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In vitro seed germination and plant regeneration of an epiphytic orchid *Aerides ringens* (Lindl.) Fischer

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Aerides ringens (Lindl.) Fischer is an epiphytic and endemic orchid of Western Ghats, India, which is popular for its beautiful flowers and fox-tail inflorescence. The present study offers novel and reproducible protocol for *in vitro* seed germination of *A. ringens*. Seed viability was determined by triphenyl tetrazolium chloride and fluorescein diacetate staining methods and 88.86%±6.7 seeds were found viable at the time of inoculation. Viable seeds were then evaluated for germination on Murashige and Skoog (MS) basal medium and Knudson C (KC) medium supplemented with variable concentrations of 6-benzyl amino purine (BAP) and peptone. Seed culture on KC medium supplemented with 4.44 μM BAP and 500 mg L^{-1} peptone exhibited best rate of seed germination (89.28±3.42%), which transformed into green colour vigorous and compact protocorms of 1.89±0.38 mm size. Protocorms were subcultured on KC medium fortified with different concentrations and combination of cytokinins [BAP, kinetin (KN) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron; TDZ) and auxin (IAA)]. Best results were obtained in the presence of Kn (9.3 μM), which produced 4.40±2.20 shoots per segment of 3.05±0.46 cm length. Seedlings were transferred to KC medium fortified with auxins (IAA, NAA & IBA) for rooting. Half strength KC with IAA (5.71 μM) produced strong and stout 4.44±1.61 roots of 3.22±0.40 cm length per plantlet. Harden and acclimatized plants were established *ex vitro* with 80% survival rate.

Keywords: *Aerides ringens*, 6-benzyl amino purine, Knudson C, Murashige and Skoog basal medium, peptone, protocorms

Introduction

Aerides ringens (Lindl.) Fischer is an epiphytic and ornamental orchid species, which is endemic to Western Ghats of southern India[1]. Three varieties of *A. ringens* are found at different elevations of Western Ghats, which differ in their flower size, leaf size and leaf pigmentation level. *A. ringens* is valuable among orchids as an ornamental plant for its beautiful pinkish-violet long-lasting flowers and inflorescence. This orchid species can be a precious member of floriculture industry, if it is made available readily. Unfortunately lack of knowledge and interest by the commercial growers and industry, and difficulties in propagation and a long maturation process have limited its market and also made it susceptible for inclusion in red listed threatened species category like *A. lawrenciae* and *A. leeanum*[2].

In vitro seed germination technique is an efficient, cost effective and fast procedure to increase population size of many orchid species for commercial as well as conservation purposes. Selection of an appropriate medium, growth regulators and additives are major factors to establish *in vitro* plantlets[3]. Knudson C and Murashige and Skoog basal media have been proven good to grow many orchid seeds including *A. odorata*[4-6]. The present study focuses on the development of reproducible protocol for *in vitro* seed germination and regeneration of *A. ringens* by manipulating concentrations and combinations of various nutrients.

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Materials and Methods

Seed Source and Initiation of Aseptic Culture

Mature capsules of the orchid plant of about 6-month age were collected from the greenhouse just before their dehiscence. The capsules were washed under running tap water, followed by liquid detergent laboline 5% (v/v) and dried under aseptic conditions inside the laminar air flow cabinet. The capsules were further surface sterilized with mercuric chloride (0.5% w/v) for 10 min, followed by washing with 70% ethanol for 2-3 min[7].

Seed Viability Test

Few seeds from the capsule were taken out in aseptic condition and kept in a 10% sucrose solution

at room temperature for 24 h to arrest the biochemical and physiological activities. The seeds were further immersed into 1% (w/v) 2,3,5 triphenyl tetrazolium chloride (TTC) solution and kept in the dark at 40°C water bath for 24 h. Colour development in intact embryo of viable seeds was observed under light microscope[8].

Further, few seeds were soaked in 1µg fluorescein diacetate (FDA) in 1 mL of acetone. The mixture was vortex to mix and incubated for 15 min at 37°C. Seeds were observed under fluorescent microscope[9]. The viability percentage was calculated using the following formula:

$$\text{Per cent viability} = \frac{\text{No. of viable seeds observed}}{\text{Total no. of seeds studied}} \times 100$$

Seed Culture Medium and Conditions

The powdery seeds were inoculated on Knudson C (KC)[10] and Murashige and Skoog (MS)[11] basal medium), fortified with randomly altered concentrations of organic nitrogen additive, peptone (250, 500, 750 mg L^{-1}), and different concentrations [4.44 (1), 8.9 (2) & 13.3 µM (3 mg L^{-1})] of growth regulator, 6-benzylaminopurine (BAP). The pH of the media was adjusted at 5.6 prior gelling with 0.8% agar. The cultures were incubated at 25±1°C with a photoperiod of 16 h at 3000 lux light intensity of cool white fluorescent light. Better medium was selected based on higher seed germination rate and size of the protocorm for further growth of seedling.

Seed Germination and Protocorm Morphogenesis

Creamy/light green coloured oval or irregular shaped early protocorms were scooped out, dispersed in one drop of water on a glass slide and observed under light microscope. The germination rate was calculated employing the formula: Germination (%) = (No. of protocorms × 100)/Total no. of seeds. Observations on different parameters, such as, days required for seed germination, early and mature protocorm formation, size, shape and texture of protocorms were observed under a stereozoom microscope[12]. Changes in morphology during different stages of protocorm development were studied thoroughly. The cultures were transferred to the same medium at 4 wk interval.

Shoots and Seedling Formation

Vigorous protocorms with leaf primordium and rhizoids were subcultured on KC medium supplemented with different concentrations and combinations of plant growth regulators (PGRs), such as, indole-3-acetic acid (IAA), BAP, kinetin (Kn) and 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (thidiazuron; TDZ), and gelled with 0.8% agar for differentiation of protocorms and induction of shoots. Numbers and size of shoots per seedling were recorded after 60-70 d of protocorms subculture.

Rooting of Shoots

Individual shoots with 2-3 expanded leaves were detached from the shoot clumps and transferred to KC medium having different concentrations of auxins (5.71, 11.42 & 17.13 µM) IAA, (4.9, 9.8 & 14.7 µM) indole-3-butyric acid (IBA) and (5.45, 9.1 & 14.55 µM) 1-naphthalene acetic acid (NAA).

***Ex Vitro* Seedling Establishment**

The healthy *in vitro* plantlets at 3 to 4 cm in size were taken out from the culture vessels and washed under running tap water. Individual plantlets were transferred to 3 types of potting mixture, vermicompost (leaf litter: cow dung 1:1v/v), sand and coconut husk (1:1 v/v) or peat-perlite-vermiculite (1:1:1 v/v), and kept in hardening chamber for 90 d. After transfer, immediately the plantlets were sprayed with fungicide captan (0.001% (w/v) and liquid MS medium without sucrose for 1 wk. The well-grown hardened plants were transferred to bigger pot containing potting medium (brick pieces: charcoal: peat mosses, 2:1:1) and maintained in the Orchid house of Bangalore University.

Experimental Design and Statistical Analysis

The experiments were set in completely randomized design. All the experiments were repeated thrice with 8-10 replicates per treatment in identical physical conditions. The data of all the experiments were subjected to one-way analysis of variance (ANOVA) and mean values were compared using Duncan's multiple range test[13].

Results

Seed Viability Test

Viable and metabolically active cells of seed embryo can change the colourless TTC into an insoluble, reddish pigment 1,3,5-triphenyl formazan (TPF). Presence of red colour in the embryo of *A. ringens* seeds confirmed its viability (Fig. 1a), while nonviable seeds remained unstained. Viable seeds stained with FDA fluoresced bright green, while nonviable seeds remained dim or nonfluorescent (Fig. 1b). In the present investigation, 88.86±6.7%

seeds were found viable at the time of inoculation by both the methods used (Fig. 2).

Seed Germination and Protocorm Morphogenesis

A. ringens seeds (Fig. 1a) were germinated on all the medium combination of MS basal and KC medium (Table 1). The germination stages were observed under light microscope at 5 d interval for 25-30 d after inoculation. It started with swelling of undifferentiated embryo (Fig. 1c), followed by cracking of the testa (Fig. 1d) and then formation of white/light green coloured, oval shaped early protocorms were achieved (Fig. 1e). The germination rate varied due to presence of variable concentrations of PGR and peptone in both the media. KC medium performed better compared to MS basal medium. Presence of BAP and peptone in both the media significantly increased seed germination rate

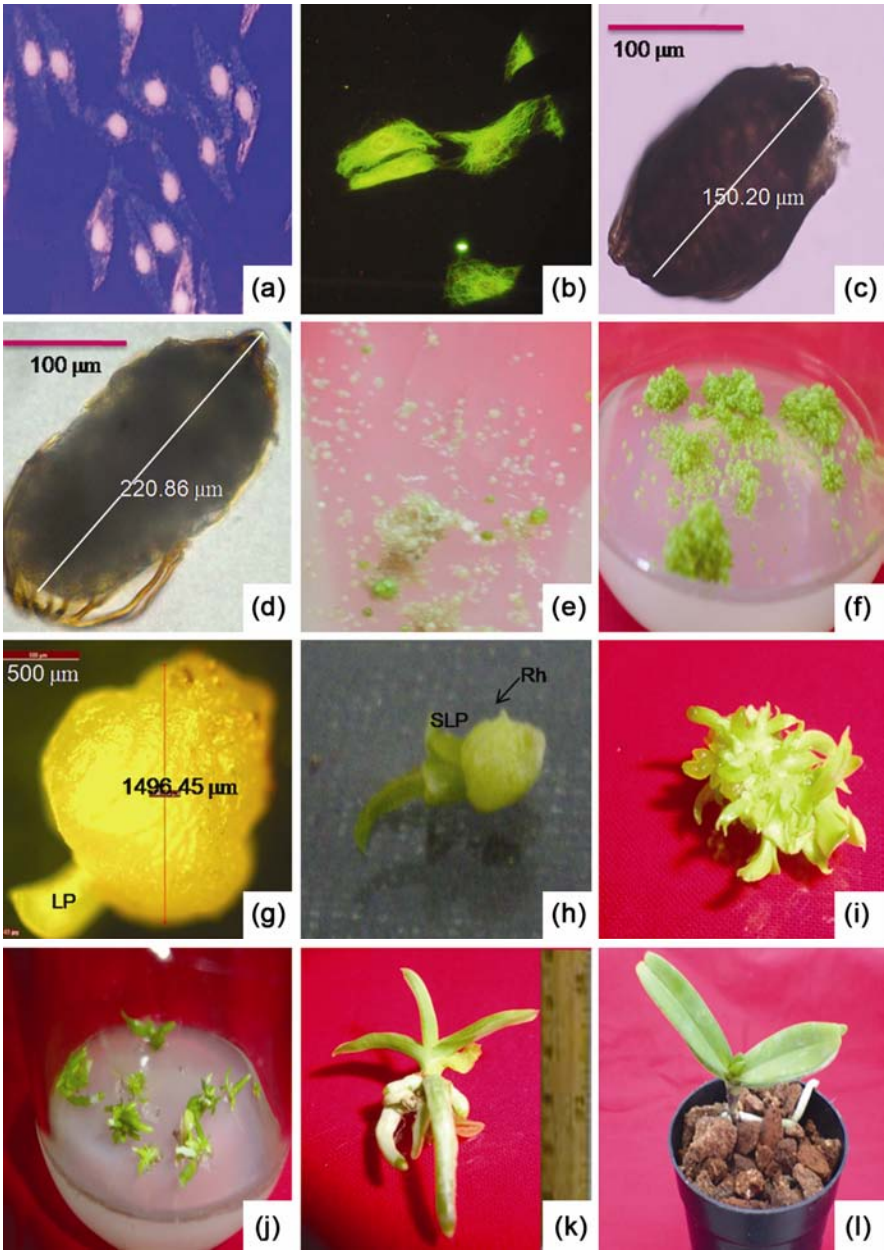

Fig 1 (a-l)—*In vitro* seed germination and plant multiplication of *A. ringens*: a. Viability of seeds tested by TTC treatment; b. Viability of seeds tested by FDA treatment; c. Swelling in seeds; d. Cracking of seed testa; e. Green and oval shaped early protocorm; f. Bigger green and conical protocorms; g. Protocorm with leaf primordium; h. Protocorm with leaf primordium and rhizoids; i. Shoot bud formation; j. Shoot segment with leaves; k. Root formation in plantlets; & l Acclimatized hardened plant in plastic pot.

at lower concentrations. The maximum seed germination rate (89.28%±3.42) was recorded in KC medium supplemented with 8.9 μM BAP and 500 mg L^{-1} peptone, followed by MS medium supplemented with 8.9 μM BAP and 250 mg L^{-1} peptone (86.96±4.56). Increase in concentration of BAP and peptone resulted in lesser germination rate on both media. Minimum germination rate (59.20%±3.42) was manifested by MS basal medium (Table 1).

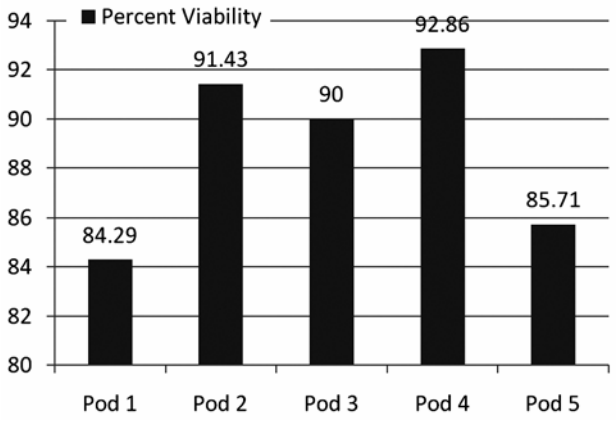

Fig. 2—Seed viability in five different pods of *A. ringens*.

The early green protocorms were gradually turned into dark green, bigger sized conical shaped structure after 55-70 d (Fig. 1f). The apical part of the protocorm, consisting of small number of cells, formed a 'tubercle', which turn into shoot apex followed by leaf primordium, and made its shape asymmetrical (Fig. 1g). Further growth was noticed by development of first and/or second leaf primordium at the upper end of protocorm and rhizoid at the lower end of protocorm (Fig. 1h). No correlation was found between time required for seed germination or protocorm formation, germination rate and size of protocorm. Both the media showed similar changes in protocorm morphogenesis. Individual presence of high concentration of BAP and peptone worked well at latter stage and exponentially increased the size protocorms but germination rate was much higher due to combined effect of BAP and peptone. Among all the combinations tried, presence of 500 mg L^{-1} peptone in KC medium induced the partially loose-textured largest protocorms (2.03±0.2 mm in diam). Further, KC medium with 8.9 μM BAP and 500 mg L^{-1} peptone was found to be the best medium for the maximum seed germination rate

Table 1—Seed germination and protocorm formation of *A. ringens* on different media composition

Medium	BAP (μM)	Peptone (mg/L)	Early protocorm formation (d)	Protocorm formation (d)	% seed germination*	Diam of protocorm* (mm)
MS	0	0	25-30	70-75	59.2±3.42^{a}	0.65±0.19^{a}
	4.4	0	28-32	72-80	63.72±5.24^{b}	0.63±0.18^{a}
	8.9	0	20-30	67-72	70.24±3.24^{d}	0.89±0.22^{b}
	13.3	0	20- 25	65-68	73.52±4.54^{e}	1.48±0.27^{f,g}
	0	250	32-35	75-85	78.24±3.41^{h,i,j}	1.82±0.17h,i
	0	500	25-30	68-75	63.81±4.93^{b}	1.42±0.29^{e,f,g}
	0	750	30-35	75-80	77.13±3.71^{g,h,i}	1.28±0.36^{d,e}
	8.9	500	30-35	75-80	67.52±3.84^{c}	1.19±0.21^{c,d}
	8.9	250	20-25	75- 80	86.96±4.56^{l}	1.54±0.31^{f,g}
KC	0	0	30-35	65-72	71.28±3.13^{d}	1.45±0.45^{e,f,g}
	4.4	0	35-38	60-70	79.96±2.75^{j}	1.56±0.22^{g}
	8.9	0	28-35	75- 80	74.64±4.10^{e,f}	1.78±0.42^{h}
	13.3	0	22-25	65-72	70.6±2.61^{d}	1.41±0.28^{e,f,g}
	0	250	18-22	60-68	79.08±3.63^{i,j}	2.01±0.23^{j}
	0	500	20-26	65-70	84.16±3.77^{k}	2.03±0.20^{j}
	0	750	20-28	68-75	77.64±3.29^{g,h,i}	1.36±0.21^{e,f}
	4.4	500	70-75	75-78	75.92±2.25^{f,g}	1.5±0.26^{f,g}
	8.9	250	22-28	70-75	78.44±3.31^{i,j}	1.97±0.21^{i,j}
	8.9	500	30-35	55- 65	89.28±3.42^{m}	1.89±0.38^{h,i}

*Mean values within a column followed by the same letters are not significantly different at p=0.05 according to Duncan's mu range test; n = 8 per treatment conducted in triplicate

(89.28%±3.42) and formation as well as fastest growth (55-65 d) of vigorous, compact and bigger sized (1.89±0.38 mm) protocorms.

Effect of Plant Growth Regulators on Shoot Formation

Shoot bud initiation or protocorm differentiation was greatly influenced by the concentrations of cytokinins and auxin. Shoot buds differentiated directly within 65-90 d of protocorm culture without an intervening callus phase. Shoot buds first appeared as small, green protuberances (Fig. 1i) from the protocorms that continued to grow and produce numerous axillary buds from the base (Fig. 1j). It was observed that the number of shoots emergence increased up to 8.9 µM BAP, 9.1 µM TDZ and 9.3 µM Kn and then declined with further increase in concentrations (Table 2). In the presence of 9.1 µM TDZ, the protocorms differentiated well and resulted in 5.40±1.78 shoot buds with 2.46±0.26 cm length per segment elongation of shoot buds. The highest elongation (3.05±0.46 cm) of shoot bud was recorded in KC medium with 9.3 µM KN, followed by 8.9 µM BAP and 9.1 µM TDZ (Table 2). The combination of 9.3 µM BAP and 5.71 µM IAA gave greater number (3.66±1.34) and size of shoot segments (2.58±0.32 cm) in comparison to other combinations. Lower number (2.20±1.29) and size (1.05±0.3 cm) of shoot buds were observed in 4.4 µM BAP and 11.42 µM IAA combination. Thus among the all combination tried, KC medium with 9.3 µM KN was found to be the best for shoot formation.

Rooting of Shoots

Rooting efficiency of shoots was stimulated by lower concentrations of auxin and nutritional stress. Half strength KC medium fortified with 2.73 µM NAA rooted well (4.12±1.17) but the roots were very thin and longer (3.94±0.18 cm), which made them fragile and prone to damage during transfer to *ex vitro*. In half strength KC medium with 2.45 µM IBA, roots were initiated well in number (4.00±1.50), but their further growth and development were not satisfactory and resulted in lean, weak and short (2.46±0.22 cm) roots. Highest number (4.44±1.61/plantlet) of roots with 3.22±0.40 cm length, having strong and stout root system, were formed in half strength KC medium supplemented with 2.85 µM IAA (Table 3; Fig. 1k), which was the best medium for rooting.

Table 2—Effect of different concentration of cytokinins and auxin on multiple shoot bud formation of *A. ringens* and their elongation

KC	BAP	TDZ	KN	IAA	No. of MSBs/ shoot segment (mean±SE)*	Length of shoots (mean cm ±SE)*
0	-	-	-	-	1.56 ±1.02^{a,b}	1.21±0.20^{a}
0.5	-	-	-	-	1.12±0.83^{a}	1.13±0.22^{a}
1	-	-	-	-	1.68±1.18^{a,b}	1.14±0.31^{a}
2	-	-	-	-	3.04±1.46^{c,d,e}	1.11±0.36^{a}
1	4.4	-	-	-	2.80±1.15^{c,d,e}	2.25±0.61^{d,e}
1	8.9	-	-	-	4.56±1.42^{g}	2.67±0.40^{g}
1	13.3	-	-	-	3.32±1.07^{d,e,f}	1.86±0.41^{b}
1	-	4.54	-	-	2.88±1.58^{c,d,e}	2.16±0.38^{c,d}
1	-	9.1	-	-	5.40±1.78^{h}	2.46±0.26^{e,f}
1	-	13.62	-	-	2.76±1.27^{c,d,e}	2.29±0.27^{d,e}
1	-	-	4.65	-	4.08±2.30^{f,g}	2.23±0.30^{d}
1	-	-	9.3	-	4.40±2.20^{g}	3.05±0.46^{h}
1	-	-	13.95	-	3.00±1.41^{c,d,e}	2.37±0.35^{d,e,f}
1	13.3	-	-	5.71	2.44±1.36^{b,c}	2.0±0.25^{b,c}
1	4.4	-	-	11.42	2.20±1.29^{b,c,d}	1.05±0.3^{a}
1	9.3	-	-	5.71	3.66±1.34^{e,f,g}	2.58±0.32^{f,g}
1	4.4	4.54	4.65	5.71	3.10±1.26^{c,d,e}	2.05±0.28^{b,c}

*Mean values within a column followed by the same letters are not significantly different at p=0.05 according to Duncan's multiple range test; n = 8 per treatment conducted in triplicate

Table 3—Effect of auxins on *in vitro* root system development of *A. ringens* after 30 d of seedling culture

KC	IAA (µM)	NAA (µM)	IBA (µM)	No. of roots per seedling (mean±SE)*	Root length (mean cm ±SE)*
0	-	-	-	1.64± 1.11^{a}	1.95±0.19^{a}
1	-	-	-	2.04± 1.21^{a,b}	2.0±0.25^{a}
2	-	-	-	1.84± 1.07^{a}	2.10±0.27^{a,b}
0.5	-	-	-	2.76±1.64^{b,c,d}	2.86±0.17^{f}
0.5	01.14	-	-	2.68± 1.07^{b,c,d}	2.6± 0.20^{e}
0.5	2.85	-	-	4.44±1.61^{g}	3.22±0.40^{h}
0.5	5.71	-	-	3.52±1.29^{d,e,f}	2.19±0.17^{b,c}
1	2.85	-	-	3.16±1.21^{c,d,e}	4.12±0.28^{k}
1	-	2.73	-	3.24±1.16^{c,d,e}	3.02±0.21^{g}
1	-	-	2.45	3.52±1.16^{d,e,f}	3.48±0.23^{i}
0.5	-	0.91	-	3.04±1.40^{c,d}	2.17±0.21^{b}
0.5	-	2.73	-	4.12±1.17^{f,g}	3.94±0.18^{j}
0.5	-	5.45	-	2.46±1.30^{a,b,c}	2.33±0.17^{c,d}
0.5	-	-	0.98	3.33±1.24^{d,e,f}	3.15±0.24^{g,h}
0.5	-	-	2.45	4.00±1.50^{e,f,g}	2.69±0.40^{e}
0.5	-	-	4.9	3.88±1.24^{e,f,g}	2.46±0.22^{d}

*Mean values within a column followed by the same letters are not significantly different at p=0.05 according to Duncan's multiple range test; n = 8 per treatment conducted in triplicate

***Ex Vitro* Plant Establishment**

The healthy *in vitro* plantlets with well-developed roots were washed under running tap water and transferred to the plastic pots containing vermicompost (leaf litter plus cow dung, 1:1 v/v). The pots with plants were kept in the hardening chamber for providing indirect sunlight and sprayed by water thrice a day. The potting mixture gave bigger green leaves and strong roots during hardening. The acclimatized plants were transferred to thumb-pot, containing charcoal at the base and above that broken brick pieces and/or tree fern roots. These pots were then transferred to Orchid House of Bangalore University where *A. ringens* plants showed 80% survival (Fig. 1l).

Discussion

For *in vitro* seed germination, species-specific medium containing PRGs has been suggested as a suitable propagation method for orchids[14,15]. KC and MS, both the media used in the present study, have different concentrations of macro- and micro-elements, vitamins, minerals as well as sucrose. KC medium contains a comparatively low amount of both macro- and micro-nutrients and lacks vitamins. Both the media were recommended for growing many epiphytic orchid species. A modified KC medium was recommended for seedling growth and development of some *Cattleya* species, *Cattleyopsis lindenii* and *Dendrobium parishii*[16]; while MS basal medium was considered the best for *A. maculosum*[17]. In the present study, KC medium gave better seed germination compared to MS medium when compared individually or along with BAP and peptone. It might be due to different nutrient compositions of KC and MS medium along with peptone and BAP, which provided required additives lacking in fully mature seeds for enhancing germination.

Formation of protocorm is considered as a peculiarity of orchid development. In the present study, peptone either individually or with BAP was found beneficial for the development of bigger sized, conical shaped protocorms in MS and KC media (Table 1). It happened possibly because of the presence of amino acids, amides and vitamin contents of peptone, while BAP provided required additives for enhancing cell division and growth of protocorms. Role of peptone and BAP in the protocorm development has also been established by the studies on other epiphytic orchids[12,18]. However, higher concentration of BAP and peptone retarded the seed germination probably because of the presence of certain amount of inactive endogenous growth regulators and enzymes in seeds.

Cytokinins have been reported to be decisive for shoot proliferation. In the present study, TDZ responded more effectively in comparison to other cytokinins for stimulating shoot bud formation and its differentiation. These results support the studies of Ket *et al*[19] and Gangadhar[20]. However further growth of shoots was more conducive with KN. High levels of cytokinins inhibited shoot formation, likely due to the formation of high levels of endogenous growth regulators in the seedling. An optimum concentration 9 μM of different cytokinins (8.9/9.3 μM BAP, 9.1 μM TDZ & 9.3 μM Kn) used in the present study was found optimum either individually or with auxin (5.71 μM IAA) for shoots initiation and multiplication (Table 2). Similar results were also obtained in other studies[21,22]. Further, acceleratory and inhibitory effects of KN were documented by many workers[3,23,]. In the present study, KN played better role in elongation of shoot buds.

Nutritional stress has been regarded as a supportive agent for *in vitro* rooting of many plants including orchids. The role of auxins to develop root system was found important in other orchids also like *Oncidium* and *Cymbidium giganteum*[12,22]. In the present study, half strength KC medium supplemented with 2.86 μM IAA was found to be the best for the development of roots. These results were found similar with the previous findings in *A. odorata*, showing positive rooting response with IAA[4].

The present protocol for *in vitro* seed germination and multiplication of *A. ringens* plants could be used on a commercial scale to increase its economic value. Successful reintroduction of cultured plant to the Orchid House would provide a method for *ex situ* conservation of this orchid species and avoid the danger of its extinction.

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