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ASYMBIOTIC SEED GERMINATION IN A MEDICINALLY IMPORTANT AND NEAR THREATENED TERRESTRIAL ORCHID, *CREPIDIUM ACUMINATUM* (D.DON) SZLACH. FROM NORTHWESTERN HIMALAYAS: A STUDY *IN VITRO*

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Abstract

The Crimson Shield Orchid, *Crepidium acuminatum* (D.Don) Szlach. (=*Malaxis acuminata* D.Don), grows in temperate and subtropical Himalayas (from Himachal Pradesh to Sikkim; 6000-7000 ft), Western Ghats, Nilgiri Hills, and Andaman & Nicobar Islands. It is an important ingredient of *Ashtavarga* drugs used in the preparation of an ayurvedic tonic *Chyavanprash* and is marketed under the trade name *Rishbhak*. Immature seeds procured from undehisced green capsules (12 wap) of *Crepidium acuminatum* were inoculated on M medium with and without different organic supplements [Yeast Extract (YE), Peptone (P), and Casein Hydrolysate (CH) (0.1% each); Coconut water (CW; 10%)] to assess the germination potential. Healthy seedlings were obtained on activated charcoal (AC) enriched basal M medium under light conditions in 33 wks. Leaf formation was observed in Peptone enriched medium under dark conditions, whereas, combinations containing other growth additives failed to induce leaf development. Early pseudobulbs formation was observed in medium containing CW under dark conditions.

Introduction

PLANTS REMAIN a major source of pharmaceuticals and over 80% of the approximately 30,000 known natural products are of plant origin. Seventy five per cent of the world's population relies on plants for traditional medicines even today and Indian Ayurveda has been using this knowledge since ages (Gómez-Galera *et al.*, 2007; Jaiswal and Williams, 2016).

Crepidium acuminatum D.Don Szlach., the Crimson Shield Orchid, is a widely distributed species found in Thailand, China, Burma, and Indo-China. In India, it is found in temperate and subtropical Himalayas (from Himachal Pradesh to Sikkim; 6000-7000 ft), Western Ghats, Nilgiri Hills, and Andaman & Nicobar Islands. It is an erect, terrestrial herb, 30 cm high having stem with few sheaths at the base (Das *et al.,* 2023; Handa, 1986; Suyal *et al.,* 2020). This species forms an important ingredient of *Ashtavarga* drugs used in the preparation of an ayurvedic tonic *Chyavanprash* and is marketed under the trade name *Rishbhak* (Handa, 1986; Sharma *et al.,* 2019). *Rishbhak* provides strength, enhances sperm formation, and makes a person free from diseases borne by *vat, pit, kaf,* and tuberculosis, breathing disorders, burning sensation, cough as a tonic, and treats general debility (Arora *et al.,* 2017; De, 2020; Gupta *et al.,* 2015; Hegde and Ingalhalli, 1988; Pathak *et al.,* 2010; Prakash and Pathak, 2019).

Conservation through reserves alone is now considered unlikely to achieve protection of plant species necessary to mitigate direct losses of habitat and the pervasive impact of global climate change. Possibilities of replenishing their natural stocks, by using tissue culture and raised plantlets are also being increasingly realized. Plant tissue culture could be one of the most suitable alternative tools to minimize the pressure on natural populations of medicinal orchids and their sustainable utilization. Though, some studies have been earlier made by a few workers to conserve different orchid species using seed and other explants *in vitro* (Anuprabha and Pathak, 2019, 2020; Anuprabha *et al.,* 2017; Arora *et al.,* 2016; Bhowmik and Rahman, 2020, 2022; Kaur *et al.,* 2017; Kumari and Pathak, 2021; Lalduhsanga *et al.,* 2021; Mutum *et al.,* 2022; Pathak *et al.*, 2016, 2017, 2022, 2023; Rahamtulla and Khasim, 2022; Sembi *et al.,* 2020; Sunita *et al.,* 2021; Thakur and Pathak, 2020, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2019, 2021), these attempts are meager in terms of size of the orchid family. The present studies were, therefore, undertaken with a view to develop protocols for propagating this medicinally important taxon, *C. acuminatum.*

Material and Methods

The plants of *C. acuminatum* were sourced from their natural habitats, Tara Devi hills (Shimla) and Kasauli hills (Solan) (Fig. 1a-b). With a view to ensuring their

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easy availability, the live plants were maintained in the Orchid House, Department of Botany, Panjab University, Chandigarh and were watered regularly. All the glassware and surgical instruments were steam sterilized in an autoclave for 40 min at a temperature of 121ºC and pressure of 1.1 $kgcm^{-2}$, and subsequently dried in an electric oven.

Culture Medium

Nutrient medium, namely M (Mitra *et al*., 1976) medium was used for seed germination of *C. acuminatum.* The medium was gelled with agar (0.85%); uniform heating and constant swirling ensured proper gelling of the medium. In some set of experiments, activated charcoal (AC) (0.2%) was used. Different organic supplements such as Yeast Extract (YE), Peptone (P), and Casein Hydrolysate (CH) (0.1% each); and Coconut water (CW; 10%) were also added to the medium.

Explanting and Surface Sterilization

Immature seeds procured from undehisced green capsules (*pods*) (12 wap) of *C. acuminatum* were used as explants for culture initiation *in vitro*. The capsules were excised from the mother plants in the morning hours, scrubbed gently with detergent (Teepol; 0.5%) using a soft brush, and washed thoroughly under running tap water for 20 min. These were further processed under aseptic conditions in a clean air laminar air-flow cabinet. These capsules served as the source for immature seeds (12 wap). The capsules were dipped in 70% ethyl alcohol for 30 sec, flamed, and then sterilized for 10 min in $HgCl_2$ solution (0.1%) with Teepol (1-2 drops) as wetting agent. These were then washed (3-4 times) with sterilized distilled water to remove all traces of $HgCl_2$. Following this, the capsules were treated with 0.05% 'Bavistin' (10-15 min), and 0.05% Streptomycin (10 min). Each step was followed by a thorough rinse with sterilized distilled water. The capsules were then split open with the help of a scalpel to scoop out the immature seeds.

Inoculation and Culture Conditions

The inoculation was performed manually under aseptic conditions in a clean air laminar air-flow cabinet using sterilized surgical instruments. The cultures were maintained at 25±2°C temperature regime and kept under a 16 hrs photoperiod, illuminated with cool fluorescent tubes at a light intensity of 25 $\mu mols^{-1}m^{-2}$.

Data Recording

The observations were made at weekly intervals and the responses were recorded on the basis of visual observations and photographs were clicked on 35 mm SLR camera (Pentax, Japan) and close up lenses (Sonia, India) on Kodak GOLD 100 film. The data was expressed as means ± standard deviation (SD) of mean and statistical analysis was performed with SPSS 14.0 for Windows Evaluation Version. Analysis of Variance (ANOVA) was performed followed by Scheffe's test for multiple comparisons. Difference with $P \leq 0.05$ was considered statistically significant.

Hardening, Deflasking, and Establishment in Greenhouse

Healthy seedlings complete with 3-4 well grown leaves and 1-2 roots (1.0-1.5 cm long) were gradually hardened *in vitro*, by sequential removal of growth supplements, vitamins, sucrose, and minor salts from the nutrient matrix at 15 days interval. Subsequently, the plantlets were taken out from the flask, washed thoroughly with lukewarm water to remove the agar, and kept in distilled water for 5-10 min to avoid drying. Then the plantlets were treated with fungicide solution (Mancozeic powder, 0.5%) for 5 min. The plantlets were then transferred to plastic or thermocol pots (2.5 inches in diameter). The pots were filled with peat moss, river sand, and silica in a ratio of 1:1:1. The plantlets and substratum were watered with half strength MS medium (only the major salts and iron source). The pots were covered with plastic covers, placed in a cool, wet environment under fluorescent light in a 30°C temperature regime. The plantlets were watered with distilled water every two days or when needed. Every week, the plants were sprayed with fungicide and half strength MS medium till one month. Plantlets were then transferred to earthen pots (2.5 inches in diameter) (Fig. 1h).

Results

In basal M medium, 55±0.82% seeds (Fig. 1c) germinated in 10.88±0.25 wks. However, under dark conditions, the germination percentage was reduced to 40±1.63%, though the time taken for spherule formation (Fig. 1d) was reduced to 8.75±1.26 wks. The spherules turned green and developed into protocorms (Fig. 1e-f) in 13.25±0.5 wks; the differentiation of leaf primordia occurred in 22.75±1.56 wks. Though the pseudobulbous shoots with 2-3 juvenile leaves were obtained, rhizogenesis failed to occur. Additional use of AC enhanced the germination percentage of seeds to 64.25±0.96%; complete healthy seedlings with 2-3 leaves and 1-2 roots were obtained in 33±0.82 wks (Fig. 1g). Addition of AC, however, did not prove beneficial under dark conditions as far as germinating percentage and rhizogenesis was concerned; though it induced leaf formation and pseudobulb multiplication.

On the supplementation of YE (1.0%) in M medium, the percentage of germinating seeds drastically declined (15±0.82%), though spherules were obtained earlier (7.5±0.41 wks). Under dark conditions, though the percentage obtained was 20±1.63%, pseudobulbs formed failed to multiply. YE enriched medium with AC also did not prove beneficial as differentiation failed to occur in the germinating entities irrespective of the light conditions provided. Fortification of M medium with P (1.0%) reduced the germination percentage; only 28±1.63% seeds germinated in 8±0.41 wks eventually leading to pseudobulb multiplication in 33.88±0.63 wks. Dark conditions slightly decreased the percentage, though these helped initiate a slightly earlier germination

Fig. 1a-h. *In vitro* asymbiotic seed germination and seedling development in *Crepidium acuminatum*: a, A plant in its natural habitat; b, Close up of inflorescence; c, Seed at the time of inoculation; d, Spherule; e, Protocorm; f, Protocorm multiplication; g, Seedling, h, Hardened seedling transferred to a pot.

Table 1. In vitro asymbiotic germination of immature seeds (12 wap) of Crepidium acuminatum on M medium and its various combinations with growth additives, under light and dark conditions.

Growth additive	L/D	Germination frequency (%)	Time taken in wks for formation of		Time taken in wks for development of						Remarks
			Spherule	Protocorm	Chlorophyll	First leaf	Pseudobulb	First root	Seedling	Pseudobulb multiplication	
-	L	55±0.82^{X,t}	10.88±0.25^{O,P,Q,o}	13.25±0.5^{Q,R,So,p}	15.13±0.63^{P,Q,R,p}	22.75±1.56^{R,q}	20±0.82^{S,q}	-	-	23.13±0.63^{R,p,q}	Healthy pseudobulbous shoots
	D	40±1.63	8.75±1.26	10±0.82	11.75±1.26	22.13±0.63	22.38±0.48	-	-	24.25±1.5	Pseudobulbous shoots
AC	L	64.25±0.96	10.88±1.03	12.5±0.58	14.75±0.96	26.38±0.48	25.63±0.95	30.13±0.86	33±0.82	28.13±0.63	Healthy seedlings
	D	20±1.63	9.53±0.41	11.88±0.63	15.5±1.73	26.25±0.87	26.75±1.04	-	-	31.75±0.5	Differentiation of leaves
YE	L	15±0.82^{R,S,s}	7.5±0.41^{R,S,To,p,q}	8.63±0.48^{R,S,T,o}	11.63±0.48^{V,p,q}	-	33.5±1.0^{U,V,q}	-	-	38.5±0.58^{S,r,s}	Healthy
	D	20±1.63	9.13±0.86	10.63±0.75	13.5±0.58	-	28.63±0.95	-	-	-	pseudobulbous shoots Pseudobulb formed
YE+AC	L	18±0.82	10.88±0.86	12.63±0.48	21±0.41	-	25.63±0.48	-	-	31.5±1.0	Pseudobulbous shoots
	D	20.5±1.92	17.75±0.65	19.13±1.44	22.75±0.5	-	24.38±0.95	-	-	30.25±1.26	Pseudobulbous shoots
P	L	28±1.63^{U,s}	8±0.41^{O,b,o,p,q}	9±0.82^{O,P,o}	12±0.41^{P,p,q}	-15.88±0.86^{O,o}	12.63±0.48^{R,q}	-	-	33.88±0.63^{S,r,s}	Pseudobulb multiplication
	D	26±0.82	7.13±0.63	8±0.82	9.88±0.63		11.75±0.5	-	-	34.38±0.95	Differentiation of leaves
P+AC	L	25.25±0.5	11.13±0.63	12±0.82	15.75±0.5	-	25.63±0.48	-	-	35.5±1.0	Pseudobulb multiplication
	D	30±1.63	10.38±0.48	11.5±0.58	15.38±0.48	-	24.5±0.58	-	-	37.38±0.48	Pseudobulb multiplication
CH	L	6±0.82^{O,s}	9.63±0.95^{O,P,Q,o,p,q}	10.63±0.95^{O,o}	12±1.41^{O,p,q}	-	27.75±0.96^{T,U,q}	-	-	32.88±0.86^{S,r,s}	Pseudobulbous shoots
	D	10.75±0.96	8.38±0.48	9.38±0.48	11.25±0.5	-	26±0.82	-	-	-	Pseudobulbous shoots
CH+AC	L	5±0.82	12.25±1.26	-	-	-	-	-	-	-	Spherules failed to differentiate further
	D	11.5±1.0	11±0.41	-	-	-	-	-	-	-	
CW	L	60±1.63^{Y,s}	11.5±1.73^{O,P,Q,R,o,p,q}	12.75±2.06^{Q,R,o}	14.5±1.29^{R,S,p,q}	-	26.25±1.26^{S,q}	-	-	28.38±1.10^{P,Q,r,s}	Pseudobulb multiplication
	D	70±0.82	7.38±0.48	9.38±0.48	10.38±0.48	-	13.38±0.48	-	-	15.13±0.25	Higher germination frequency
CW+AC	L	40±0.82	12.13±0.25	13.38±0.48	17±0.82	-	28.38±0.48	-	-	-	-
	D	52.25±1.71	10.13±0.63	11.75±0.5	19.38±0.75	-	24.75±0.5	-	-	-	

L, Light conditions; D, Dark conditions; *, Medium containing AC; Figures mentioned as subscripts indicate the concentrations of the growth adjuncts used. o-z are means for groups in homogenous subsets are displayed using Scheffe's Post hoc tests. Similar superscripts denote similar subsets. Post hoc tests are not performed for AC/nAC and Light/Dark because there are fewer than three groups or error term has zero degrees of freedom. #, Original observation quoted.

response (7.13±0.63 wks) and eventually steering the formation of leaf and pseudobulbs. Supplementation of AC in the medium slightly increased the germination percentage under dark conditions (30±1.63%), though no leaf formation occurred. When CH (1.0%) was added to M medium, the germination percentage was extremely affected (6±0.82%); it, however, favoured pseudobulb multiplication. Dark conditions slightly improved the germination (10.75±0.96%). Additional utilization of AC did not prove beneficial either; arresting the development at spherule stage. With the addition of CW (10%) in the medium, as many as 60±1.63% seeds germinated in 11.5±1.73 wks and pseudobulbs multiplied in 28.38±1.10 wks. Dark conditions proved beneficial by promoting 70±0.82% seed germination as well as initiating an earlier response (7.38±0.48 wks). Pseudobulbs multiplied as early as 15.13±0.25 wks. However, the addition of AC to the above medium reduced the germination percentage to 40±0.82% and 52.25±1.71% under light and dark conditions, respectively. It also failed to induce pseudobulb multiplication.

Discussion

Presently, though successful asymbiotic seed germination was achieved on M medium and its combinations with growth additives in *Crepidium acuminatum,* the frequency of germination and time taken for various morphogenetic stages varied depending upon growth stimulus in the nutrient regime and light or dark conditions. Perusal of literature reveals that complex growth supplements have been used earlier in order to enrich the culture media for orchid seed germination; some such supplements are apple juice, banana homogenate, beef extract, casein hydrolysate, coconut water, extract of silkworm pupae, fish extract, honey, peptone, potato extract, tomato juice, yeast extract *etc.* (Arditti, 1967; Pathak *et al.,* 2001). Various organic compounds, *i.e.* coconut water, peptone, banana homogenate, potato homogenate, chitosan, tomato juice, and yeast extract can promote seed germination and growth and development of various orchids. They also stimulate seedling development, formation of protocorm-like bodies (PLBs), plantlet growth, and multiple shoot formation. The addition of organic compounds to culture media, individually or in combination, accelerates seed germination, and seedling development. Organic nutrients are known as a source of vitamins, amino acids, fatty acids, carbohydrates, peptides, and growth factors, which all facilitate growth (Utami and Hariyanto, 2020). In the present study, the effect of CH/CW/P/YE was assessed on seed germination and subsequent seedling development. Leaf formation was observed in Peptone enriched medium under dark conditions, whereas, combinations containing other growth additives failed to induce leaf development.

In the present studies, the embryos followed a uniform pattern of morphogenetic changes; their swelling and emergence from the seed coat formed spherules. The spherules developed into protocorms which eventually differentiated leaf and root primordia. Similar observations were reported by some authors (Kanase and Takano, 1995; Mitra, 1971; Pathak *et al*., 2001; Yamazaki and Miyoshi, 2006). In *C. acuminatum,* the chlorophyll development was a post-protocorm phenomenon. However, chlorophyll development was a pre-protocorm phenomenon in *Vanda amesiana* (Devi *et al*., 2006). Inconsistency in the stage of development of chlorophyll in terrestrial orchids has been ascribed to ecological and/or genetic factors (Stoutamire, 1974; Vij *et al*., 1988). In the present study, leaf formation did not occur in most of the combinations while pseudobulbs were invariably obtained excepting for AC supplemented CH containing nutrient combinations. Similar observations of bulb formation instead of leaves were reported in *Aerides multiflora* (Katiyar *et al*., 1987). Presently, CW was effectively used at concentration (10%) for germination. Enrichment of the medium with CW has been utilized since long for obtaining growth and organ differentiation in orchid cultures. CW induces division of the otherwise non-dividing cells (Goh *et al*., 1975) and multiplication of protocorms (Intuwong and Sagawa, 1973). Its stimulatory effect has been attributed partly to zeatin riboside content (cf. Bejoy *et al*., 2004; cf. Devi *et al*., 2006). Earlier researchers have also successfully used this growth supplement for the germination of *Dendrobium* hybrid (Lekha Rani *et al*., 2005), *Peristeria elata* (Bejoy *et al*., 2004), and *Vanda coerulea* (Seeni and Latha, 2000). Enhancement of foliar growth by the addition of coconut water has also been reported by Lekha Rani *et al*. (2005). Gupta (2003) reported the advancement of the onset of morphogenetic changes leading to seedling development in *Cymbidium eburneum* and *Paphiopedilum spicerianum,* in its presence. However, it could not promote good germination in some cases (Devi *et al*., 1990; Mitra, 1987). During the present study, in medium containing CW under dark conditions though up to 70% seeds germinated and early pseudobulbs formation was promoted, seedling formation was not achieved. Similarly, CW at 40% was found to be inhibitory for seedling growth in *Vanda testacea* (Naha *et al.,* 2013). *Dimorphorchis rossii* protocorms grown on medium containing 10% CW promoted the best complex additive for the development of protocorms to seedlings with 78% and 66% of the explants produced leaf and root respectively (Gansau *et al.,* 2015).

A water soluble protein hydrolysate with high amino acid content, P (0.1%) was beneficial for inducing leaf formation and pseudobulb multiplication; though germination percentage was reduced during the present studies. In accordance with the present study, P impaired germination in *Dactylorhiza maculata* (Van Waes and Debergh, 1986) and *Vanda tessellata* (Roy and Banerjee, 2002). However, P has been shown to be beneficial for better germination in *Cattleyopsis lindenii* and *Dendrobium parishii* (Buyun *et al*., 2004), *Crepidium acuminatum* (Gupta, 2003), *Epidendrum ibaguense* (Hossain, 2008), *Vanda* hybrid (Johnson and Kane, 2007), *V. amesiana* (Devi *et al*., 2006), and *V. teres* (Sinha and Roy, 2004); it also promoted the formation of healthy seedlings in *V. amesiana* (Devi *et al*., 2006). Naha *et al.* (2013) observed that 0.1% Peptone stimulated seedling growth in *Vanda testacea*. The above data indicate that efficacy of P varies with the species and the developmental stage of the germinating entities.

During the present investigation, CH (0.1%) supported only marginal germination per cent in *C. acuminatum*, however, pseudobulb multiplication was induced. In consonance with the present results, CH impaired germination in *Saccolabium papillosum* (Znaniecka *et al*., 2005). It was, similarly, shown to be inhibitory to organogenesis and rhizogenesis in *Cymbidium eburneum* and *Crepidium acuminatum*, respectively (Gupta, 2003) and germination in *Herminium lanceum* (Mahant, 1991). However, the beneficial role during germination and seedling formation has been demonstrated in *Aerides multiflora, Rhynchostylis retusa, Saccolabium papillosum,* and *Vanda testacea* (Vij *et al*., 1981). In contrast to the present study, CH was shown to be constructive in germination and callus mediated protocorm multiplication and earlier seedling formation in *Crepidium acuminatum* (Sharma, 2009). The variable effect of CH during germination may be credited to its compositions, which vary with the extent of hydrolysis of the nitrogenous substances present in the casein (Arditti *et al*., 1982).

YE is also an essential source of reduced nitrogen and has been efficiently utilized in germination and proliferation in various orchid species. In the present investigation, the use of YE (0.1%) reduced germination and leaf and root formation; though pseudobulb formation and multiplication was supported. In consonance with present results, it was proven to be inhibitory to organogenesis in *Cymbidium eburneum* and rhizogenesis in *Crepidium acuminatum* (Gupta, 2003), and shoot development in *Cattleya* (Kusumoto, 1979). In contrast, YE has been demonstrated to be favourable for germination in several orchids (cf. Mahant, 1991; Pathak *et al*., 2001). Similarly, YE was found to be beneficial for germination and protocorm multiplication in *Eulophia dabia* and *C. acuminatum,* and for germination and seedling development in *Habenaria commelinifolia* (Sharma, 2009), whereas YE was shown to be inhibitory to PLB development in *Vanda amesiana* (Devi *et al*., 2006) and to seedling growth in *Vanda testacea* (Naha *et al*., 2013). YE proved beneficial for protocorm proliferation of *Dimorphorchis rossii* (Gansau *et al*., 2015).

During the present studies, basal M medium proved to be the optimal nutritional combination for successful *in vitro* asymbiotic seed germination and healthy seedling formation in a medicinally important and near threatened terrestrial orchid, *Crepidium acuminatum*. Such studies may be extended to other closely related taxa.

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