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APPLICATION OF ORGANIC ADDITIVES FOR THE ENHANCEMENT OF SEED GERMINATION AND SEEDLING DEVELOPMENT IN AN ENDANGERED AND MEDICINAL ORCHID, *RHYNCHOSTYLIS RETUSA* (L.) BLUME THROUGH ASYMBIOTIC CULTURE

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Abstract

Rhynchostylis retusa, a valuable ornamental, medicinal, and endangered orchid is rich with bioactive compounds which are potential raw materials for pharmacological utilization. In the present study, application of organic additives for the enhancement of seed germination and seedling formation in the species was assessed through asymbiotic culture and an efficient protocol for its rapid propagation using immature seeds procured 8 wks after pollination (wap) was developed. Four different nutrient media namely Mitra *et al.* (M, 1976), Modified Knudson C (KC, 1946), Murashige and Skoog (MS, 1962), and Vacin and Went (VW, 1949) were used to investigate the effects of different organic additives *i.e.* Yeast Extract (YE; 1 gl^{-1}), Peptone (P; 1 gl^{-1}), Casein Hydrolysate (CH; 1 gl^{-1}), and Activated Charcoal (AC; 2 gl^{-1}) during asymbiotic seed germination and subsequent seedling development. The seeds of the presently investigated species successfully germinated in all the nutrient media used. Highest seed germination (88.00%) and early seedling formation (129.50±0.64 days) was observed in VW medium followed by M (87.25%; 137±0.47 days), MS (79.25%; 148±0.40 days), and KC (73.75%; 157.25±0.47 days) media respectively. Based on present results, the optimal nutritional combination during germination, growth and subsequent development into seedlings of *R. retusa* is suggested as VW medium supplemented with P (1 gl^{-1}). An efficient propagation protocol formulated for the species may be utilised as a useful guide for similar studies in other related orchids.

Introduction

ORCHIDS HAVE intrigued scientists and enthusiasts by their astonishing appearance, endless structural variability, and complex survival strategies including above and below ground interaction with other organisms (Darwin, 1862; Kaushik, 1983; Pal *et al.,* 2019; Pathak *et al.,* 2001; Shefferson *et al*., 2020; van der Cingel, 1995). These interactions involve host trees (69% of orchid species are epiphytes; Zotz, 2013), fungi (orchid species have seeds that in nature cannot germinate without forming a symbiotic relationship with one or more species of fungus; Manoharachary, 2019; Phillips *et al*., 2011; Singh *et al*., 2019), and a diverse array of pollinators (most orchids depend upon pollinators to achieve seed production; Micheneau *et al*., 2009; Prakash and Pathak, 2020). Orchids are r-strategist plants with Type III survival strategy characterized by high fecundity and high mortality at early stages of life (Charintonidou and Halley, 2020); the high fecundities of orchids do not shield them neither bestow immunity to extinction but may cause them to be more vulnerable to extinction because any increase in fecundity is balanced by an increase in pre reproductive mortality. These plants are represented by 28,484 species in the world (Govaerts *et al*., 2017) spanning 736 orchid genera (Chase *et al*., 2015). In India, orchids are represented by 1256 species which belong to 155 genera (Singh *et al*., 2019). The Indian orchids have extensive utility in varied system of local medicine since the Vedic period (Kaushik, 1983; Kumari and Pathak, 2020; Pathak *et al.,* 2001, 2010; Prakash and Pathak, 2019).

Rhynchostylis retusa (L.) Blume, a monopodial, epiphytic, and endangered orchid, also known as Queen of orchids is native to Tropical Asia and India (Chhattisgarh, Himachal Pradesh, Kerala, Meghalaya, Odisha, Sikkim, Tamil Nadu, Uttarakhand). *R. retusa* is state flower of Assam, Arunachal Pradesh, and Andhra Pradesh indicating its high cultural value. The epiphytic orchids are unique in that they have velamen roots which contain wide varieties of symbiotic endophytic fungal species (Deepthi and Ray, 2018, 2020; Molina and Echem, 2018). Many of such fungi secrete secondary metabolites including growth hormones (Shah *et al*., 2019). Because of the beautiful flowers that are arranged in racemose inflorescences, this species ranks amongst the important ornamental orchid. Inflorescences are commercially utilized for decorating hair by local women during Bihu festival in Assam (Buragohain *et al*., 2015). The plant has been widely used by the tribal communities of different regions for the treatment of cuts and wounds, fracture, fever, inflammation, paralysis, piles, malarial fever, and menstruation disorders (Akhter *et al*., 2017; Pant and Raskoti, 2013; Rohani *et al*., 2018; Tiwari *et al*., 2012). The dried flowers of the plant can be used as an insect

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repellent (Subedi *et al*., 2013). Hossain (2011) reported that the plant has significant antibacterial activity against *Bacillus subtilis* and *Escherichia coli*.

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; McCormick and Jacquemyn, 2014). Orchid trade which is largely unreported is threatening its populations in wild (Phelps and Webb, 2015; Subedi *et al*., 2013). 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 *R. retusa* 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, 2020; Gurudeva, 2019; Pathak *et al*., 2011; Thakur and Pathak, 2020; Vasundhra *et al*., 2019).

As the presently investigated *R. retusa* 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 organic additives 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 immature seeds procured 8 week after pollination (wap) was developed.

Material and Methods

Sample Collection

Immature seeds procured from green unripe capsules (8 wap) used as an explant were collected in month of July, 2019 from Sarkaghat region of District Mandi, Himachal Pradesh, India. The mass propagation experiments 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 96% of the seeds were viable as evidenced from microscopic examinations.

Media Preparation

In the present study, four different nutrient media [Mitra *et al.* (M, 1976), Modified Knudson C (KC, 1946), Murashige and Skoog (MS, 1962), and Vacin and Went (VW, 1949)] used for seed germination and seedlings development were augmented with sucrose (2%; Daurala Sugar works, Uttar Pradesh), agar powder (0.8%; Himedia, Mumbai) and different growth additives *i.e.* Activated Charcoal (AC; 2 gl^{-1}; Thermo Fisher Scientific, Mumbai), Yeast Extract (YE; 1 gl^{-1}; Himedia, Mumbai), Peptone (P; 1 gl^{-1}; Himedia, Mumbai), and Casein Hydrolysate (CH; 1 gl^{-1}; Himedia, Mumbai). The pH of all nutrient media was adjusted to 5.7 with either HCl or 0.1 N NaOH. The test tubes containing nutrient media were autoclaved at 121°C with 15 psi pressure for 20 min.

Surface Sterilization and Culture Conditions

Green capsules were washed in running tap water using Teepol (detergent) for 20 min and then, rinsed with distilled water. The washed capsules were taken inside a laminar air flow hood where these were treated with Bavistin (0.01%; 8 min), Streptomycin (0.01%; 10 min), and Mercuric Chloride (0.1%; 5 min). After each treatment, capsules were rinsed thrice with autoclaved distilled water. The capsules were flame sterilized with ethyl alcohol (70%) for 2-3 sec and then, left on a sterile filter paper in a sterile petriplate to absorb excess water. The immature capsules were then split open vertically with sterile surgical blade and the powdery seeds were inoculated on nutrient medium in the test tubes, each containing 25 ml medium. The cultures were maintained under a 12 hrs photoperiod of 3500 lux light intensity and a temperature of 25±2°C and observed regularly.

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 matrix 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.

Statistical Analysis

The experiments were designed following complete randomize block design (CRD) to determine the effects of growth additives on germination response and seedlings development. The experiments were performed using factorial analysis, with significant difference being accepted at the $p<0.05$ level. All the experimental manipulations were carried out under aseptic conditions and for each experiment, at least four replicates were used. The data was analysed statistically using one-way analysis of variance (SPSS, 16.0 version), and the data Mean±Standard Error of the experiments was compared using Tukey's test.

Results and Discussion

In the present experiment, immature seeds of *R. retusa* were successfully used as explants for mass propagation (Fig. 1B). As the seed coat is transparent, changes in the seeds during culture were directly observed under the microscope. The process of seed germination consisted of swelling of embryo to the width of seed coat followed by emergence of embryo from seed coat (Fig. 1C-D). When the embryo was completely discharged from the seed, the structure so formed is known as spherule. Spherule formation was followed by protocorm stage which subsequently developed into seedlings (Fig. 2). The time taken by the seeds to germinate *in vitro* was determined by the type of nutrient medium, the growth additives used, and the species concerned.

The seeds of the presently investigated species successfully germinated in all the nutrient media used. Highest seed germination (88.00%) and early seedling formation (129.50±0.64 days) was observed in VW medium followed by M (87.25%; 137±0.47 days), MS

Fig. 1. A-F. Application of organic additives for the enhancement of seed germination and seedling formation in *Rhynchostylis retusa*: A, Plant in bloom; B, Immature seeds at the time of inoculation; C, Swelling of embryos; D, Rupturing of seed coat; E-F, Protocorm growth and development (VW+AC).

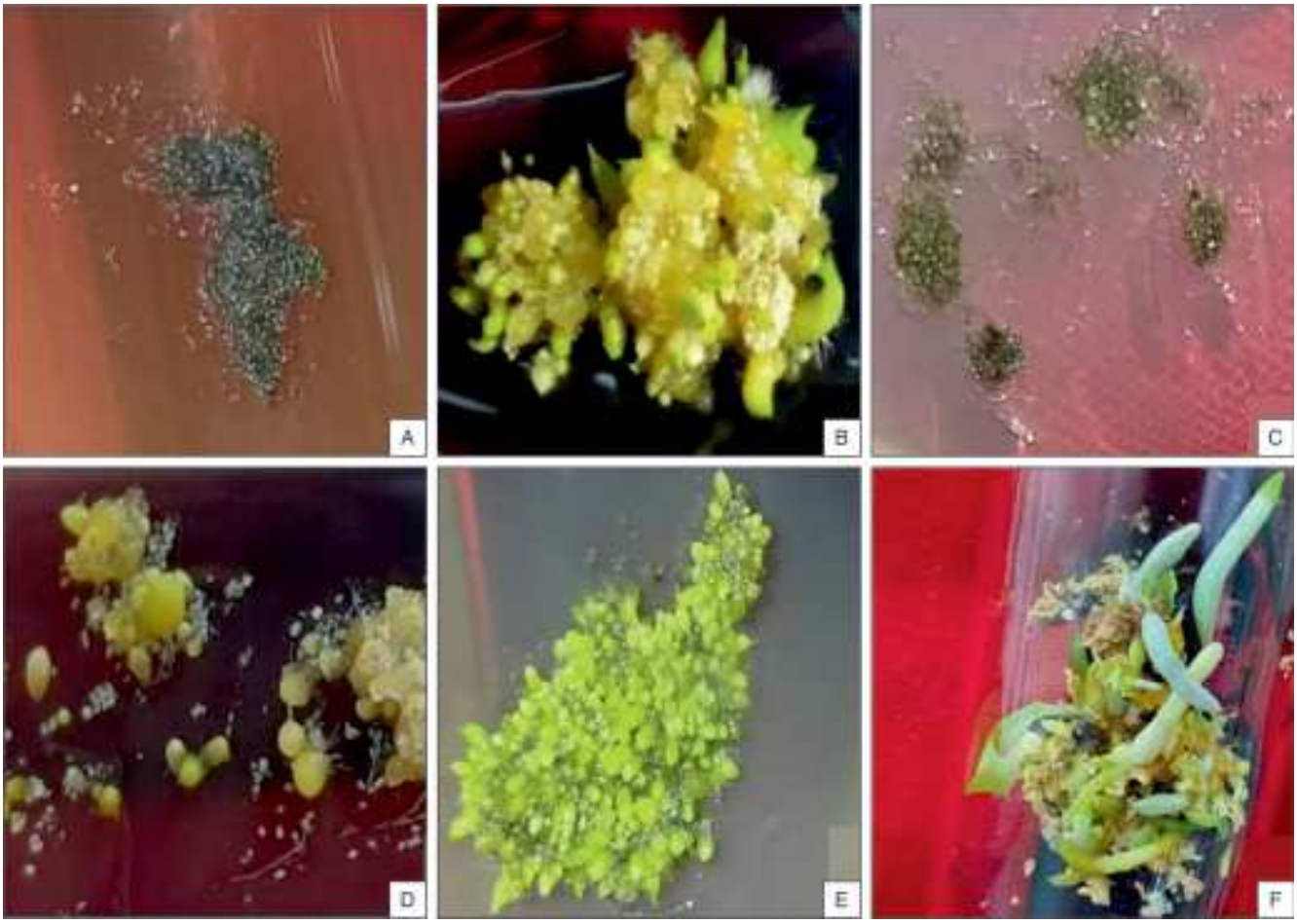

Fig. 2. A-F. Application of organic additives for the enhancement of seed germination and seedlings formation in *R. retusa* (contd.): A, Suppressed growth of protocorms (MS+YE); B, Appearance of leaf primordia (MS+AC); C, Appearance of small protocorms (KC+P); D, Protocorm growth (KC+AC); E, Development of protocorms (M+P); F, Healthy seedlings (M+AC).

(79.25%; 148±0.40 days), and KC (73.75%; 157.25±0.47 days) media respectively (Figs. 1-2; Tables 1-4). The nutritional requirements of different orchid species depend upon their inherent genetic makeup; some have very stringent needs while others can grow on a wide range of nutritional regime (Anuprabha and Pathak, 2012; Arditti, 1967; Mitra, 1986; Pathak *et al.,* 2001)

Addition of AC (2 gl^{-1}) in VW, M, and MS media enhanced germination frequency; rapid protocorm multiplication was also observed in former two nutrient media. The positive effects of AC on *in vitro* seedling growth was supported earlier by many researchers (Druart and Wulf, 1993; Fridborg and Eriksson, 1975; Ket *et al*., 2004; Thomas and Michael, 2007). The use of AC is advantageous in media because it adsorbs inhibitory substances such as polyphenols which can be harmful to plant growth and proliferation (Fridborg and Eriksson, 1975) and helps in reduction of light at the base of explants, which provides an environment conductive to the accumulation of auxin or cofactors (Druart and Wulf, 1993). According to Pierik *et al.* (1988), AC is not necessary during the early stages of seedling growth but had a critical role in further development of the seedlings.

In VW medium, though protocorm proliferation was observed in medium augmented with YE (1 gl^{-1}), the combination remained ineffective during early stages of germination. Interestingly, addition of P (1 gl^{-1}) not only proved beneficial for enhanced seed germination (96.25%) but also advanced protocorm development (14.50±0.28 days), differentiation thereof and subsequent seedling formation (124.50±1.04 days). VW medium augmentation with CH (1 gl^{-1}) enhanced seed germination (93.75%). In M medium, addition of YE and CH delayed onset of germination whereas additional presence of P in medium slightly increased germination frequency. In MS medium, additional presence of organic additives (YE, P, CH) reduced germination frequency and delayed onset of germination, and protocorm growth and development. However, the presence of these additives in KC medium not only reduced germination frequency, but also invariably

Table 1. Effect of organic additives on seed germination of *Rhynchostylis retusa* on VW medium.

Growth additives	Germination Frequency (%)	Onset of germination (days)	Development of					Remarks
			Spherules (days)	Protocorms (days)	First leaf primordium (days)	First root primordium (days)	Seedlings (days)	
Control	88.00%	5.50±0.28^{abc}	11.75±0.25^{bc}	18.75±0.25^{bc}	29.00±0.40^{bc}	89.50±0.64^{bc}	129.50±0.64^{b}	Healthy seedlings
AC (2 gl^{-1})	92.00%	4.50±0.28^{ab}	10.50±0.28^{ab}	17.50±0.28^{b}	28.50±0.25^{ab}	89.50±0.28^{bc}	130.25±0.47^{b}	High rate of multiplication of protocorms
YE (1 gl^{-1})	89.00%	6.00±0.40^{bc}	12.25±0.47^{bcd}	19.25±0.47^{bcd}	29.75±0.75^{bc}	90.25±0.47^{bcd}	130.00±0.40^{b}	Proliferation of protocorms
P (1 gl^{-1})	96.25%	3.00±0.40^{a}	8.00±0.40^{a}	14.50±0.28^{a}	24.50±0.28^{a}	84.50±1.04^{a}	124.50±1.04^{a}	Seedlings with narrow and elongated leaves
CH (1 gl^{-1})	93.75%	3.75±0.25^{ab}	9.75±0.25^{ab}	17.25±0.47^{b}	27.50±0.50^{ab}	88.25±0.47^{b}	128.50±0.64^{b}	Healthy seedlings

Entries in column no. 3 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%.

delayed protocorm differentiation and seedling development. Earlier, P was added as the Carbon and Nitrogen source in the media but later, it was suggested that at an efficient concentration, P can promote the growth of explants (Chen and Chang, 2002; Shekarriz *et al.,* 2014; Sivakumar *et al*., 2005). CH can be a source of Nitrogen, several micronutrients, vitamins, mixture of amino acids, and some unknown growth promoting factor (Bister-Miel *et al*., 1985). YE, the water-soluble portion of autolyzed yeast, supplies essential vitamins, Nitrogen, amino acids, peptides, and carbohydrates (Sommer, 1996). Supplementation of other organic additive such as banana powder (5%) in the medium proved highly effective for root induction (Sinha and Jahan, 2012) and coconut water (CW; 10-20%) showed good results for callus induction in *R. retusa* (Parab and Krishnan, 2012; Permatasari *et al*., 2021; Thomas and Michael, 2007).

The role of different organic additives in asymbiotic orchid germination is uncertain, and responses of growth regulators are often species specific. The small size of orchid seed is a major obstacle in understanding the role of exogenous and endogenous

Table 2. Effect of organic additives on seed germination of *Rhynchostylis retusa* on M medium.

Growth additives	Germination Frequency (%)	Onset of germination (days)	Development of					Remarks
			Spherules (days)	Protocorms (days)	First leaf primordium (days)	First root primordium (days)	Seedlings (days)	
Control	87.25%	7.50±0.64^{cd}	13.25±0.47^{cd}	20.25±0.47^{cd}	30.25±0.47^{bcd}	92.25±0.47^{cde}	137.25±0.47^{cd}	Proliferation of protocorms
AC (2 gl^{-1})	90.00%	5.75±0.47^{bc}	11.25±0.75^{bc}	18.25±0.75^{bc}	28.25±0.75^{ab}	89.75±0.47^{bc}	134.75±0.47^{c}	Rapid multiplication of protocorms
YE (1 gl^{-1})	85.50%	12.50±0.28^{fg}	17.50±0.28^{f}	24.50±0.28^{f}	34.50±0.28^{def}	94.50±0.28^{efg}	139.50±0.28^{d}	Seedlings with elongated leaves
P (1 gl^{-1})	89.75%	9.75±0.47^{de}	14.75±0.47^{de}	21.75±0.47^{de}	31.75±0.47^{bcde}	92.00±0.40^{cde}	137.00±0.40^{cd}	Seedlings with narrow and elongated leaves
CH (1 gl^{-1})	88.00%	11.50±0.64^{ef}	16.50±0.64^{ef}	23.50±0.64^{ef}	34.25±0.47^{def}	93.75±0.62^{def}	138.75±0.62^{d}	Healthy seedlings

Entries in column no. 3 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%.

Table 3. Effect of organic additives on seed germination of *Rhynchostylis retusa* on MS medium.

Growth additives	Germination Frequency (%)	Onset of germination (days)	Development of					Remarks
			Spherules (days)	Protocorms (days)	First leaf primordium (days)	First root primordium (days)	Seedlings (days)	
Control	79.25%	11.50±0.64^{ef}	17.75±0.47^{f}	24.75±0.47^{f}	35.00±0.40^{ef}	98.00±0.40^{ghi}	148.00±0.40^{ef}	Achlorophyllous protocorms
AC (2 gl^{-1})	81.00%	9.75±0.47^{de}	16.00±0.40^{ef}	23.00±0.40^{ef}	33.00±0.40^{cde}	96.00±0.40^{fgh}	146.00±0.40^{e}	Multiplication of protocorms
YE (1 gl^{-1})	73.75%	20.00±0.40^{i}	25.50±0.28^{hi}	32.50±0.28^{hi}	42.50±0.28^{h}	102.50±0.28^{ijk}	152.50±0.28^{g}	Suppressed growth of spherules
P (1 gl^{-1})	78.00%	14.75±0.85^{gh}	20.75±0.85^{g}	27.50±0.86^{g}	37.50±0.86^{fg}	99.50±1.19^{hij}	150.50±1.19^{fg}	Achlorophyllous protocorms
CH (1 gl^{-1})	72.00%	17.00±0.40^{h}	23.00±0.40^{gh}	30.00±0.40^{gh}	40.75±0.62^{gh}	101.25±0.85^{ijk}	151.75±0.62^{fg}	Achlorophyllous protocorms

Entries in column no. 3 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%.

plant growth regulators in promoting/inhibiting germination (Arditti *et al.,* 982 a,b; Madhavi and Shankar, 2019; Pathak *et al.,* 2001). During the present investigation, immature seeds (8 wap) invariably showed germination response in all the organic additives (YE, P, CH) enriched media, the frequency and onset of germination, protocorm growth and development, and subsequent seedling development varied with the nutrient medium used and quality of the growth additive. Though the use of growth additives to augment seed germination in *R. retusa* has been tested by a few researchers earlier (Bhattacharjee and Islam, 2015; Devi and Neelashree, 2018; Kumar and Palni, 2003; Oliya *et al*., 2021; Parab and Krishnan, 2012; Sharma, 2019; Thakur and Dongarwar, 2019; Thomas and Michael, 2007), their effect varies not only with the nutrient medium, quality and quantity of the growth additive (growth regulator/organic additive) used but also with the stage of development of the seed (Arditti *et al.,* 1982 a,b; Pathak *et al.,* 2001).

Based on present results, the optimal nutritional combination during germination, growth and subsequent development into seedlings of *R. retusa* is suggested as VW medium supplemented with P (1 gl^{-1}). Amongst all the growth additives used, rapid protocorm multiplication and proliferation was observed in AC supplemented VW medium.

Table 4. Effect of organic additives on seed germination of *Rhynchostylis retusa* on KC medium.

Growth additives	Germination Frequency (%)	Onset of germination (days)	Development of					Remarks
			Spherules (days)	Protocorms (days)	First leaf primordium (days)	First root primordium (days)	Seedlings (days)	
Control	73.75%	14.50±0.64^{gh}	20.50±0.64^{g}	27.50±0.64^{g}	37.50±0.64^{fg}	97.25±0.47^{fgh}	157.25±0.47^{h}	Achlorophyllous protocorms
AC (2 gl^{-1})	71.25%	11.25±0.47^{ef}	17.25±0.47^{ef}	24.25±0.47^{ef}	34.50±0.28^{def}	96.75±0.85^{fgh}	156.75±0.85^{h}	Achlorophyllous protocorms
YE (1 gl^{-1})	68.75%	20.25±0.47^{i}	26.25±0.47^{i}	33.25±0.47^{i}	43.25±0.47^{h}	103.25±0.47^{k}	165.25±0.47^{i}	Small achlorophyllous protocorms
P (1 gl^{-1})	69.25%	16.25±0.47^{h}	22.00±0.57^{g}	29.00±0.57^{g}	44.25±2.21^{h}	103.50±1.32^{k}	163.75±1.18^{i}	Suppressed growth of spherules
CH (1 gl^{-1})	70.00%	16.75±0.47^{h}	22.75±0.47^{g}	30.00±0.40^{gh}	43.00±2.04^{h}	107.50±0.95^{l}	166.50±0.64^{i}	Small achlorophyllous protocorms

Entries in column no. 3 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%.

Conclusion

The conservation priorities for orchid species seem to be in disarray and encompass huge challenges as all the interactions with different groups of organisms are not affected in the same way by anthropogenic activities and other environmental perturbations. Mass propagation of orchids through asymbiotic seed culture techniques has received greater attention; plant-specific bioactive compound production has also very high application values in horticulture, fragrance, and pharmaceutical industries. An efficient *in vitro* mass scale propagation protocol using immature seed was successfully developed presently for the conservation of this endangered and medicinal orchid and this may be utilised as a useful guide for similar studies in other related orchids.

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