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ASYMBIOTIC SEED GERMINATION AND SEEDLING DEVELOPMENT OF *SATYRIUM NEPALENSE* D.DON: AN *IN VITRO* APPROACH FOR OPTIMIZATION OF CULTURAL REQUIREMENTS FOR A MULTI-FACETED THREATENED INDIAN ORCHID

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Abstract

Satyrium nepalense D.Don is a terrestrial, multi-purpose orchid with therapeutic, nutritional, and aesthetic benefits as well as aroma. Its extensive elemental composition which contains triterpenes, alkaloids, flavonoids, and unsaturated sterols, makes it eligible to be classified as a medicinal orchid. The juice of the plant is used to treat fever as well as cuts and wounds, and the dried tubers are used as a dysentery preventative. The survival of the species is threatened by natural factors including habitat loss triggered by undulating topography and landslides sparked by heavy rains. Its multiplication by conventional and micropropagation methods, as well as its restoration into natural habitats and niche regions will prove to be a significant conservation effort. Mature seeds (20 wap) procured from dehisced capsules (pods) of *S. nepalense* were cultured on MS Medium with or without growth hormones [Auxins (Indole-3-Acetic acid, IAA and 1-Napthalene Acetic acid, NAA; 0.5 mgL^{-1} each); Cytokinins (6-Benzylaminopurine, BAP, Kinetin, KN, and Thidiazuron, TDZ; 0.5 mgL^{-1} each); and activated charcoal (0.2%)]. MS medium fortified with AC+BAP (0.5 mgL^{-1}) proved an optimal nutritional combination during germination and seedling development. However, the medium supplemented with NAA (0.5 mgL^{-1})+BAP (1.0 mgL^{-1}) proved beneficial for rapid protocorm multiplication. The *in vitro* seed culture protocol established from the present study may be employed for large scale propagation of this commercially important *S. nepalense* and other related taxa.

Introduction

ORCHIDS, THE doyens amongst ornamentals, are one of the most important global cut flower and pot plants, and their sheer beauty has enchanted and fascinated people since early times. These plants belong to the family Orchidaceae which is represented by 29,481 species distributed in 693 genera (POWO, 2025; WFO, 2023). In India, orchids are represented by 1,256 species which belong to 155 genera (Singh *et al.*, 2019). The orchids stand distinct from other flowering plants in having spectacular array of adaptations that are linked to several innovative features including zygomorphic flowers with well-developed gynostegium, elaborated perianth and resupinated ovary, presence of pollinia, specialized pollination mechanisms, production of microscopic and non-endospermic seeds with undifferentiated embryos, symbiotic association with mycorrhizal fungi, colonization of epiphytic habitats, velamenous roots, and crassulacean acid metabolism (Kaushik, 2019; Pathak *et al.*, 2001; Prakash and Pathak, 2022). Though, most orchids have a long juvenile period, slow growth rate, and low photosynthetic capacity, yet they are of great value in ornamental, medical, conservation, and evolutionary research (Janakiram and Baskaran, 2018). The physiology of seed germination has made the family Orchidaceae most interesting as their seeds are unique and adaptive in several aspects.

Orchid seeds are microscopic, dust-like, highly fragile, and are produced in very large numbers. The percentage germination of these, in nature, is very low *i.e.* 0.2-0.3% (cf. Lal *et al.*, 2021) as these seeds have reduced embryos and suppressed endosperm. A close association with a specific fungal partner, the orchid mycorrhiza is a pre-requisite for their seed germination. The propagation and cultivation of orchids was revolutionized after the discovery by Knudson (1922) that orchid seeds can be germinated on a simple sugar-containing medium. His work showed for the first time that the seed germination in orchids was possible *in vitro* without fungal association. Since then, some of the commercially important and/or rare, endangered, and threatened (RET) orchid species have been successfully raised *in vitro* (Anderson, 1990; Anuprabha and Pathak, 2019; Anuprabha *et al.*, 2017; Arora *et al.*, 2016; Bhatti *et al.*, 2017; Bhowmik and Rahman, 2020, 2022, 2023; Chen *et al.*, 2015; Dhillon *et al.*, 2023; Gangaprasad *et al.*, 2024; Gurudeva, 2019; Kaur *et al.*, 2017; Pathak *et al.*, 2017; Sibin and Gangaprasad, 2016; Singh *et al.*, 2006; Sunita *et al.*, 2021; Thakur and Pathak, 2020, 2021; Vasundhra *et al.*, 2021).

The members of the genus *Satyrium* including 92 species, is almost entirely restricted to continental Africa, with just five species found in Madagascar and four species in Asia (Van der Niet and Linder, 2005). Its presence in alpine meadows and temperate forest

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borders at elevations of 1200-4500 m amsl with a habitat of Alpine Pinus forest and grassy slopes has been reported in Bhutan, China, India, and Nepal on the Asian subcontinent (Wu *et al*., 2019). *Satyrium nepalense* (commonly called as Nepal *Satyrium*) is a multi-faceted threatened medicinal orchid found in the Indian Himalayan Region from Shimla eastward, the Khasia Mountains, and the Deccan Peninsula surrounding Travancore generally between 1800-2300 m. It is a remarkable orchid since it is one of the few with a distribution that spans both the Himalayas and the Western Ghats (Babbar and Singh, 2016). The cultural and medicinal uses of *S. nepalense* are truly intriguing. The indigenous people of the upper Nilgiris- the Todas utilizes the dried and powdered tubers of this terrestrial orchid as an energizing tonic. Tubers and whole plant are eaten by Mopa tribe for curing Malaria and Dysentry. Also, the stem of this plant is used to nourish the blood, support the kidneys, reinforce the loins, and calm the mind (Teoh, 2016). *Satyrium* species are used as edibles and are essential to human sustenance in the Nagaland region (Deb, 2013).

Despite the well-developed legal trade, orchids are widely and illegally collected from the wild for local, regional, and international trade. As a result of various interaction with other groups, specificity of habitat and speciation, these plants occur at low densities with limited range and are vulnerable to over harvest (De and Pathak, 2018). Orchid trade which is largely unreported is threatening its populations in wild. This is compounded by various other anthropogenic activities such as deforestation, forest fire, random construction of roads, overgrazing and thus making many orchid species including *S. nepalense* threatened, vulnerable or endangered. As a result of all these factors, the entire family has been placed in the Appendix-II of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora), while a number of selected species have been placed in its Appendix-I to prevent the wild harvesting for illegal trade and export (CITES, 2017). The conservation priorities for orchid species seem to be in disarray. Plant tissue culture is the most reliable mean for mass propagation and sustainable utilization of plant materials, thus leading to effective conservation (Anuprabha and Pathak, 2019; Pathak *et al*., 2017; Thakur and Pathak, 2020; Sunita *et al*., 2021; Vasundhra *et al*., 2019)

As the presently investigated *S. nepalense* is a promising orchid having high value in the medicinal and ornamental market, a reliable propagation method for its mass production is required for its commercialization. In the present study, application of plant growth regulators for the enhancement of seed germination and seedlings formation in the species was assessed through asymbiotic culture and an efficient protocol for its rapid propagation using mature seeds procured 20 weeks after pollination (wap) was developed.

Material and Methods

Collection of Plant Material

The mature seeds (20 wap) procured from ripe and dehisced capsules were used as explants during the present investigation. For this purpose, the inflorescences of *S. nepalense* (Fig. 1a,b,c) with mature capsules (Fig. 1d) were collected from Tara Devi Hills, Shimla, Himachal Pradesh, India during the last week of August and first week of September 2022. The mass propagation experiment was performed at Orchid Laboratory, Department of Botany, Panjab University, Chandigarh, India.

Seed Viability Test

Seed viability was determined by the ability to reduce 2,3,5- Triphenyltetrazolium chloride (TTC) to the red coloured formazan (Brewer, 1949). The orchid embryos either turned red or stayed colourless after TTC staining. Seeds with TTC reduction ability (red coloured) were scored as viable and 98 % of the seeds were viable as evidenced from microscopic examinations.

Sterilization of Explant

Seeds were treated with 4% sodium hypochlorite (NaOCl) and 2-3 drops of Tween-20 (Hi Media, India) for 15-20 min and these were rinsed thrice with autoclaved distilled water (to remove the 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. The cultures were maintained under a 12 hr photoperiod of 3500 lux light intensity and a temperature of 25±2°C and observed regularly.

Culture Media

In the present investigation, MS medium (Murashige and Skoog, 1962) was prepared containing 2% sucrose and (0.8% w/v) agar as a solidifying agent. 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), 1 naphthalene acetic acid (NAA)] and Cytokinins [Kinetin (KN), Thidiazuron (TDZ), 6-benzylaminopurine (BAP)] 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.

Acclimatization

Healthy seedlings with 2-3 well grown leaves and 1-2 roots were gradually hardened *in vitro*, by sequential elimination of organic additives, vitamins, sucrose, and minor salts from the nutrient medium at 15 days interval. The well rooted seedlings were taken out from culture vessels and thoroughly washed under running tap water for removal of agar attached to root surface and transferred to pots containing a potting mixture of brick pieces, moss, and bark in 1:1:1 ratio

Data Recording and Statistical Analysis

The experiment was conducted using 4 replicates per treatment. The cultures were examined at regular intervals and data was recorded on the basis of average number of days for onset of germination, spherule and protocorm formation, chlorophyll synthesis, emergence of first leaf and root primordia, and subsequent seedling development. The data recorded from the present investigation was subjected to one way ANOVA

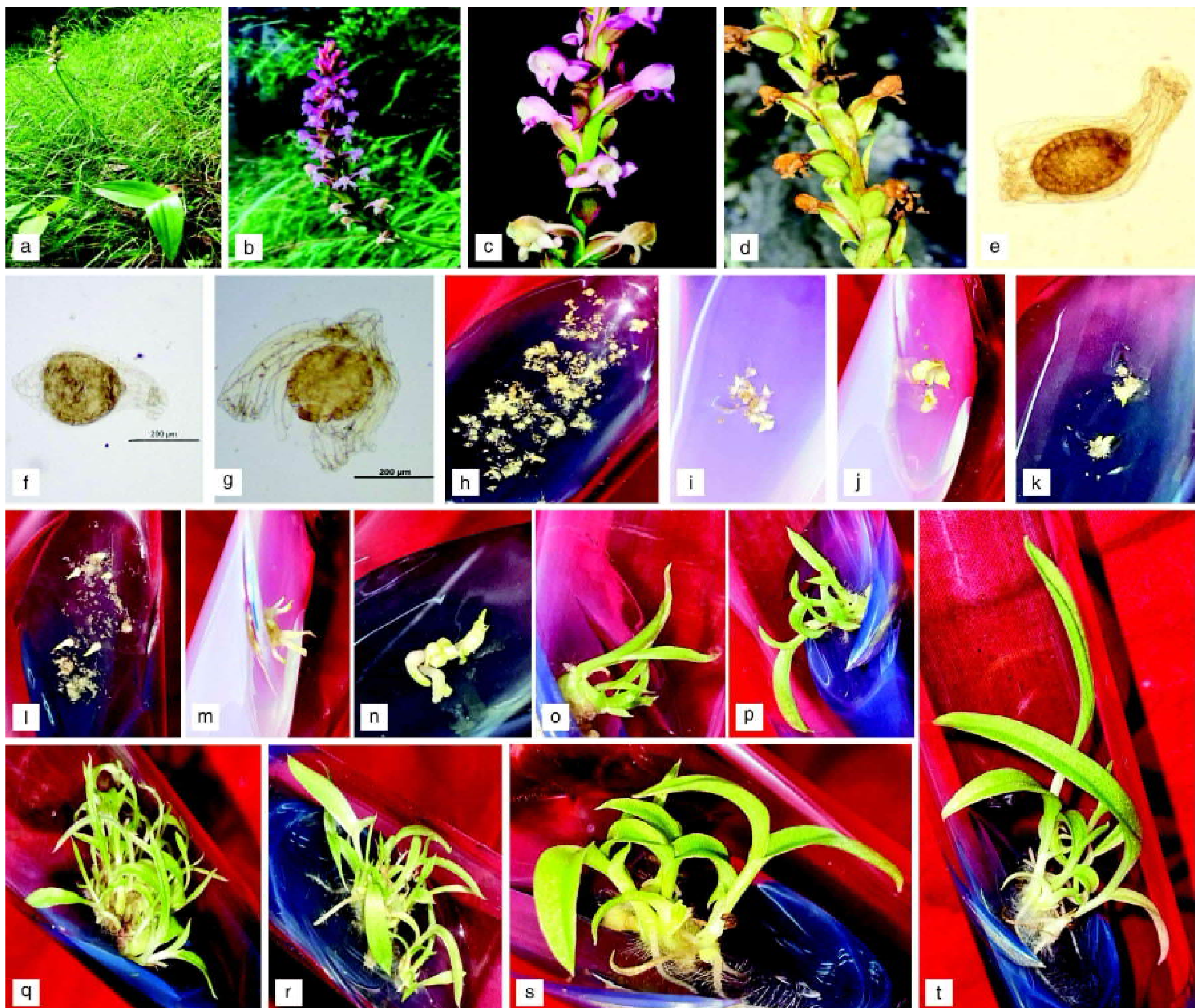

Fig. 1. a-t. *In vitro* asymbiotic seed germination and seedling development in *Satyrium nepalense:* a, Plant in its natural habitat; b-c, Inflorescence bearing flowers; d, linflorescence with mature capsules (20 wap); e, Mature seed at the time of inoculation; f, Swollen embryo 10×; g, rupturing of seed coat; h, Achlorophyllous protocorms with profuse growth of absorbing hair (MS+AC+BAP (0.5 mgL^{-1}); i-m, Formation of leaf primordium in MS+TDZ (0.5 mgL^{-1}), MS+NAA (0.5 mgL^{-1})+BAP (1.0 mgL^{-1}), MS+AC+BAP(0.5 mgL^{-1}), MS+AC+TDZ (0.5 mgL^{-1}), and basal medium; n, Formation of root primordium in MS+AC+BAP (0.5 mgL^{-1}); o-p, Seedlings development in M+AC+TDZ (0.5 mgL^{-1}) and MS+AC+BAP (0.5 mgL^{-1}); q-t, Healthy seedlings with profuse growth of absorbing hair at base in MS+AC, M+AC+TDZ (0.5 mgL^{-1}), MS+AC+NAA (0.5 mgL^{-1}), and MS+AC+BAP (0.5 mgL^{-1}).

(Analysis of Variance) and significant differences were determined by employing Tukey's Test at 5% significance level. The statistical data analysis was performed using SPSS Software Program (SPSS Inc.).

Results and Discussion

In the present study, the mature seeds (Fig. 1e) procured from ripe and dehisced capsules (20 wap) of *Satyrium nepalense* were aseptically inoculated on agar-gelled MS (Murashige and Skoog, 1962) medium. The efficacy of different growth additives (NAA, IAA, KN, TDZ, BAP) with and without activated charcoal (AC) was also tested on the onset of germination, spherule formation, development of protocorm, first leaf primordium, first root primordium, and subsequent seedling development (Table 1; Fig. 1a-t). In the basal medium, onset of seed germination started within 19.00±0.91 days. The embryonal masses emerged out of the ruptured seed coats as spherules within 33.50±1.32 days and soon developed into protocorms in 47.75±0.85 days. Protocorm multiplication and differentiation into leaf and root primordium thereof were observed in 89.50±1.04 and 108.00±0.70 days, respectively. Complete healthy seedlings with 3-4 leaves and 3-4 roots were obtained in 149.75±1.88 days. During the present study, initially the protocorms were achlorophyllous which subsequently acquired chlorophyll. The protocorms in the terrestrial orchids of the open grasslands, well drained shady and seasonally dry soils are achlorophyllous (cf. Gayatri *et al.,* 2006). Morphologically, a protocorm is considered as a state

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

Growth additives	Time taken in days						Remarks
	Onset of germination	Spherule formation	Protocorm formation	1st leaf primordium	1st root primordium	Complete seedling	
Basal	19.00±0.91^{def}	33.50±1.32^{fg}	47.75±0.85^{ef}	89.50±1.04^{d}	108.00±0.70^{cde}	149.75±1.88^{de}	Healthy seedlings
AC	16.25±0.75^{bcd}	28.00±0.91^{cde}	43.75±1.25^{de}	75.75±1.18^{b}	105.00±1.77^{bcd}	139.25±0.85^{abc}	Healthy seedlings with profusely absorbing hair at base
$BAP_{0.5}$	13.50±0.86^{ab}	24.75±1.49^{abc}	34.00±0.81^{ab}	73.75±0.85^{ab}	102.00±1.22^{ab}	136.00±1.29^{abc}	Healthy seedlings
$KN_{0.5}$	16.75±1.18^{bcd}	29.25±0.47^{def}	41.75±1.18^{cd}	77.50±0.86^{bc}	107.50±0.95^{bcde}	141.25±0.85^{bc}	Healthy seedlings
$TDZ_{0.5}$	14.25±0.75^{abc}	26.25±0.47^{bcd}	37.25±0.47^{bc}	71.25±0.94^{a}	103.50±1.25^{abc}	137.00±2.73^{abc}	Healthy seedlings
$NAA_{0.5}$	20.50±0.86^{def}	34.50±0.95^{g}	54.75±0.94^{h}	96.75±1.03^{e}	111.25±2.92^{de}	152.00±3.13^{e}	Healthy seedlings
$IAA_{0.5}$	23.50±1.32^{fg}	34.75±1.10^{g}	53.25±0.75^{gh}	98.50±0.64^{e}	113.75±1.93^{e}	154.75±1.18^{ef}	Healthy seedlings
AC+$BAP_{0.5}$	11.25±0.62^{a}	21.25±0.47^{a}	34.50±1.04^{ab}	75.00±0.70^{b}	100.75±0.75^{ab}	133.00±1.77^{a}	Healthy seedlings with profusely absorbing hair at base
AC+$KN_{0.5}$	14.50±1.32^{abc}	27.25±1.25^{bcd}	-	-	-	-	-
AC+$TDZ_{0.5}$	16.50±0.75^{bcd}	29.25±0.47^{def}	43.50±0.95^{de}	80.25±2.92^{c}	109.50±1.70^{cde}	143.00±2.16^{bcd}	Healthy seedlings
AC+$NAA_{0.5}$	19.50±0.86^{def}	32.50±1.04^{efg}	46.50±0.64^{ef}	85.75±0.62^{d}	107.75±1.03^{bcde}	144.25±1.10^{cde}	Healthy seedlings with profusely absorbing hair at base
AC+$IAA_{0.5}$	21.25±0.62^{efg}	34.50±0.95^{g}	48.75±1.18^{fg}	90.00±1.22^{d}	116.50±0.95^{f}	157.50±0.86^{f}	Healthy seedlings
$NAA_{0.5}$+$BAP_{1.0}$	13.25±0.62^{ab}	22.75±1.49^{ab}	32.50±1.04^{a}	74.75±1.43^{b}	101.00±1.22^{ab}	135.25±1.65^{ab}	Rapid protocorm multiplication
$NAA_{0.5}$+$KN_{1.0}$	-	-	-	-	-	-	No germination
$NAA_{0.5}$+$TDZ_{1.0}$	14.00±0.91^{abc}	26.00±0.70^{bcd}	37.75±1.10^{bc}	74.50±0.86^{b}	103.75±1.03^{abc}	137.75±1.18^{abc}	Healthy seedlings
$IAA_{0.5}$+$BAP_{1.0}$	13.25±0.62^{ab}	22.25±0.47^{ab}	33.50±1.04^{ab}	76.75±0.75^{b}	101.75±1.18^{ab}	135.00±1.22^{ab}	Healthy seedlings
$IAA_{0.5}$+$KN_{1.0}$	18.25±0.75^{cde}	30.75±0.85^{defg}	43.25±1.10^{de}	81.00±1.22^{c}	97.75±1.03^{a}	144.50±2.10^{cde}	Healthy seedlings
$IAA_{0.5}$+$TDZ_{1.0}$	16.75±1.18^{bcd}	29.75±1.49^{def}	41.25±1.10^{cd}	76.25±1.54^{b}	105.75±1.43^{bcd}	140.00±1.22^{abc}	Healthy seedlings

Entries in column no. 2 to 7 are Mean±S.E.; same alphabetical letter in superscript denotes that the corresponding means are in same group Tukey's Test at 5% level.

between an undifferentiated tissue and shoot primordium. Functionally, it is believed to behave like a cotyledon for supplying nutrition to developing embryo as well its subsequent growth into seedling (Stewart and Kane, 2006). Additional presence of AC in the medium enhanced the onset of germination, protocorm formation, and subsequent seedling formation. Activated charcoal has widely been used to darken the medium used for seed/organ (leaf/root) culture. Perusal of literature revealed a beneficial role of this darkening agent in better germination and healthy seedling development earlier in some orchid species (Pathak *et al*., 2001). One possible explanation of the effects of activated charcoal is that it improves aeration and second possibility is that the charcoal adsorbs ethylene which can inhibit growth and proliferation (Ernst, 1975). The seedlings grown in AC supplemented medium have also been reported to have more fresh weight because of its better light absorbing ability and enhanced availability of energy quantum per unit of plant material, in its presence (Werckmeister, 1971).

Plant Growth Regulators (PGRs) such as auxins, cytokinins, gibberellins, ethylene, brassinosteroides *etc*. are employed to enrich the culture medium for orchid seed germination and subsequent seedling development. Presently, the effect of auxins (IAA, NAA) and cytokinin (BAP, KN, TDZ) was assessed during germination and seedling development. Early onset of germination (11.25±0.62 days) and advanced morphogenetic events leading to seedling development (133.00±1.77 days) were best observed in AC supplemented BAP (0.5 mgL^{-1}) nutritional combination. Similar effects of AC+BAP during seed germination and subsequent seedling germination were also reported by Ket *et al.* (2004) and Mulgund *et al.* (2011). 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 formosum, D. hookerianum* (Dohling *et al*., 2008) and *Herminium lanceum* (Thakur and Pathak, 2020) in which seeds require higher salt content medium for germination. Cytokinins have been reported to be decisive for shoot proliferation. In earlier report (Chen and Piluek, 1995), TDZ responded more effectively in comparison to other cytokinins for stimulating shoot bud formation and its differentiation which supports our present investigation in which medium fortified with TDZ (0.5 mgL^{-1}) showed early leaf primordium formation (71.25±0.94 days) followed by BAP (0.5 mgL^{-1}) in 73.75±0.85 days. The better combined effect of auxin and cytokinin ($IAA_{0.5}$+$KN_{1.0}$) was found to be effective in formation of early root primordium (97.75±1.03 days). This combined effect has also been reported earlier in *Cymbidium bicolor* (Mahendran *et al*., 2013), *Phaius tankervilleae* (Pant *et al*., 2011), and *Vanda spathulata* (Decruse *et al*., 2003). When medium was fortified with AC+KN (0.5 mgL^{-1}), spherules failed to develop into protocorms and showed no further growth and development. KN was reported to retard germination frequency in *Coelogyne punctulata* (Sharma and Tandon, 1990) and impair seedling development in *Dendrobium amoenum* (Arora *et al*., 2016) but was effectively utilized to enhance seed germination and protocorm multiplication in *Vanda stangeana* (Bembemcha *et al*., 2016), *Aerides multiflora,* and *Pholidota articulata* (Pathak, 1989). Additional presence of AC+IAA (0.5 mgL^{-1}) in the medium, delayed the seedling formation (157.50±0.86days). Similar inhibitory effects of IAA on seed germination and seedling development have been reported in *Coelogyne viride* (Hadley, 1970), *Cymbidium lowianum* (Sood, 1984), *Dendrobium chrysanthum*, and *Rhynchostylis retusa* (Pathak, 1989). On contrary, IAA enhanced seedling development in *Coelogyne stricta* (Parmar and Pant, 2016), *Malaxis acuminata* (Arenmongla and Deb, 2012), and *Vanda testacea* (Naha *et al*., 2013).

The present study highlights the intriguing responses of mature seeds in in vitro cultures, examining key stages such as seed germination, spherule formation, protocorm development, differentiation, and seedling growth. These stages were assessed in relation to various combinations of plant growth regulators (PGRs) in the nutrient medium, shedding light on how different hormonal environments influence the development of seeds from germination through to seedling establishment. MS medium fortified with AC+BAP (0.5 mgL^{-1}) proved as an optimal nutritional combination during germination and seedling development. However, the medium supplemented with NAA (0.5 mgL^{-1}) + BAP (1.0 mgL^{-1}) proved beneficial for rapid protocorm multiplication. The *in vitro* seed culture protocol established from the present study may be employed for large scale propagation of this medicinally important *Satyrium nepalense* and other related taxa.

Conclusion

The conservation of orchid species faces significant challenges due to the complex interactions between these plants and various organisms, all of which are impacted differently by human activities and environmental changes. Recent efforts have focused on the mass propagation of orchids through asymbiotic seed culture techniques, which are gaining attraction for their potential in horticulture, fragrance, and pharmaceuticals. A successful *in vitro* protocol for mass propagation using mature seeds has been developed for an endangered and medicinal orchid species.

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