this study, liquid media was optimized which gives us four times increased fold of biomass as compared to solid media as shown in table 2, which could be used as a platform for its conservation and its mass propagation. This technique can be utilized by pharmaceutical industries. This technology will help not only in

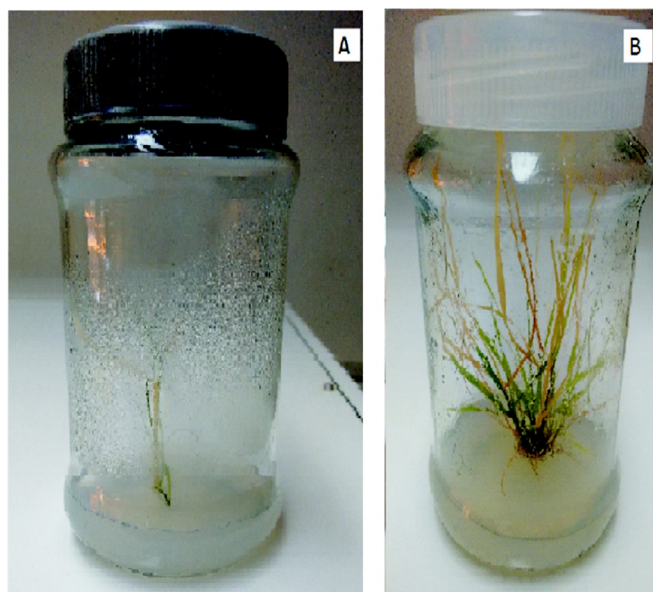

Figure 2: (A) Cultured micro-shoot on solid MS media containing IBA(3 mg/L) and KN(1 mg/L) on first day. (B) Complete plantlet formation on 31st day of culturing.

Figure 3: (A) and (B) shows in-vitro plantlet formation on 28th day of culturing on liquid MS media IBA (4mg/L) and BAP (3mg/L) having roots and shoots.

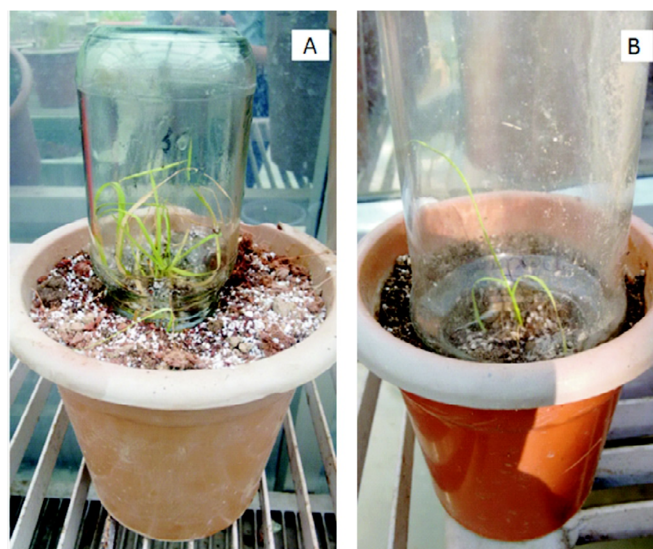

Figure 4: (A) Transplantation of plantlet obtained from liquid medium, kept in green house for acclimatization. (B) Transplantation of plantlet obtained from solid medium kept in green house for acclimatization.

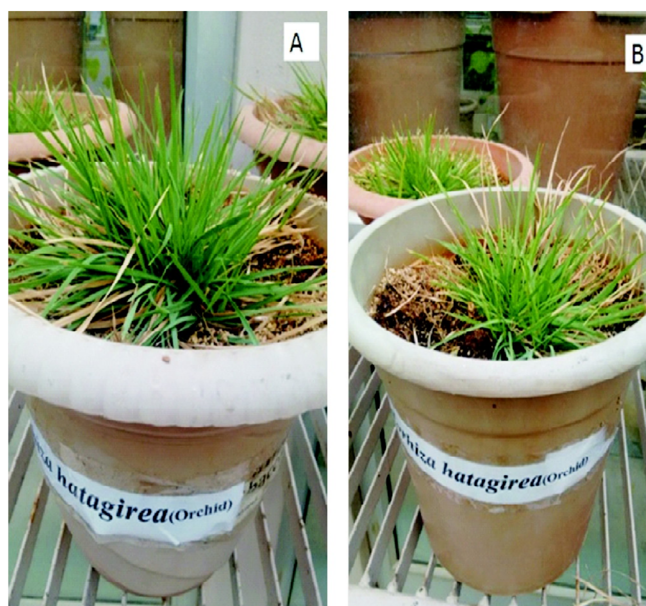

Figure 5: (A) and (B) show mass multiplied *Dactylorhiza hatagirea* ready for transfer to field conditions.

multiplying the plantlets but can also play a major role in the conservation of this endangered orchid species.

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