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ASYMBIOTIC SEED GERMINATION AND SEEDLING DEVELOPMENT IN A COMMERCIALLY IMPORTANT AND ENDEMIC ORCHID OF WESTERN GHATS, *AERIDES CRISPA* LINDL.- A STUDY *IN VITRO*

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Abstract

The natural populations of many splendid orchids are declining from their natural habitats at an alarming rate and their conservation is becoming a matter of global concern. The present study was intended to facilitate *ex situ* conservation of *Aerides crispa*, an epiphytic orchid having tremendous ornamental and medicinal value. Asymbiotic seed germination potential of immature seeds procured from unripe green capsules was assessed on agar-gelled MS Medium with and without various combinations of plant growth regulators [Auxins (IAA, NAA) and Cytokinins (KN, BAP, TDZ)] and activated charcoal (AC; 0.2%). The onset of germination initiated within 6.25±0.47 days of culturing on MS Medium fortified with IAA+TDZ (0.5 mgl^{-1} each). The percentage germination response showed significant differences in majority of combinations used; it was highest (99.50±0.25%) in the medium supplemented with AC. Early protocorm development (23.25±0.47 days) and their rapid multiplication was observed when medium was supplemented with NAA+KN (0.5 mgl^{-1} each). First leaf and root primordia were developed within 30.05±0.47 and 82.75±1.10 days of culturing, respectively on medium supplemented with IAA+TDZ (0.5 mgl^{-1} each); seedling development was recorded in 99.00±1.29 days, in the medium containing NAA+TDZ (0.5 mgl^{-1} each). MS medium supplemented with IAA+TDZ (0.5 mgl^{-1}each) proved an optimal nutritional combination during germination and seedling development.

Introduction

THE ORCHIDS, belonging to family Orchidaceae, rank amongst the most significant ornamental plants and are known for their beauty, colour combinations, and shape of their flowers; these have always been interesting to evolutionary biologists because of their remarkable floral forms and diversity in pollination systems (Prakash and Pathak, 2020a, 2022). The family comprises 29,481 species (WFO, 2023) in 705 genera (POWO, 2023). They are distributed worldwide, barring a few isolated islands and frozen continent of the Antarctica but their major diversity occurs in Tropical American, Indo-Malayan, and the East Himalayan regions of India (Chen *et al.,* 2009; Prakash and Pathak, 2019). However, their distribution is not uniform, even in tropics or in subtropics and it varies widely between continents and within regions. In India, orchids are represented by 1,256 species which belong to 155 genera (Singh *et al.,* 2019). Their stunningly beautiful flowers with gorgeous colours, unique shapes, curious ornamentation, and prolonged shelf-life have made orchids one of the most sought after flowering plants (Prakash and Pathak, 2020b). 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 % (Lal *et al.,* 2021; Vij, 2002) 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. Subsequently, he proposed a new nutrient solution for germination of orchid seeds (Knudson, 1946). 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; Bhowmik and Rahman, 2020, 2022; Chen *et al.,* 2015; Gurudeva, 2019; Kaur *et al.,* 2017; Lalduhsanga *et al.,* 2021; Mutum *et al.,* 2022; Pathak *et al.,* 2016, 2017, 2022; Singh *et al.,* 2006; Verma, 2016; Thakur and Pathak, 2020, 2021; Tripura *et al.,* 2022; Vasundhra *et al.,* 2021). The application of tissue culture techniques which helps in achieving round the year rapid mass propagation of desired genotypes have opened up new possibilities of their conservation as well as commercialization.

The genus *Aerides* belonging to family Orchidaceae, subfamily Epidendroideae, tribe Vandeae, sub-tribe Aeridinae, was described by Joao De Loureiro in 1790

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in his *Flora Cochinchinensis* and described later in 1990 by Juan Fourcrio. The genus comprises of approximately 21 South East Asian species distributed from India to Papua New Guinea (Kocyan *et al.,* 2008) with main centre of species diversity in mainland tropical Asia. Plants are medium to large sized epiphytes and lithophytes, with mostly pinkish coloured heavily scented flowers. *Aerides* is one of the commercially prospective orchids with great medicinal significance and highly valued in flori-trade (Anuradha and Prakash, 1998; Ghanaksh and Kaushik, 1999; Kishor *et al.,* 2006). *Aerides crispa* Lindl., commonly called the Curled *Aerides*, is a vandaceous orchid with handsome, fragrant, and medium-size flowers, valued for its medicinal and ornamental properties. The species is endemic to the broad leaved evergreen and semi-evergreen forests of Western Ghats and Nilgiri Hills. The species regenerates poorly in nature and is unable to ameliorate the detrimental effect of anthropogenic pressures on the size and frequency of its natural populations. Therefore, *in vitro* propagation technique is an efficient procedure to increase population size of many orchid species for commercial as well as conservation purposes (Anuprabha and Pathak, 2020; Behera *et al.,* 2011; Hossain, 2008; Kumari and Pathak, 2021; Martin and Madassery, 2006, Pant and Gurung, 2005; Pathak *et al.,* 2023; Sharma and Tandon, 1990; Sunita *et al*., 2021; Teng *et al.,* 1997; Vasundhra *et al*, 2019; Vij, 1993; Wimber, 1965). Selection of appropriate medium, growth regulators, and additives are major factors to establish *in vitro* seedlings. Therefore, the asymbiotic germination potential was presently tested for rapid *in vitro* propagation of *A. crispa* by using immature seeds procured from unripe green capsules.

Material and Methods

Sample Collection and Experimental Site

Immature seeds procured from unripe green capsules (18 wap) used as explant were collected from Western Ghats.

Source and Sterilization of the Explant

The cultures were initiated using immature seeds procured from unripe, green, and undehisced capsules. The capsules ('pods') were washed thoroughly under the running tap water for 15 min to remove dust particles. These were then scrubbed with Teepol for 2-3 min and washed with distilled water. Further, the capsules were surface sterilized with Bavistin (0.02 mgl^{-1}) for 8-10 min and Streptomycin (0.02 mgl^{-1}) for 5-8 min, followed by Mercuric chloride (0.1 mgl^{-1}) for 2-3 min. These were then rinsed 5-6 times with autoclaved distilled water, so as to remove all the traces of sterilizing agents. The sterilized capsules were then split open longitudinally with the help of sharp sterilized surgical blade to scoop out the seeds in laminar airflow cabinet under aseptic conditions.

Culture Media and Culture Conditions

Murashige and Skoog, 1962 (MS) basal medium and MS medium supplemented with different growth regulators such as auxins: Indole-3-acetic acid (IAA), 1-Naphthalene acetic acid (NAA); cytokinins: 6-Benzylaminopurine (BAP), Kinetin (KN), Thidiazuron (TDZ) (0.5 mgl^{-1} each); and activated charcoal (0.2%; Thermo Fischer Scientific, Mumbai) were used to test the germination potential of seeds. Medium was solidified with agar (0.8%; Himedia, Mumbai) and pH of the medium was adjusted to 5.6-5.8 using 0.1N HCl or NaOH. A carbohydrate source, Sucrose (2%; Daurala Sugar works, Uttar Pradesh) was also added to the nutrient medium. Approximately, 25 ml of the medium was poured in each test tube and the tubes were plugged with cotton buns before autoclaving at 15 psi pressure and at 121°C temperature for 20 min. The cultures were maintained at 25±2°C temperature and exposed to 12 hr illumination of 3500 lux light intensity.

Data Recording and Statistical Analysis

The experiment was conducted using 4 replicates per treatment. The cultures were examined at regular intervals and data were recorded on the basis of germination frequency and time taken in days for onset of germination, spherule and protocorm formation, chlorophyll synthesis, emergence of leaf and root primordia, and seedling development. The seed germination frequency was derived using the following formula:

$$\text{Seed germination frequency} = \frac{\text{Number of seeds successfully germinated}}{\text{Total number of seeds inoculated}} \times 100$$

The data recorded from the present investigation was subjected to one way ANOVA (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, immature seeds procured from undehisced green capsules of *Aerides crispa* were aseptically grown on agar-gelled MS medium with and without various combinations of Plant Growth Regulators (IAA, NAA, KN, BAP, TDZ, IAA+KN, IAA+BAP, IAA+TDZ, NAA+KN, NAA+BAP, NAA+TDZ; 0.5 mgl^{-1} each) and activated charcoal (AC; 0.2%). Seeds germinated successfully with varied germination

Table 1. *In vitro* asymbiotic seed germination and seedling development in *Aerides crispa* on MS (Murashige and Skoog, 1962) medium.

Additives	Germination frequency(%)	Time taken in days for						Remarks
		Onset of germination	Spherule formation	Protocorm formation	1st Leaf primordium	1st root primordium	Complete seedling	
Basal	94.75±0.25	11.25±0.47^{b}	20.25±0.47^{bcd}	28.50±0.28^{c}	35.25±0.47^{c}	92.50±0.95^{d}	111.25±0.47^{cd}	Healthy seedlings
AC	99.50±0.25	10.75±0.25^{b}	15.75±0.70^{a}	24.75±0.40^{b}	35.00±0.40^{c}	91.25±0.47^{d}	107.00±0.40^{c}	Enhanced germination frequency
IAA	89.75±0.85	18.00±0.40^{d}	27.25±0.47^{e}	37.75±0.47^{d}	-	-	-	Browning of protocorms
NAA	91.50±1.50	10.50±0.64^{b}	21.50±0.64^{cd}	39.25±0.47^{d}	48.25±0.47^{e}	98.00±0.40^{e}	120.00±0.40^{e}	Delayed seedling formation
KN	87.25±1.93	10.75±0.47^{b}	20.50±1.04^{bcd}	-	-	-	-	-
BAP	93.75±0.25	14.75±0.25^{c}	21.00±0.57^{cd}	27.25±0.47^{c}	36.25±0.62^{c}	86.50±0.64^{c}	108.00±0.40^{c}	Healthy seedlings
TDZ	93.50±0.50	16.50±0.28^{cd}	22.75±0.62^{d}	28.00±0.40^{c}	37.00±0.40^{cd}	91.25±0.75^{d}	112.75±0.47^{d}	Healthy seedlings
IAA+KN	92.75±0.47	15.25±0.25^{c}	21.00±0.70^{cd}	28.00±0.40^{c}	37.50±1.19^{cd}	91.25±0.47^{d}	108.75±0.47^{c}	Healthy seedlings
IAA+BAP	90.50±0.28	14.75±0.25^{c}	21.50±0.50^{cd}	27.25±0.47^{c}	-	-	-	Browning of protocorms
IAA+TDZ	98.25±0.85	6.25±0.47^{a}	14.75±0.62^{a}	24.00±0.40^{b}	30.05±0.47^{b}	82.75±1.10^{b}	101.50±1.55^{b}	Rapid protocorm multiplication; early seedling formation
NAA+KN	94.25±0.62	11.00±0.40^{b}	19.25±0.25^{bc}	23.25±0.47^{b}	35.00±0.40^{c}	92.00±0.40^{d}	108.50±0.64^{c}	Rapid protocorm multiplication
NAA+BAP	90.75±0.47	10.75±0.47^{b}	16.00±0.70^{a}	24.00±0.47^{b}	39.25±0.47^{d}	98.25±0.47^{e}	111.25±0.25^{cd}	Rapid protocorm multiplication; healthy seedlings
NAA+TDZ	97.50±0.64	10.00±0.40^{b}	17.75±0.25^{ab}	27.00±0.40^{c}	30.75±0.47^{b}	87.75±0.47^{c}	99.00±1.29^{b}	Early seedling formation

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

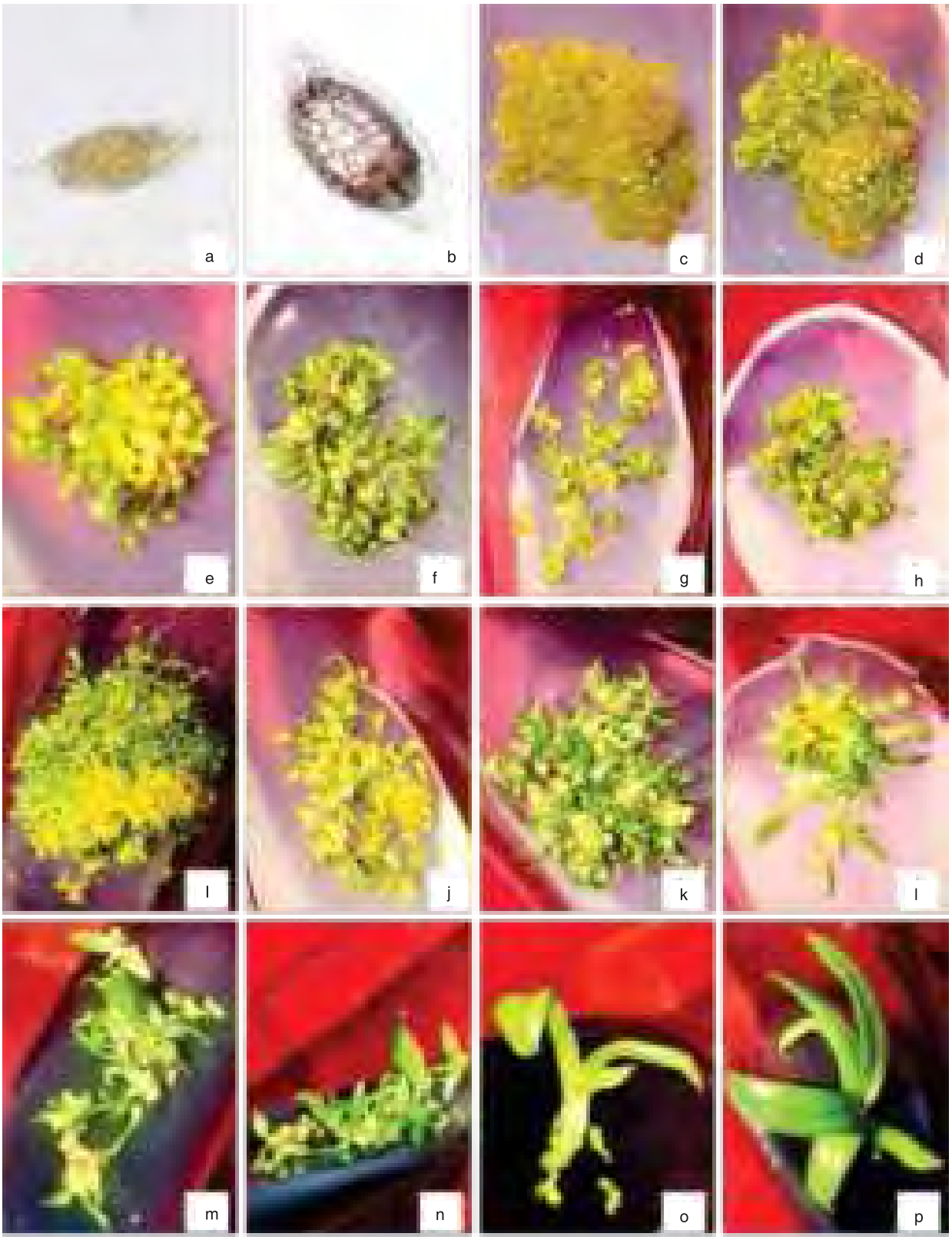

Fig. 1. a-p. *In vitro* asymbiotic seed germination and seedling development of *Aerides crispa* on MS Medium and its combinations with growth additives: a, Immature seed at the time of inoculation; b, Swollen embryo in the seed; c-d, Rapid multiplication of protocorms (M+NAA+BAP; M+NAA+KN); e-h, Protocorms with leaf primordia (M+IAA+TDZ; M+NAA+TDZ; M+BAP; M+NAA+KN); I, Rapid protocorm multiplication (M+IAA+ TDZ); j-k, Development of seedlings (M+TDZ; M+BAP); l, Seedlings (M+NAA+KN); m-p, Healthy seedlings (M+AC).

frequency in different nutritional combinations (Table 1; Fig. 1). Germination of immature seeds was, however, initiated within 6.25±0.47 days of culturing in medium fortified with IAA+TDZ (0.5 mgl^{-1} each). The swelling of embryo and breaking of seed coat was the first sign of successful germination process. The ability of the orchid seeds to germinate prior to reaching maturity has been mentioned earlier by few authors (Arditti *et al.,* 1982; Pathak *et al.,* 2001; Piri *et al.,* 2013) and such a culture of immature seeds is referred to as *Green Pod Culture*. This technique is very useful as it follows an easy sterilization procedure and helps in reduction of time lapse between flower pollination and seed sowing (Alouffa *et al.,* 1998; Pyati and Murthy, 1995). Immature seeds exhibit better germination because of their distended testa cells, metabolically awakened embryos, and absence of dormancy factors (Arditti *et al.,* 1981). In the present study, differences were observed in time taken for initiation of germination response in various nutritional combinations (6.25±0.47-18.00±0.40 days). This indicates that orchid seeds can germinate in a wider variety of nutritional regimes but germination frequency varies with respect to actual chemical stimulus present in the culture medium. Presently, basal medium invoked germination response in 94.75±0.25% seeds and it was enhanced (99.00±0.25%) in medium fortified with AC. 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 (Arditti and Ernst, 1984; Pathak *et al.,* 2001, Vij *et al.,* 1981; Yam *et al.,* 1989). 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, 1970).

The swelled embryos emerged out of seed coats as spherules. Spherules obtained as early as in 14.75±0.62 days in the medium fortified with IAA+TDZ (0.5 mgl^{-1} each), became green with the development of chlorophyll and acquired polarity to develop into pear shaped structures, protocorms (24.00±0.40 days). Pre-protocorm development of chlorophyll is almost universal in epiphytic orchids (Vij *et al.,* 1981; Stoutamire, 1974). Morphologically, a protocorm is considered as a state between an undifferentiated tissue and shoot primordium (Kanase and Takano, 1995). Functionally, it is believed to behave like a cotyledon for supplying nutrition to developing embryo as well its subsequent growth into seedling (Johnson *et al.,* 2007; Lee and Lin, 1987; Stewart and Kane, 2006). A synergistic effect in the form of rapid protocorm multiplication was observed when medium was supplemented with NAA+KN (0.5 mgl^{-1} each). The protocorms obtained in the medium fortified with IAA+TDZ (0.5 mgl^{-1} each) developed first leaf and root primordia within 30.75±0.47 and 82.75±1.10 days, respectively. Seedlings were obtained in 99.00±1.29 days when medium was supplemented with NAA+TDZ (0.5 mgl^{-1} each).

Several Plant Growth Regulators (PGRs) such as auxins, cytokinins, gibberellins, ethylene, brassinosteroides *etc.* are routinely employed to enrich the culture medium for orchid seed germination and subsequent seedling development. Presently, the effect of auxins (IAA, NAA) and cytokinins (KN, BAP, TDZ) was assessed during germination and seedling development. Additional presence of auxin (NAA, 0.5 mgl^{-1}) in the medium delayed the seedling formation (120.00±0.40 days). Similar inhibitory effects of NAA on seed germination and seedling development have been reported in *Coelogyne viride* (Hadley, 1970), *Cymbidium lowianum* (Sood, 1984), *Dendrobium nobile* (Miyazaki and Nagamastu, 1965), *D. chrysanthum*, and *Rhynchostylis retusa* (Pathak, 1989). On contrary, NAA enhanced germination in *Coelogyne stricta* (Parmar and Pant, 2016), *Cymbidium aloifolium* (Deb and Pongener, 2011), *Malaxis acuminata* (Arenmongla and Deb, 2012), and *Vanda testacea* (Naha *et al.,* 2013). Additional presence of BAP in the medium advanced rhizogensis and promoted healthy seedling development. Similar effect of BAP during seedling development was also reported by Ket *et al.* (2004) and Mulgund *et al.* (2011). When the medium was supplemented with KN, 87.25±1.93% seeds showed germination and the germinating entities failed to show further growth and development. KN was reported to retard germination frequency in *Coelogyne punctulata* (Sharma and Tandon, 1990) and impair seedling development in *Dendrobium heterocarpum* (Arora, 1990) but was effectively utilized to enhance seed germination and protocorm multiplication in *Vanda stangeana* (Bembemcha *et al.,* 2016), *Aerides multiflora, Eria spicata,* and *Pholidata articulata* (Pathak, 1989). KN or TDZ, when used in combination with IAA proved beneficial for inducing further development in the germinating entities leading to seedling formation; additional presence of NAA in KN/BAP enriched combination showed synergistic effect in the form of rapid protocorm multiplication and the former combination proved effective during differentiation. The better combined effect of auxin and cytokinin in enhancing germination frequency and seedling development has been reported earlier in *Cattleya aurantiaca* (Smith *et al*., 1994), *Cymbidium aloifolium*

(Deb and Pongener, 2011), *C. bicolor* (Mahendran *et al.,* 2013), *C. elegans* (Pant and Pradhan, 2010), *C. iridiodes* (Pongener and Deb, 2009), *Phaius tankervilleae* (Pant *et al.,* 2011), and *Vanda spathulata* (Decruse *et al.,* 2003).

The present investigation brings forward the exciting revelations of varied *in vitro* culture behaviour of immature seeds in terms of seed germination, spherule formation, protocorm development, differentiation thereof and seedling development in response to different combinations with PGRs in the nutrient medium. MS medium supplemented with IAA+TDZ (0.5 mgl^{-1}each) proved an optimal nutritional combination during germination and seedling development. However, the medium supplemented with NAA+KN (0.5 mgl^{-1} each) 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 *Aerides crispa* and other related taxa.

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