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IN VITRO ASYMBIOTIC SEED GERMINATION AND REGENERATION COMPETENCE OF LEAF EXPLANTS IN *SATYRIUM NEPALENSE* D.DON, A MEDICINALLY IMPORTANT, AND AN ENDANGERED TERRESTRIAL ORCHID OF KASAULI HILLS, HIMACHAL PRADESH (NORTHWESTERN HIMALAYAS)

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Abstract

Satyrium nepalense, a medicinally important terrestrial orchid, thrives in the Himalayan foothills including Kasauli hills of Himachal Pradesh (NorthWestern Himalayas). Terrestrial orchids are uncommon and vulnerable as they flourish in specialized, sparsely dispersed environments and many important terrestrial species are being destroyed as a consequence of commercial collection pressures and anthropogenic impacts on their specific habitats. Due to its endangered status, mass multiplication protocols were successfully established using *in vitro* asymbiotic seed germination technique and regeneration potential for this species. Mitra *et al.,* 1976 medium was used for both the experiments of seed germination and regeneration. Auxins [Indole 3 Acetic Acid (IAA), Indole 3 Butyric Acid (IBA), 1-naphthalene acetic acid (NAA)] and cytokinins [Kinetin (KN), Thidiazuron (TDZ), 6-benzylaminopurine (BAP)], each at a concentration of 1 mgl^{-1}, were also employed individually or in combinations. Activated Charcoal was also added to the culture media at concentration 2 gl^{-1} in order to track its impact on the growth and development of cultures. The mature seeds from ripe and dehisced capsules were used during the present investigation. Early onset of germination was observed within 10.00±0.40 days on medium containing AC+BAP. IBA when used in AC enriched medium, synergistic action was apparent; seeds germinated within 18.50±0.28 days and healthy seedlings were obtained in 70.25±0.47 days. For regeneration, leaves (0.4-0.6 cm long) procured from 16 wks old *in vitro* grown plantlets were used as explants. Interestingly, regeneration occurred through shoot buds, PLBs and callusing, depending upon the nutrient combination used. The combination containing IAA and AC proved as the optimal nutrient combination for inducing regeneration via both shoot buds and PLBs formation and subsequent development of healthy plantlets with 2-3 tubers (40.25±0.47 days). The main objective of the present study was to ensure better frequency of germination of seeds and reduce the time laps between pollination and sowing of seeds. Leaf explants have been effectively used for *in vitro* propagation of the presently investigated species, *Satyrium nepalense* and such studies may be extended to other related orchid taxa.

Introduction

THE GREAT taxonomic and morphological diversity of orchids is escorted by an incredible array of pollinators and pollination systems (Buragohain and Chaturvedi, 2016; Pal *et al.,* 2019; Prakash and Pathak, 2020b). They have enormous horticultural values and also help to maintain the forest ecosystem's equilibrium (De and Pathak, 2018, 2020; Hegde, 2016; Janakiram and Baskaran, 2018; Kaushik, 1983). They are one of the most cherished plants, ranking first amongst all blooming plants appreciated for cut flower output as well as for potted plants, fetching a premium price on the worldwide market (Kaveriamma *et al.,* 2018; Patnaik *et al.,* 2017; Prakash and Pathak, 2020a; Thammasiri, 2020). In India, there are about 1,256 species and 155 genera of orchids (Singh *et al.,* 2019). Orchids are high in phytochemicals and have long been utilized in traditional healing systems such as Ayurveda, Chinese *etc*. (Balkrishna *et al.,* 2020; Devi *et al.,* 2018; Kumar *et al.,* 2018). Their use as an aphrodisiac and restorative medication, as well as a source of food, gums, glues, and narcotics, is widely known (Arditti, 1992; Kumar *et al.,* 2019; Kumari and Pathak, 2020; Pathak *et al.,* 2010; Prakash and Pathak, 2019). Medicinal plants are of considerable interest to biotechnology researchers since the majority of drug businesses rely on plants for the production of pharmaceutical chemicals (Chand *et al.,* 1997; Jhansi *et al.,* 2019; Joseph *et al.,* 2018). Many popular medications used in clinical practice in many countries today have their origins in herbal remedies.

Satyrium nepalense D.Don commonly called Nepal *Satyrium* is an endangered medicinal orchid found in India's highlands, abundant in the Himalayas from Shimla Eastwards, as well as in the Khasia Mountains and the Deccan Penisula near Travancore. The Todas, who live in the higher Nilgiris, name it *Ezhtkwehhdr*, which means *bullock's horns*. The twin spurs of the blooms resemble bullock horns, giving the flower its name. The dried and powdered tubers of the terrestrial orchid are used as an energy tonic by the Todas of the Nilgiris. Malaria and Diarrhoea are treated using tubers and the entire plant

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by the Mopa tribe (Mahendran and Bai, 2009). As the orchid seeds are devoid of endosperm and the necessary machinery to use their own lipid food reserves, they require a sufficient fungal stimulus for their germination in nature (Hajong and Kapoor, 2016; Lal and Pathak, 2020; Manoharachary, 2019; Thakur *et al.,* 2015); only 0.2-0.3% of them can germinate when they come in contact with an appropriate substratum (Bhatti *et al.,* 2017; Lekshmi and Decruse, 2018). Furthermore, traditional methods of propagation have lost favour in commercial operations since vegetative multiplication by division and back bulb culture is slow-moving and yields just a few plantlets even after 5-6 years; hence this may drive the species to extinction (CITES, 2012; 2013). This constraint will be overcome by *in vitro* asymbiotic seed germination technique utilizing the plant tissue culture technique, which will provide the required inorganic and organic nutrients for seed germination. During the last few years, this technique has been exploited for mass propagation of some of the orchid species (Anuprabha and Pathak, 2019; Arditti *et al.,* 1982; Bhowmik *et al.,* 2020; Decruse and Gangaprasad, 2018; Gurudeva, 2019; Kaur *et al.,* 2017; Madhavi and Shankar, 2019; Pathak *et al.,* 2001, 2010; Thakur and Pathak, 2020). With advancement in plant tissue culture techniques, the current focus is on the utilization of alternative vegetative explants for *in vitro* multiplication of medicinally and economically important species which is especially beneficial in outbreeding plants like orchids, which produce a lot of heterozygozity in their offsprings. The successful regeneration is impacted a lot by the source and physiological age of donor tissue, as well as the nutritional formula used (Vij and Pathak, 1997). There are some reports on evaluating the possibility for plant regeneration utilizing shoot meristems, leaves, roots, rhizomes, and other explants (Anuprabha and Pathak, 2020; Anuprabha *et al.,* 2017; Arora *et al.,* 2016; Bhattacharjee and Hossain, 2015; Chauhan *et al.,* 2015; Kaur and Pathak, 2015; Pathak *et al.,* 2017; Sembi *et al.,* 2020; Vasundhra *et al.,* 2019). The main objective of the present study was to ensure better frequency and early germination of seeds and assess the utility of leaf explants for *in vitro* propagation of the presently investigated species *i.e. Satyrium nepalense*.

Material and Methods

Collection of Plant Material

The mature seeds from ripe and dehisced capsules were used as explants during the present investigation. For this purpose, the inflorescences of *S. nepalense* with mature capsules (Fig. 1A-C) were collected from Kasauli Hills, Himachal Pradesh, India during the last week of August and first week of September 2019. These inflorescences with mature capsules were kept at 4°C for 1 month for further experimentation. For another experiment on regeneration, leaves (0.4-0.6 cm long) excised from 3 months old aseptic cultures were used as explants.

Asymbiotic Seed Germination

Initially, mature seeds procured from ripe and dehisced capsules were treated with 1% (w/v) 2,3,5-triphenyl tetrazolium chloride to determine seed viability. A light microscope was used to examine the treated seeds and determine if they were viable (with red embryos) or non-viable. Seeds were treated with 4% sodium hypochlorite (NaOCl) and 2-3 drops of Tween-20 (Hi-Media, India) for 15-20 min and and these were rinsed three times with autoclaved distilled water (removal of traces of chemicals) under aseptic conditions, in laminar air flow hood. The seeds were sown by spreading thinly as possible over the surface of the culture medium in the test tubes with each tube containing 25 ml of medium.

Culture Medium and Incubation Condition

In the present investigation, M medium (Mitra *et al*., 1976) was prepared containing 2% sucrose and (0.8% w/v) agar as a solidifying agent for both the experiments. After the sucrose had been adequately dissolved, the pH was adjusted to 5.8. Activated charcoal was also added to the culture medium at a concentration of 2 gl^{-1} in order to assess its impact on the growth and development of cultures. Auxins [Indole 3 Acetic Acid (IAA), Indole 3 Butyric Acid (IBA), 1-naphthalene acetic acid (NAA)] and cytokinins [Kinetin (KN), Thidiazuron (TDZ), 6-benzylaminopurine (BAP)], each at a concentration of 1 mgl^{-1}, were also employed individually or in combinations. Cotton plugs were used to firmly seal the cultured test tubes, and the medium was autoclaved for 20 min at 121°C at 15 psi pressure. After inoculation, the cultures were incubated at a temperature of 25±2°C and a light intensity of 3500 lux.

Sub-Culturing and Acclimatization

The process involved the transfer of well grown seedlings/plantlets with fully developed leaves and roots to full strength culture medium devoid of any plant growth regulators and culturing them for 2-3 wks. Subsequently, the sucrose and vitamins were removed from culture medium and the plants were grown in the same conditions for another 2-3 wks. The well rooted healthy seedlings/plantlets with tubers were taken out from culture vessels using sterile forceps, washed with lukewarm water to remove agar sticking to these and transferred to culture flasks containing potting mixture

of sterile sand and vermiculite (1:1). The flasks were covered with porous transparent polybags for initial wks and kept under aseptic condition for one week. These flasks with plantlets were kept at room temperature for 1 wk; the plantlets taken out from the flasks were treated with 0.01% fungicide solution for 15-20 min, prior to their transfer into pots with potting mixture of sand and vermiculite (1:1).

Experimental Design

Both experiments were performed in a randomized design and repeated twice. Each treatment was used with four replicates. Seed germination and regeneration response of leaf explants were evaluated after regular interval of 3 days. In the former case, average number of days for onset of germination, development of protocorm formation, chlorophyll synthesis, first leaf primordium, first root primordium, and seedling development were recorded. In case of regeneration experiment, days taken for initiation of regeneration response, number of meristematic loci, days for further differentiation and completion of regeneration cycle were recorded.

Statistical Analysis

All data were subjected to analysis of variance (one way ANOVA) and significance ($P<0.05$) was determined with Tukey's honest significance test. Statistical tests were performed with the help of Statistical Package for the Social Science (SPSS) 16.0 Version. The results are presented as mean±standard error of both the experiments.

Results and Discussion

Asymbiotic Seed Germination

In the present study, the mature seeds (Fig 1D) from ripe and dehisced capsules (18 wap) were inoculated

Table 1. *In vitro* asymbiotic seed germination and seedling development on M medium, in *Satyrium nepalense*.

Growth additives*	Onset of germination	Time taken in days for						Remarks
		Spherule formation	Protocorm formation	Chlorophyll synthesis	Emergence of first leaf primordium	Emergence of first root primordium	Formation of complete seedling	
-	51.00±0.40^{m}	63.75±0.47^{i}	78.00±0.40^{j}	86.75±0.25^{m}	91.75±0.47^{k}	112.75±1.10^{l}	123.25±0.47^{l}	Healthy seedlings
AC	28.00±0.40^{e}	32.00±0.40^{b}	39.75±0.25^{c}	48.50±0.28^{c}	57.00±0.40^{c}	63.50±0.64^{b}	78.50±0.28^{b}	Healthy seedlings
IAA	45.25±0.62^{l}	58.75±0.4^{gh}	64.00±0.40^{g}	76.25±0.25^{jk}	81.25±0.47^{h}	93.00±0.00^{h}	103.00±0.40^{i}	Healthy seedlings
IAA + AC	24.75±0.25^{d}	32.00±0.40^{b}	37.00±0.40^{b}	45.75±0.25^{b}	57.25±0.47^{c}	67.25±0.25^{c}	81.50±0.28^{c}	Healthy seedlings
IBA	45.00±0.40^{kl}	55.50±0.64^{fg}	63.00±0.40^{g}	74.25±0.47^{ij}	79.00±0.40^{fg}	90.25±0.47^{g}	100.25±0.47^{gh}	Healthy seedlings
IBA+ AC	18.50±0.28^{c}	24.75±0.47^{a}	32.00±0.40^{a}	39.50±0.28^{a}	43.00±0.40^{a}	58.25±0.47^{a}	70.25±0.47^{a}	Healthy seedlings
NAA	43.00±0.70^{jk}	55.50±0.64^{fg}	68.00±0.40^{h}	73.25±0.47^{i}	79.75±0.25^{gh}	94.25±0.62^{h}	106.50±0.28^{j}	Healthy seedlings
NAA+ AC	28.25±0.25^{e}	37.25±0.25^{c}	50.00±0.40^{e}	58.75±0.25^{e}	73.50±0.28^{e}	86.50±0.28^{f}	97.25±0.25^{ef}	Healthy seedlings
TDZ	49.25±0.47^{m}	56.75±2.28^{fgh}	66.75±0.47^{h}	75.00±0.40^{ij}	86.50±0.28^{ij}	97.00±0.40^{i}	108.75±0.47^{j}	Callus formation
TDZ+AC	31.00±0.40^{fg}	54.50±0.50^{ef}	71.50±0.28^{i}	82.25±0.47^{l}	87.75±0.25^{j}	99.00±0.40^{ij}	136.75±0.47^{m}	Callus formation
KN	42.25±0.25^{j}	51.75±0.47^{e}	60.00±0.40^{f}	69.25±0.47^{h}	79.00±0.40^{fg}	87.00±0.40^{f}	99.25±0.47^{fgh}	Healthy seedlings
KN +AC	14.50±0.28^{b}	24.25±0.47^{a}	31.25±0.47^{a}	40.25±0.47^{a}	50.00±0.40^{b}	62.75±0.47^{b}	70.25±0.25^{a}	Healthy seedlings
BAP	47.00±0.40^{l}	60.25±0.47^{hi}	72.75±0.25^{i}	83.75±0.47^{l}	90.75±0.25^{k}	103.00±0.40^{k}	117.50±0.28^{k}	Healthy seedlings
BAP+AC	10.00±0.40^{a}	28.50±0.28^{b}	43.75±0.47^{d}	47.00±0.40^{bc}	57.25±0.47^{c}	71.25±0.25^{d}	80.25±0.75^{bc}	Healthy seedlings
IAA+TDZ	38.25±0.47^{h}	46.25±0.62^{d}	51.25±0.47^{e}	59.25±0.47^{ef}	61.50±0.28^{d}	73.00±0.40^{d}	87.00±0.40^{d}	Healthy seedlings
IAA +KN	41.00±0.40^{ij}	51.50±0.86^{e}	59.25±0.47^{f}	63.75±0.25^{g}	77.25±0.47^{f}	85.50±0.28^{f}	98.25±0.85^{fg}	Healthy seedlings
IAA +BAP	33.00±0.40^{g}	45.00±0.40^{d}	51.75±0.47^{e}	61.25±0.47^{f}	71.50±0.28^{e}	79.25±0.25^{e}	95.00±0.40^{e}	Healthy seedlings
IBA + TDZ	43.00±0.40^{jk}	51.25±0.47^{e}	67.75±0.25^{h}	77.75±0.47^{k}	88.00±0.40^{j}	99.75±0.75^{j}	121.25±0.47^{l}	Healthy seedlings
IBA +KN	39.00±0.40^{hi}	53.50±0.28^{ef}	60.00±0.40^{f}	71.00±0.40^{h}	85.50±0.28^{i}	92.75±0.47^{gh}	101.25±0.25^{hi}	Healthy seedlings
IBA +BAP	29.25±0.25^{ef}	36.50±0.28^{c}	43.75±0.25^{d}	52.25±0.47^{d}	60.25±0.47^{d}	71.25±0.75^{d}	88.50±0.28^{d}	Healthy seedlings

Entries in column number 2 to 8 are Mean±S.E.; same alphabetical letter in the superscript denotes that the corresponding mean are in the same group using Tukey test at 5%.

*All the growth additives were used at concentration of 1 mgl^{-1}.

Fig. 1. A-L. *In vitro* seed culture in *Satyrium nepalense*: A, A flowering plant with terminal raceme; B, Inflorescence bearing flowers; C, Inflorescence with mature capsules; D, Mature seed at the time of inoculation; E, Swelling of embryos; F, Rupturing of seed coat and emergence of spherule; G, Protocorm formation ($M+IAA_1$); H-I, Formation of leaves ($M+IBA_1$; $M+AC+IAA_1$); J-K, Healthy seedling with 2-3 leaves and 1-2 hairy roots ($M+AC+IAA_1$); L, Seed callusing and formation of seedlings ($M+AC+TDZ_1$).

Fig. 2. A-I. Regeneration of plantlets through leaf explants in *Satyrium nepalense*: A, Aseptically grown plantlets-source of leaf explants; B, Leaf explants at the time of inoculation; C, Formation of meristematic loci (M+AC+IAA_1); D, Protocorm like bodies (PLBs) formation (M+AC+KN_1); E-F, Direct shoot bud induction at the basal part of the leaf explants (M+AC); G, Healthy plantlets bearing 2-3 leaves and 1-2 roots (M+AC+IBA_1); H, PLBs formation from callus (M+AC+IBA_1); I, Plantlet bearing 2-3 tubers (M+AC+IAA_1).

on Mitra *et al*., 1976 (M) medium. The efficacy of different growth additives with and without activated charcoal (AC) was also tested on the onset of germination, spherule formation, protocorm development, first leaf primordium, first root primordium, and subsequent seedling development (Table 1). In the basal medium, onset of seed germination started within 51.00±0.40 days (Fig. 1E). The embryonal masses emerged out of the apically/ vertically ruptured seed coats as a spherule within 63.75±0.47 days (Fig. 1F) and soon developed into protocorms in 78.00±0.40 days (Fig. 1G). Protocorm multiplication and differentiation thereof were observed in 91.75±0.47 and 112.75±1.10 days respectively. Complete healthy seedlings with 2-3 leaves and 2-3 roots were obtained in 123.25±0.47 days (Fig. 1J). During the present study, initially, the protocorms were achlorophyllous which subsequently acquired chlorophyll. This agrees with the findings of Sheelavanthmath and Murthy (2001) and Gayatri *et al.* (2006) who reported that the protocorms in the terrestrial orchids of the open grasslands, well drained shady and seasonally dry soils are achlorophyllous. Additional presence of AC in the medium not only enhances the frequency of germination but also advanced the onset of germination, protocorm formation and subsequent seedling formation.

Growth additives like IAA, IBA, NAA, TDZ, KN, and BAP (1 mgl^{-1}) when used singly enhanced the onset of germination, spherule formation, protocorm development,

Table 2. Regeneration competence of leaf explants and plantlet formation on M medium, in *Satyrium nepalense*.

Growth additives*	Time taken in days for initiation of response	Number of meristematic loci invoked /explant	Regeneration pathway	Time taken in days for emergence of first leaf primordium	Time taken in days for emergence of first root primordium	Time taken in days for completion of regeneration of cycle	Remarks
-	16.25±0.25^{j}	1.25±0.25^{bc}	PLBs	65.00±0.40^{j}	81.75±0.47^{l}	91.50±0.28^{k}	Healthy plantlets
AC	4.50±0.57^{bc}	1.25±0.25^{bc}	Sb	20.00±0.40^{bc}	38.25±0.47^{d}	47.00±0.40^{c}	Healthy plantlets
IAA	14.50±0.28^{j}	2.25±0.25^{cd}	Sb	55.75±0.47^{h}	78.00±0.40^{k}	86.00±0.40^{j}	Healthy plantlets
IAA+ AC	3.75±0.50^{b}	1.25±0.25^{bc}	Sb/PLBs	18.00±0.40^{b}	29.00±0.00^{b}	40.25±0.47^{b}	Early (2-3) tubers formation
IBA	12.25±0.62^{i}	2.00±0.00^{bcd}	Callus/Sb	54.00±0.40^{h}	68.00±0.40^{j}	82.50±0.64^{i}	PLBs formation through callus
IBA+AC	3.25±0.50^{b}	1.00±0.00^{bc}	PLBs	20.75±0.47^{cd}	35.25±0.25^{c}	51.75±0.25^{d}	Healthy plantlets
NAA	14.50±0.64^{j}	1.25±0.25^{bc}	Callus/Sb	62.25±0.62^{i}	-	-	Browning
TDZ	9.75±0.25^{gh}	1.00±0.00^{bc}	Callus	-	-	-	No further growth
KN	8.00±0.40^{efg}	1.50±0.28^{bc}	PLBs	31.75±0.45^{e}	49.75±0.47^{g}	69.00±0.40^{g}	Multiple PLBs formation
BAP	10.75±0.25^{hi}	2.25±0.47^{cd}	PLBs/Sb	37.00±0.40^{f}	54.50±0.28^{i}	73.75±0.47^{h}	Healthy plantlets
IAA+TDZ	7.75±0.25^{ef}	2.75±0.25^{d}	Sb/Callus	22.00±0.40^{cd}	45.50±0.28^{f}	-	No further growth
IAA +KN	5.75±0.25^{cd}	2.00±0.40^{bcd}	PLBs/Sb	22.25±0.47^{d}	40.75±0.25^{e}	54.50±0.28^{e}	Healthy plantlets
IAA+BAP	7.75±0.47^{ef}	1.25±0.25^{bc}	Sb	30.25±0.47^{e}	52.00±0.00^{h}	64.50±0.64^{f}	Healthy plantlets
IBA+TDZ	-	-	-	-	-	-	-
IBA +KN	6.50±0.28^{de}	2.00±0.00^{bcd}	PLBs/Sb	30.00±0.40^{e}	42.00±0.40^{e}	56.25±0.47^{e}	Healthy seedlings
IBA+BAP	9.00±0.40^{fgh}	1.00±0.00^{bc}	PLBs	39.75±0.47^{g}	51.75±0.47^{h}	63.50±0.28^{f}	Healthy plantlets

Entries in column number 2, 3 and 5 to 7 are Mean±S.E.; same alphabetical letter in the superscript denotes that the corresponding mean are in the same group using Tukey test at 5%. PLBs, Protocorm like Bodies; Sb, Shoot bud.

*All the growth additives were used at concentration of 1 mgl^{-1}.

first leaf primordium, and first root primordium as compared to basal medium (Table 1). Presence of KN / or IBA (1 mgl^{-1}) in activated charcoal supplemented M medium advanced seedling development in 70.25±0.25 and 70.25±0.47 days respectively. Early onset of germination (10.00±0.40 days) was best seen in AC+BAP (1 mgl^{-1}) nutritional combination. Advanced morphogenetic events leading to seedling development were observed in AC supplemented IBA (1 mgl^{-1}) medium. According to Mahendran and Bai (2009), IBA was found to be the best for root induction in *S. nepalense*. The effectiveness of IBA in rooting has been reported for medicinal orchids like *Cymbidium pendulum* (Nongdam *et al.,* 2006) and *Vanilla planifolia* (Girdhar *et al*., 2001). The seed germination of terrestrial orchids under natural conditions has been found more difficult than that of epiphytic species (Arditti and Ernst, 1984). There are several reports on certain orchids like *Dendrobium longicornu, D. formosum, D. hookerianum* (Dohling *et al.,* 2008) and *Herminium lanceum* (Thakur and Pathak, 2020) in which seeds require higher salt content medium for germination. M medium has differential amount of micro and macro elements and is highly enriched with vitamins. The use of M medium has been well documented for a number of orchid species in literature (Deb and Temjensangba, 2006; Hossain *et al.,* 2010; Mitra, 1986; Pathak *et al.,* 1992, 2001, 2011; Vij, 1995). Cytokinins have been reported to be decisive for shoot proliferation. In earlier reports, TDZ responded more effectively in comparison to other cytokinins for stimulating shoot bud formation and its differentiation (Mulgund *et al.,* 2011) which supports our investigation and in addition to this callus formation was also observed in medium containing TDZ. The promotory effect of AC on asymbiotic seed germination and mass multiplication were reported by several researches (Pathak *et al.,* 2001; Sungkumlong and Deb, 2008). One possible explanation of the effects of charcoal is that it improves aeration and second possibility is that the charcoal adsorbs ethylene which can inhibit growth and proliferation (Ernst, 1974, 1975).

Optimal nutrient combination for protocorm formation, chlorophyll synthesis, emergence of first leaf and first root, and complete seedling formation was observed as M+ KN+AC.

Regeneration of Leaf Explants

Regeneration potential of whole leaf was tested on Mitra *et al*., 1976 (M medium) with and without growth additives either singly or in combination (1 mgl^{-1} each). Leaves (0.4-0.6 cm long) procured from 16 wks old *in vitro* grown plantlets were used as explants. The explants in general, regenerated *via* shoot bud, protocorm like bodies (PLBs), and callus; the regeneration response was invariably initiated mostly at the bases of the leaf explants. In basal medium, the explants showed initiation of regeneration response in 16.25±0.25 days; these regenerated through PLBs formation and healthy plantlets were observed in 91.50±0.28 days. The effect of darkening of medium using AC in the basal nutrient medium also proved effective for inducing early regeneration and plantlet development; the regeneration response was however via shoot bud formation. Highest number of meristematic loci was formed in IAA and TDZ containing combination. Interestingly, regeneration occurred through shoot buds alone or with additional PLBs /or callus was found in nutrient medium having IAA either alone or in combination. In our investigation, TDZ induced early regeneration response via callus formation (9.75±0.25 days); the callus however failed to undergo organogenesis, turned brown and ultimately perished. Literature studies also revealed that TDZ induced callus formation in various explants, when TDZ was used along with either NAA or 2,4-D (Huan and Tanaka, 2004; Teixeira da Silva *et al.,* 2006). TDZ, which has strong cytokinin activity (Mok *et al.,* 1982), induces *in vitro* morphogenesis in several orchid species [*Doritaenopsis* (Ernst, 1994), *Oncidium* (Chen *et al*., 1999), *Phalaenopsis* (Chen and Piluek, 1995), and *Spathoglottis* (Nayak *et al.,* 1998)]. In *Oncidium* 'Gower Ramsey', TDZ alone was efficient to induce direct somatic embryogenesis from the leaf explants (Chen *et al*., 1999) but in contrast in our study, TDZ promoted callus formation and was ineffective for shoot induction. The authors suggest that specific stringent requirements to achieve morphogenesis for mass propagation on specific explants from single elite plant need to be identified indicating thereby that the regeneration abilities of the explants vary with cultivar genotype. According to Thakur and Dongarwar (2017), BAP has been one of the most successfully used cytokinin for tuberization induction in several species; BAP (5 mgl^{-1}) showed benign effect to increase the tuber length and width, however, some effects on the shoot height and leaf length and width were also observed but that was not significantly different. However, in our studies early tuberization took place in medium containing AC supplemented IAA combination. AC, in general has beneficial and harmful effects in culture medium, depending upon the type of medium, explants, and plant growth regulator(s) used. AC often promotes shoot proliferation and it has been reported to promote multiple shoot formation in nodal segments of *Anoectochilus formosanus* (Ket *et al.,* 2004). Presently, BAP (1 mgl^{-1}) in the medium was favourable for inducing regeneration via both shoot buds and PLBs formation. Similarly, Hasegawa *et al.* (1985) recommended the use of BAP (0.1-1 mgl^{-1}) for the shoot regeneration in *Cymbidium faberi* explants. During the present investigation, *Satyrium nepalense* shoots on transfer to cytokinin free medium, rooted well. Similar results were shown in *Coelogyne fimbriata* (Anuprabha and Pathak, 2020); *Crepidium acuminatum* (Vasundhra *et al.,* 2019); and *Cymbidium eburneum* (Sembi *et al.,* 2020). The optimal nutrient combination for regeneration competence of *S. nepalense* leaf explant and plantlet formation was M+AC+IAA (1 mgl^{-1}).

Conclusion

The present experimental work ensured better frequency of seed germination, reduced the time lapse between pollination and sowing of seeds, and helped in mass propagation of *Satyrium nepalense,* a medicinally important, and an endangered terrestrial orchid. The regeneration competence of leaf explants in the investigated species was successfully tested and the efficacy of these explants for *in vitro* propagation objective may be investigated in other related orchid species as well.

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References

Anuprabha and Promila Pathak. 2019. *In vitro* asymbiotic seeed germination and seedling development in *Coelogyne fimbriata* Lindl. *J. Orchid Soc. India,* **33**: 83-89.

Anuprabha and Promila Pathak. 2020. Micropropagation of *Coelogyne fimbriata* Lindl. using pseudobulb explants. *J. Orchid Soc. India,* **34**: 131-36.

Arditti, J., 1982. *Orchid Biology: Reviews and Perspectives* II. Cornell University, Ithaca, New York, U.S.A.

Arditti, J. 1992. *Fundamentals of Orchid Biology*. John Wiley & Sons, New York, U.S.A.

Arditti, J. and R. Ernst. 1984. Physiology of germinating orchid seeds- A manual. *In: Orchid Biology-Reviews and Perspectives* Vol. III (ed. J. Arditti). pp. 177-222. Cornell University Press, Ithaca, New York, U.S.A.

Arora, S. K., Promila Pathak, Shivani Verma, Ankush Prakash, Kriti Dhiman, and K. C. Mahant. 2016. Mass propagation of *Dendrobium amoenum* Wall. ex Lindl. through stem nodal explants: A study *in vitro. J. Orchid Soc. India*, **30**: 51-55.

Balkrishna Acharya, Rajesh Juyal, Reema Devi, Jitender Kumar, Ankush Prakash, Promila Pathak, Ved Priya Arya, and Ashwani Kumar. 2020. Ethnomedicinal status and pharmacological profile of some important orchids of Uttarakhand (NorthWestern Himalayas), India. *J. Orchid Soc. India,* **34**: 137-47.

Bhattacharjee, D. K. and M. M. Hossain. 2015. Effects of plant growth regulators and explants on propagation of a monopodial and sympodial orchid: A study *in vitro. J. Orchid Soc. India*, **29**: 91-102.

Bhatti, S. K., Jagdeep Verma, Jaspreet K. Sembi, and Promila Pathak. 2017. Symbiotic seed germination of *Aerides multiflora* Roxb.- A study *in vitro. J. Orchid Soc. India,* **31:** 85-91.

Bhowmik, Tapash Kumar and Md Mahbubur Rahman. 2020. *In vitro* seed germination and micropropagation of *Dendrobium chrysotoxum* Lindl. (Golden Bow): A highly fragrant orchid species of Bangladesh. *J. Orchid Soc. India,* **34**: 69-77.

Buragohain, B. and S. K. Chaturvedi. 2016. Deceptive pollination in an endangered orchid, *Vanda coerulea* Griff. ex Lindl. (Orchidaceae). *J. Orchid Soc. India*, **30**: 31-35.

Chand, S., A. K. Sahrawat, and D. V. S. S. R. Prakash. 1997. *In vitro* culture of *Pimpinella anisum* L (anise). *J. Plant Biochem. Biotechnol.,* **6**: 1-5.

Chauhan, Shaveta, Promila Pathak, Anuprabha, and Sanjay Sharma. 2015. Regeneration of *Eulophia dabia* through rhizome explants and flowering: A study *in vitro. J. Orchid Soc. India*, **29**: 61-65.

Chen, Y. and C. Piluek. 1995. Effects of thidiazuron and N^6-benzylaminopurine on shoot regeneration of *Plalaenopsis*. *Plant Growth Regul*., **16**: 99-101.

Chen, J. T., C. Chang, and W. C. Chang. 1999. Direct somatic embryogenesis on leaf explants of *Oncidium* 'Gower Ramsey' and subsequent plant regeneration. *Plant Cell Rep*., **19**: 143-49.

CITES. 2012. *Convention on International Trade in Endangered Species of Wild Fauna and Flora,* Appendices I, II and III. http://www.cites.org.

CITES. 2013. *Convention on International Trade in Endangered Species of Wild Fauna and Flora,* Appendices I. Geneva, Switzerland.

De, L. C. and Promila Pathak. 2018. Conservation, management, and utilization of orchid genetic resources. *J. Orchid Soc. India,* **32**: 81-91.

De, L. C. and Promila Pathak. 2020. Good agricultural practices of *Dendrobium* orchids. *J. Orchid Soc. India,* **34:** 35-43.

Deb, C. R. and S. Temjensangba. 2006. *In vitro* propagation of threatened orchid, *Malaxis khasiana* Soland ex Swartz through immature seed culture. *Indian J. Exp. Biol.,* **44**: 762-66.

Decruse, S. W. and A. Gangaprasad. 2018. Restoration of *Smithsonia maculata* (Dalz.) Saldanha, an endemic and vulnerable orchid of Western Ghats through *in vitro* propagation. *J. Orchid Soc. India,* **32**: 25-32.

Devi, Kaushalya, S. S. Samant, Sunil Puri, and S. Dutt. 2018. Diversity, distribution pattern and indigenous uses of Orchids in Kanawar Wildlife Sanctuary of Himachal Pradesh, North Western Himalaya. *J. Orchid Soc. India*, **32**: 17-23.

Dohling, S., S. Kumaria, and P. Tandon. 2008. Optimization of nutrient requirements for asymbiotic seed germination of *Dendrobium longicornu* and *D. formosum. In: Proc. of the Indian National Science Academy*, **74**: 167-71.

Ernst, R. 1974. The use of activated charcoal in asymbiotic seedling culture of *Paphiopedilum. Amer. Orchid Soc. Bull*., **43**: 35-38.

Ernst, R. 1975. Studies in asymbiotic culture of orchids. *Amer. Orchid Soc. Bull*., **44**: 12-18.

Ernst, R. 1994. Effect of thidiazuron on *in vitro* propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae). *Plant Cell Tiss. Organ Cult.,* **39**: 273-75.

Gayatri, M. C., R. Kavyashree, R. Usha, and S. P. Dhananjaya. 2006. *In vitro* seed germination of *Pecteilis gigantea*- A rare and endangered orchid from Western Ghats. *Plant Cell Tiss. Organ Cult.,* **90**: 313-23.

Giridhar, P., B. Obul Reddy, and G. A. Ravishankar. 2001. Silver nitrate influences *in vitro* shoot multiplication and root formation in *Vanilla planifolia*. Abdr. *Curr. Sci*., **81**: 1166-70.

Gurudeva, M. R. 2019. Ontogeny and organization of female gametophyte in triandrous orchid, *Neuwiedia veratrifolia* Blume (Orchidaceae)- A Re-investigation. *J. Orchid Soc. India*, **33**: 49-53.

Hajong, S. and R. Kapoor. 2016. Orchid mycorrhizal symbiosis: Evolution, molecular mechanism and role in orchid distribution. *J. Orchid Soc. India*, **30**: 19-30.

Hasegawa, A., H. Ohashi, and M. Goi. 1985. Effects of BA, rhizome length, mechanical treatment and liquid sharking culture on the shoot formation from rhizome in *Cymbidium faberi* Rolfe. *Acta Hort*., **166**: 25-40.

Hegde, S. N. 2016. Commercial potential of orchids in India and societal benefits. *J. Orchid. Soc. India*, **30**: 43-49.

Hossain, M. M., M. Sharma, Jaime A. Teixeira da Silva, and Promila Pathak. 2010. Seed germination and tissue culture of *Cymbidium giganteum* Wall. ex Lindl. *Sci. Hortic*., **123**: 479-87.

Huan, L. V. T. and M. Tanaka. 2004. Callus induction from protocorm like body segments and plant regeneration in *Cymbidium* (Orchidaceae). *J. Hortic. Sci. Biotechnol*., **79**: 406-10.

Janakiram, T. and V. Baskaran. 2018. Commercialisation and conservation aspects of orchids. *J. Orchid Soc. India*, **32**: 55-61.

Jhansi, K., R. Venkatesh, and S. M. Khasim. 2019. Pharmcological studies in *Luisia zeylanica* Lindl. *J. Orchid Soc. India,* **33**: 95-103.

Joseph, M., L. Jose, and S. Sequeria. 2018. A comparative phytochemical screening of four epidendroid orchids of Kerala, India. *J. Orchid Soc. India*, **32**: 41-43.

Kaur, S. and Promila Pathak. 2015. Reversion of reproductive phase to vegetative phase in the inflorescence segments of *Saccolabium papillosum* Lindl. - A study *in vitro*. *J. Orchid Soc. India*, **29**: 75-79.

Kaur, S., Promila Pathak, Ankush Prakash, Anamika, and Aakanksha Sharma. 2017. *Ex situ* conservation of floriculturally and medicinally important endangered orchid, *Coelogyne cristata* Lindl. *J. Orchid Soc. India*, **31**: 15-22.

Kaushik, P. 1983. *Ecological and Anatomical Marvels of the Himalayan Orchids*. Today and Tomorrow's Printers and Publishers, New Delhi, India.

Kaveriamma, M. M., P. K. Rajeevan, P. K. Valsalakumari, and C. K. Geetha. 2018. Effect of inflorescence pruning in *Phalaenopsis* orchid. *J. Orchid Soc. India*, **32**: 99-101.

Ket, N., E. Hahn, S. Park, D. Chakrabarty, and K. Paek. 2004. Micropropagation of an endangered orchid *Anoectochilus formosanus*. *Biol. Plant,* **48**: 339-44.

Kumar, Ashish, S. S. Samant, L. M. Tewari, and Shiv Paul. 2018. Diversity, distribution and indigenous uses and status of Orchids in Kalatop - Khajjiar Wildlife Sanctuary, Chamba district Himachal Pradesh. *J. Orchid Soc. India,* **32**: 93-98.

Kumari, Anamika and Promila Pathak. 2020. Medicinal orchids of Shimla hills, Himachal Pradesh (NorthWestern Himalayas), threats, and conservation measures. *J. Orchid soc. India,* **34**: 45-56.

Lal Roshan and Promila Pathak. 2020. Substratum analysis of some therapeutically significant and/or endangered orchids of Shimla hills (Himachal Pradesh), NorthWestern Himalayas and their conservation. *J. Orchid Soc. India,* **34**: 101-11.

Lekshmi, S. and S. W. Decruse. 2018. *In vitro* symbiotic seed germination of *Vanda spathulata* (L.) Spreng., a vulnerable orchid of Western Ghats. *J. Orchid Soc. India,* **32**: 113-19.

Madhavi, M. and P. C. Shankar. 2019. Effects of different growth additives on seed germination of *Vanda tessellata* (Roxb.) Hook. ex G. Don- A medicinal orchid. *J. Orchid Soc. India,* **33**: 105-12.

Mahendran, G. V. and Narmatha Bai. 2009. Mass propagation of *Satyrium nepalense* D.Don.- A medicinal orchid via seed culture. *Sci. Hortic.*, **119**: 203-07.

Manoharachary, C. 2019. Orchids- Mycorrhizae. *J. Orchid Soc. India*, **33**: 23-29.

Mitra, G. C. 1986. *In vitro* culture of orchid seeds obtaining seedlings. *In: Biology, Conservation, and Culture of Orchids* (ed. S. P. Vij) pp. 401-12. Affiliated East-West Press, New Delhi, India.

Mitra, G. C., R. N. Prasad, and A. Roy Chowdhury. 1976. Inorganic salts and differentiation of protocorms in seed callus of an orchid and correlated changes in its free amino acid content. *Indian J. Exp. Biol.*, **14**: 350-51.

Mok, M. C., D. W. S. Mok, D. J. Armstrong, K. Shudo, Y. Isogai, and T. Okamoto. 1982. Cytokinin activity of N-phenyl-N'-(1,2,3,-thiadiazol-5-yl)-urea (thidiazuron). *Phytochemistry*, **21**: 1509-11.

Mulgund, Gangadhar S., K. Nataraja, Ravindra B. Malabadi, and S. Vijaya Kumar. 2011. TDZ induced *in vitro* propagation of an epiphytic orchid *Xenikophyton smeeanum* (Reichb. f.). *Plant Biol.*, **1**(4): 7-15.

Nayak, N. R., S. P. Rath, and S. Patnaik. 1998. High frequency plant regeneration from alginate encapsulated protocorm-like bodies of *Spathoglottis plicata* Bl., a terrestrial orchid. *Phytomorphol.: Int. J. Plant Morphol.,* **48**: 179-86.

Nongdam, P., C. Nirmala, and R. Tewari. 2006. *In vitro* multiplication of *Cymbidium pendulum* orchids via embryo culture. *Plant Cell Biotechnol. Mol. Biol.,* **7**: 145-50.

Pal, Ram, D. R. Singh, and Promila Pathak. 2019. Pollination biology of orchids: An unexplored area of research in India. *J. Orchid Soc. India,* **33**: 79-82.

Pathak, Promila, J. Verma, and S. P. Vij. 2001. 'Isubgol' as an alternative gelling agent for orchid embryo culture *In: Proc. National Seminar on Orchid Diversity in India: Science and Commerce*. The Orchid Society of India, Chandigarh, India.

Pathak, Promila, S. P. Vij, and K. C. Mahant. 1992. Ovule culture in *Goodyera biflora* (Lindl.) Hk.f.: A study *in vitro*. *J. Orchid Soc. India*, **6**(1-2): 49-53.

Pathak, Promila, Shivani Verma, Ankush Prakash, and K. C. Mahant. 2017. Regeneration competence of an ornamentally important epiphytic orchid, *Rhynchostylis gigantea* (Lindl.) Ridl. through leaf segments: A study *in vitro*. *J. Orchid Soc. India*, **31**: 97-101.

Pathak, Promila, Hossein Piri, S. P. Vij, K. C. Mahant, and S. Chauhan, 2011. *In vitro* propagation and mass scale multiplication of a critically endangered epiphytic orchid, *Gastrochilus calceolaris* (Buch.-ham ex J.E. Sm.) D.Don. using immature seeds. *Indian J. Exp. Biol.,* **49**: 711-16.

Pathak, Promila, A. Bhattacharya, S. P. Vij, K. C. Mahant, Mandeep K. Dhillon, and H. Piri. 2010. An update on the medicinal orchids of Himachal Pradesh with brief notes on their habit, distribution, and flowering period. *J. Non Timber Forest Products,* **17**(3): 365-72.

Patnaik, A., M. Kannan, M. Ganga, and S. Vincent. 2017. Studies on influence of nutrient and growth regulator interactions on growth, yield and quality of *Dendrobium* orchid cv. Sonia 17. *J. Orchid Soc. India*, **31**: 9-14.

Prakash, Ankush and Promila Pathak. 2019. Orchids of Water Catchment Wildlife Sanctuary, Shimla (Himachal Pradesh), NorthWestern Himalayas: Their diversity, status, indigenous uses, and conservation status. *J. Orchid Soc. India*, **33**: 65-77.

Prakash, Ankush and Promila Pathak. 2020a. Effects of different concentrations of NPK on vegetative growth parameters of a floriculturally important epiphytic orchid, *Dendrobium chrysanthum* Wall. ex Lindl. *J. Orchid Soc. India,* **34**: 117-21.

Prakash, Ankush and Promila Pathak. 2020b. Ant facilitated pollination of *Herminium lanceum* (Thumb. ex Sw.) Vuijk

(Orchidaceae)- An endangered terrestrial orchid of NorthWestern Himalayas. *J. Orchid Soc. India,* **34**: 11-15.

Sembi, Jaspreet K., Promila Pathak, and J. D. Verma. 2020. Regeneration competence of leaf explants in *Cymbidium eburneum* Lindl. (Orchidaceae). *J. Orchid Soc. India,* **34**: 17-21.

Sheelavanthmath, S. S. and H. N. Murthy. 2001. *In vitro* propagation of a terrestrial orchid *Habenaria marginata* Coleb. *J. Orchid Soc. India,* **15**: 85-88.

Singh, S. K., D. K. Agarwala, J. S. Jalal, Paramjit Singh, and A. A. Mao. 2019. *Orchids of India: A Pictorial Guide.* Botanical Survey of India, Kolkata, India.

Sungkumlong and C. R. Deb. 2008. Effect of different factors on immature embryo culture, PLBs differentiation and mass multiplication of *Coelogyne sauveolens* (Lindl.) Hook. *Indian J. Exp. Biol.,* **46**: 243-48.

Teixeira da Silva, J. A., D. T. T. Giang, M. T. Chan, A. Sanjaya Norikane, M. L. Chai, J. Chico-Rui'z, S. Penna, T. Granstrom, and M. Tanaka. 2007. The influence of different carbon sources, photohetero, photoauto and photo mixtrophic conditions on protocorm like body organogenesis and callus formation in thin cell layer culture of hybrid *Cymbidium* (Orchidaceae). *Orchid Sci. Biotechnol.*, **1**: 15-23.

Thakur, Babita and Promila Pathak . 2020. *In vitro* propagation of *Herminium lanceum* (Thunb. ex Sw.) Vuijk (Orchidaceae), through asymbiotic seed germination: A therapeutically important and endangered orchid from Northwestern Himalayas. *J. Orchid Soc. India,* **34**: 61-67.

Thakur, U. and N. Dongarwar. 2017. Asymbiotic seed germination, seedling development and tuberization of *Peristylus lawii* Wight (Orchidaceae), a rare terrestrial orchid. *Hislopia J.,* **10**(1): 59-72.

Thakur, J., A. Madan, M. D. Dwivedi, and P. L. Uniyal. 2015. What drives orchids toward myco-heterotrophy? *J. Orchid Soc. India*, **29**: 23-30.

Vasundhra, Promila Pathak, and Ankush Prakash. 2019. *In vitro* shoot induction and regeneration potential of floral buds in *Crepidium acuminatum* (D.Don) Szlach., a medicinal ayurvedic plant from NorthWestern Himalayas. *J. Orchid Soc. India,* **33**: 43-48.

Vij, S. P. 1995. Genetic resources of orchids. *In: Advances in Horticulture* Vol. 12-*Ornamental Plants* I (eds. K. L. Chadha and S. K. Bhattacharjee) pp. 153-81. Malhotra Publishing House, New Delhi, India.

Vij, S. P. and Promila Pathak. 1997. On the utility of green pod culture in *Satyrium nepalense* D.Don (Orchidaceae): A study *in vitro. In: Proc. 1st Punjab Science Congress.* pp. 90. Punjabi University, Patiala, India.