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Research Article

ROLE OF PLANT GROWTH REGULATORS ON ASYMBIOTIC SEED GERMINATION AND SEEDLING DEVELOPMENT OF *VANDA COERULEA* GRIFF. EX LINDL. AN ENDANGERED ORCHID

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ABSTRACT
The endangered epiphytic orchid *Vanda coerulea* Griff. ex Lindl. is widely known for its high floristic value and medicinal properties. Considering the poor seed germination under natural condition, the role of NAA, IBA, 2-iP and BAP on the asymbiotic seed germination and protocorm development of this orchid was assessed in Knudson's C basal medium supplemented with 0.1% peptone. Each plant growth regulator (PGR) was tested at various concentrations i.e. 0.5, 1, 2, 4, and 8 mg L^{-1}. For initiation of germination, preference for particular PGR was not so apparent and optimal germination was recorded at the higher concentration of all PGR tested (up to 92%). Seedling survival was least affected with IBA, but significantly retarded with increasing concentration of NAA, 2-iP and BAP. NAA was stimulatory for seedling growth while 2-iP and BAP retarded this process. However, IBA at higher concentration accelerated seedling growth up to rooted stage. For optimal rooting of the seedlings, higher concentration of NAA and IBA was satisfactory. In all media containing 2-iP and BAP, very low frequency of the seedlings reached up to rooted stage and most of them endured at globular and leaf primordial stage. Lower concentration of NAA and IBA resulted in unorganized growth of seedlings to form callus. Multiple protocorm body formation was observed in most of the treatments including basal medium. These data support the wide suitability of simple PGRs in supporting the germination and seedling development of *V. coerulea* and also for establishing cultures of difficult to germinate endangered orchids.

Keywords: *Asymbiotic Germination, Callus, Protocorms, Seedling Development, Vanda Coerulea*

INTRODUCTION
Vanda coerulea Griff. ex Lindl. commonly known as blue *Vanda* is the origin of a vast variety of floriculturally significant hybrids because of its qualities such as flower colour, size and cold tolerance. However, this particular species is undergoing a steady decline due to extensive collection, habitat destruction, deforestation ultimately resulting into restricted small populations existing in remote areas. Therefore, the long term sustainability of enduring population is in question. In general, most of the orchids are propagated clonally and very few are by seeds.

The seeds of orchids are tiny, devoid of endosperm and possess rudimentary undifferentiated embryos. Germination of some orchid seeds in nature is slow, relatively inefficient process that required a long period of time (Sagawa, 1990).

Following Knudson's (1946) success of asymbiotic seed germination, production and commercialization of many orchids have been expanded tremendously. Since then, *in vitro* seed culture protocols have been established for many orchid species, and a number of media, salts, and plant growth regulators have been used for germination and propagation (Arditti and Ernst, 1993). Thus, for conservation of orchid (*Vanda coerulea* Griff. Ex Lindl) from extinction and to increase the population size, *in vitro* techniques can play a significant role (Lauzer *et al.*, 2007). Asymbiotic seed germination that has been applied for the conservation of endangered and threatened taxa may be useful in the reintroduction of this orchid to its natural habitat (Stewart and Kane, 2006). Therefore, there is a need to modify and enrich the culture medium for achieving successful propagation of certain Orchids. The present study evaluates the cultural requirements for *in vitro* seed germination, protocorm growth and seedling development of this

endangered species in presence of various exogenous plant growth regulators. In addition to that this study will help to achieve the suitable medium for asymbiotic seed germination and seedling development of this endangered orchid.

MATERIALS AND METHODS

Plant Material, Sterilization and Culture Media

Cultures were established with the seeds of *V. coerulea*, collected from 8-months old yellowish green capsules. For surface sterilization, the freshly collected capsules were rinsed in 90% alcohol for 20-30 seconds, followed by treatment with 0.1% (w/v) mercuric chloride for 10 minutes and finally washed thrice with sterile distilled water. The sterilized capsules were excised longitudinally and the seeds were scooped out and placed in a conical flask with 100 ml sterile distilled water and stirred for 5 min. Subsequently 100 µl (containing about 200 seeds) of the seed suspension was inoculated into sterilized culture tubes (25x 150 mm) containing 20 ml nutrient medium. For each treatment five replicate cultures were raised.

The germination media were based on inorganic salts of Knudson's (1946) C, with some modifications. In the media, the original iron source and micronutrients was replaced with iron-EDTA (Ethylene Diamine Tetra Acetate) and micronutrients in half strength respectively as used in Murashige and Skoog's (1962) medium. In addition, media supplemented with 2% sucrose served as carbon source and 0.1% peptone as organic supplement. The Plant growth regulator treatments consisted of various grades of α-Napthalene acetic acid (NAA), Indole-3-butyric acid (IBA), 6-γ,γ-dimethyl allyl-amino purine (2-iP) and 6-Benzyl amino purine (BAP) procured from Sigma. The pH of the media was adjusted to 5.2 prior to autoclaving. The media were solidified with 0.9% (w/v) agar and autoclaved at 1.02 kg/cm for 20 minutes. The cultures were maintained at $25^{o} \pm 2^{o}$C under a 10 hr photoperiod provided by Philips white fluorescent lights of 3000-lux intensity. The cultures were incubated for three months and thereafter well developed seedlings were transferred to culture media with or without PGR for plantlet development.

Data Collection

Data on morphogenetic responses of seed cultures were collected after 3 months of culture initiation. The emergence of embryo through the ruptured seed testa was considered as the first sign of germination (De Pauw *et al.*, 1995). Growth rate of seedlings in seed cultures was expressed through quantitative assessment of the different stages of seedling development, such as seedlings with or without visible shoot apex, with expanded leaves, and with roots.

RESULTS AND DISCUSSION

Seed Germination, Protocorm Development and Seedling Survival

Germination of the microscopic seeds (Figure 2a) was initiated about three weeks after culture initiation. The *in vitro* seedling development of *V. coerulea* took place in a sequence like most of the other orchid species. The germination was achieved by testa rupture, i.e. appearance of globular protocorms designated as stage 1 seedling (Figure 2b). By week four, some protocorms develop further to Stage 2 with slightly elongated apical regions defining the protomeristem (Figure 2c). The effects of different PGRs on seed germination and protocorm necrosis are shown in Table 1. Rhizoids were occasionally observed on Stage 2 protocorms.

With further development, the protomeristem developed an angular opening from which the first true leaf emerged (Stage 3, Figure 2e). Shoot elongation was accompanied with root development at the final stage seedlings (Stage 4, Figure 2h).There was no significant difference in the germination percentage among the treatments, and it ranged from 79 to 93%. Optimal germination was recorded in presence of 0.1% peptone, containing NAA 4 mg/L and BAP 8 mg/L. Germination not hindered with any of the PGRs, but lower concentrations (0.5-1 mg/L) of NAA resulted in reduction in germination rate. It has been variously studied that the effects of peptone (or other additives) may vary according to basal media used, since the response in many cases depends on the nitrogen level in the basal solution (Ichihashi and Islam, 1999; Roy and Banerjee, 2002; Naha *et al.*, 2013).

Table 1: Effects of PGRs on Germination and Morphogenetic Responses of Protocorms of *Vanda Coerulea* after Three Months of Inoculation

Treatments (mg/L)	Germination (%)	Necrosis (%)	MPB (%)	Callus (%)
PGR-Free	84.65 ± 1.86	63.80 ± 6.32	8.09 ±2.94	14.94 ± 5.99
NAA 0.5	79.00 ± 6.60	63.28 ± 6.11	2.57 ±1.82	40.91 ± 8.89
NAA 1	79.41 ± 2.70	66.95 ± 1.00	1.86 ±1.48	28.02 ± 5.12
NAA 2	83.35 ± 1.86	65.52 ± 4.34	3.26 ±1.89	0 ± 0.00
NAA 4	92.69 ± 2.26	76.45 ± 2.87	2.56 ±1.69	0 ± 0.00
NAA 8	91.45 ± 0.74	88.25 ± 1.75	6.24 ±3.51	0 ± 0.00
2iP 0.5	86.00 ± 2.45	90.77 ± 4.64	15.79 ±7.09	3.40 ± 2.69
2iP 1	89.58 ± 2.35	80.79 ± 5.78	7.42 ±4.77	6.93 ± 6.01
2iP 2	85.90 ± 4.18	80.93 ± 10.27	1.79 ±2.31	9.52 ± 8.47
2iP 4	90.73 ± 1.54	73.80 ± 4.19	7.65 ±2.32	4.28 ± 1.92
2iP 8	83.03 ± 3.12	86.49 ± 2.37	12.79 ±3.89	1.04 ± 0.82
IBA 0.5	87.39 ± 1.94	75.72 ± 1.53	6.40 ±2.40	26.54 ± 7.76
IBA 1	85.23 ± 3.03	69.76 ± 2.60	11.55 ±1.59	23.03 ± 3.13
IBA 2	82.84 ± 2.60	71.06 ± 4.57	13.91 ±1.59	30.23 ± 3.53
IBA 4	91.76 ± 1.25	53.78 ± 5.26	8.60 ±0.81	17.68 ± 2.00
IBA 8	85.01 ± 0.96	60.10 ± 3.44	5.12 ±0.79	12.74 ± 2.53
BAP 0.5	89.39 ± 2.93	73.31 ± 4.54	8.64 ±2.97	7.50 ± 2.00
BAP 1	84.52 ± 3.39	76.00 ± 5.88	11.32 ±3.65	7.33 ± 1.53
BAP 2	81.57 ± 2.17	81.60 ± 3.95	13.56 ±3.45	2.33 ± 1.88
BAP 4	90.86 ± 1.43	85.40 ± 4.54	9.10 ±5.49	0 ± 0.00
BAP 8	92.78 ± 1.92	85.11 ± 2.23	2.58 ±2.04	16.34 ± 5.35

Data shown are the mean of five replicates ± SE (Standard error).

In the present findings, total nitrogen content in modified KC medium and peptone seems to be satisfactory for germination of *V. coerulea*, but application of additional PGRs resulted in enhancement in germination. Seedling survival was poor in most of the treatments including the control. 2-iP resulted in severe protocorm necrosis upto 90.77% in 0.5 mg/L concentration. The necrosis of protocorms was mostly observed during Stage 1, with occasional incidences in later stages of development. In general, death of protocorms in the early stages of germination is a common phenomenon in orchid seed cultures (Harvais, 1982; De Pauw *et al.*, 1995; Roy *et al.*, 2011). According to Stoutamire (1974) such a phenomenon could be related to lack of adequate nutrient conditions and/or essential growth regulating substances. Our results corroborate this suggestion.

Seedling Growth and Morphogenetic Responses

The type and concentration of growth regulators play an important role in development of many orchid species *in vitro* (Arditti and Ernst, 1993). Seedlings in media containing NAA grew well beyond at least Stage 2.

Higher concentration of NAA (2-8 mg/L) results in significant rooting; best frequency of rooted seedling (49.85%) is achieved with NAA 2 mg/L (Table 2). Higher concentration of IBA (4-8 mg/L) also showed moderate rooting compared to the basal medium where maximum seedlings remained at Stage 1 and 2. Optimum number of leaves (2.05) and roots (2.42) are observed with NAA 2mg/L (Figure 1a and 1b). Rooting of the seedlings hindered with BAP and 2-iP; and high frequency of seedlings could not proceed beyond Stage 2. Significant enhancement in unorganized callus formation (Figure 2f) was observed in

lower concentration of NAA and IBA. Lower concentrations of 2iP, IBA and BAP accelerate MPB formation (Figure 2g).

Table 2: Effects of PGRs on Seedling Growth of *Vanda Coerulea* after Three Months of Inoculation

Treatments (mg/L)	Stage 1 (%)	Stage 2 (%)	Stage 3 (%)	Stage 4 (%)
PGR-Free	23.60 ± 4.01	28.82 ± 1.99	17.31 ± 3.32	7.24 ± 4.95
NAA 0.5	15.94 ± 4.06	22.54 ± 2.84	11.70 ± 4.92	6.34 ± 7.13
NAA 1	23.28 ± 6.25	32.36 ± 6.49	9.28 ± 5.31	5.2 ± 5.01
NAA 2	6.54 ± 1.55	18.97 ± 5.04	21.38 ± 4.14	49.85 ± 6.68
NAA 4	10.18 ± 1.31	20.18 ± 3.64	17.74 ± 4.36	49.34 ± 6.27
NAA 8	9.42 ± 3.28	19.91 ± 3.06	18.45 ± 4.05	45.98 ± 8.58
2iP 0.5	11.78 ± 6.62	67.90 ± 7.57	1.14 ± 1.03	0 ± 0.00
2iP 1	30.84 ± 9.05	52.18 ± 7.80	1.86 ± 1.15	0.78 ± 1.01
2iP 2	22.52 ± 1.73	60.32 ± 5.72	5.85 ± 2.50	0 ± 0.00
2iP 4	21.49 ± 2.85	61.55 ± 3.25	5.03 ± 1.15	0 ± 0.00
2iP 8	24.24 ± 1.47	60.50 ± 4.54	1.44 ± 0.76	0 ± 0.00
IBA 0.5	22.46 ± 2.92	18.77 ± 1.12	22.80 ± 3.65	3.02 ± 3.90
IBA 1	13.95 ± 3.59	23.87 ± 1.50	14.61 ± 3.05	13.00 ± 5.76
IBA 2	18.36 ± 2.47	19.70 ± 4.14	15.89 ± 2.74	1.89 ± 1.79
IBA 4	19.26 ± 4.33	23.27 ± 2.09	14.42 ± 3.71	16.77 ± 5.79
IBA 8	18.59 ± 2.64	27.04 ± 3.46	11.79 ± 3.11	24.72 ± 2.87
BAP 0.5	26.43 ± 2.20	56.01 ± 2.52	1.42 ± 1.37	0 ± 0.00
BAP 1	18.78 ± 3.42	60.28 ± 6.15	2.29 ± 1.87	0 ± 0.00
BAP 2	14.89 ± 1.92	68.21 ± 3.91	1.0 ± 1.29	0 ± 0.00
BAP 4	29.14 ± 1.74	54.83 ± 3.86	6.93 ± 5.51	0 ± 0.00
BAP 8	41.47 ± 7.27	39.61 ± 7.21	0 ± 0.00	0 ± 0.00

Data shown are the mean of three replicates ± SE (Standard error).

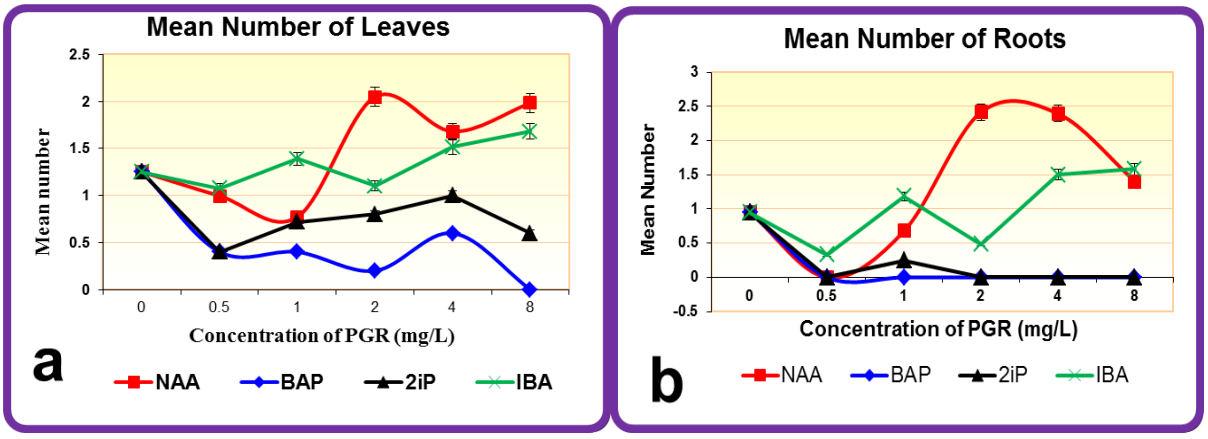

Figure 1: Growth Profile of the Seedlings of *V. Coerulea*: (a) Mean Number of Leaves and (b) Root, under the Influence of Plant Growth Regulators

Orchid needs auxins and/or cytokinins for PLB formation and plantlet development. In *V. coerulea*, NAA and IBA at its lower levels enhanced unorganized callus formation. In presence of NAA, seedling growth was faster and all seedlings at least formed leaf primordia. Kumaria and Tandon (2000) studied the effect of PGRs on peroxidase, polyphenol oxidase and IAA oxidase activities and phenolic content during initiation and development of protocorm of *Dendrobium fimbriatum*. They proposed that PGRs at low

concentration in the medium might act like symbiotic fungal stimulant and conveyed the physiological changes for protocorm development. However, with application of BAP and 2-iP, both frequency of rooted seedling and mean number of root were restrained and majority of seedling growth was checked within stage 1 and stage 2. This growth arresting effect of PGRs on seedling could be efficiently used for *in vitro* conservation.

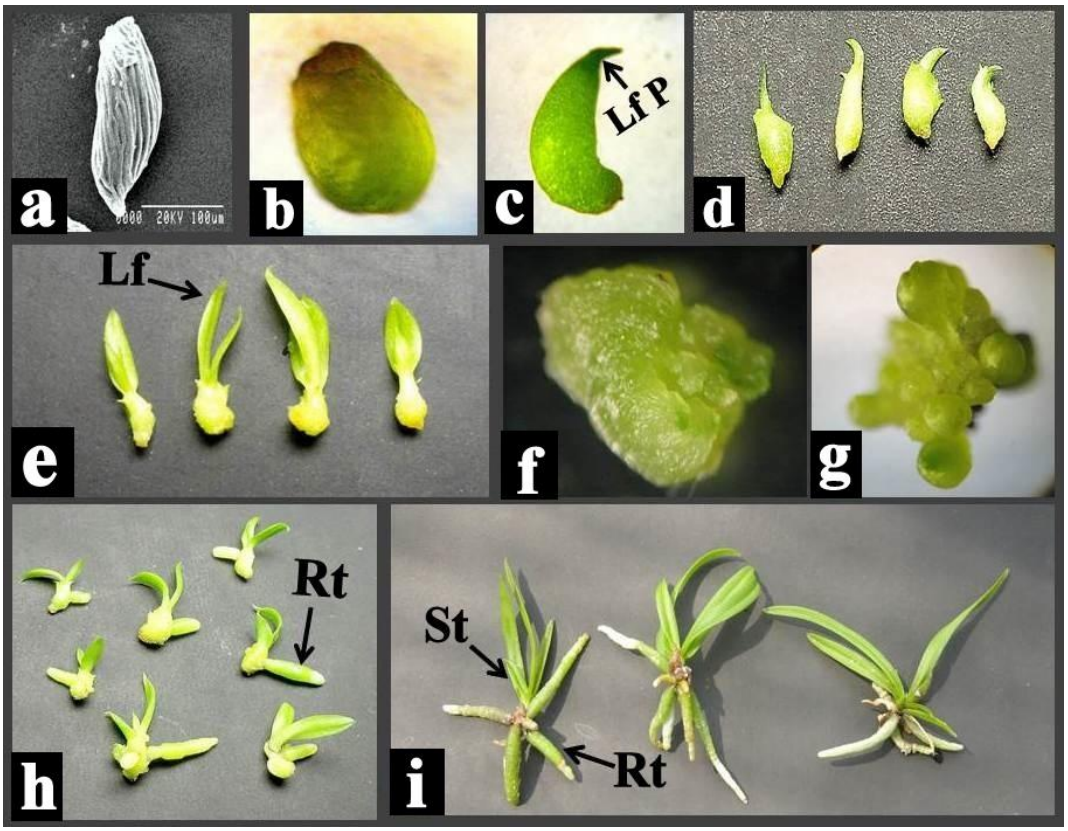

Figure 2: Asymbiotic Seed Germination and Seedling Development of *Vanda Coerulea*: (a) Scanning Electron Micrograph of Seed (b) Stage 1 Early Globular Stage, (c) & (d) Stage 2 Protocorm Showing Initiation of Leaf Primordium, (e) Protocorms with Distinct Leaf (Stage 3), (f) Unorganized Callus, (g) Multiple Protocorm Bodies, (h) Stage 4 Seedling Showing Developed Shoot and Root, (i) Well Developed Plants (Lf P- Leaf Primordial, Lf- Leaf, Rt- Root, St- Shoot).

Conclusion

During asymbiotic seed germination and seedling development of *V. coerulea*, three different morphogenetic pathways led to the formation of complete plant. Firstly, it depicted normal seedling growth, second pathway is the multiple protocorm body formation and third one is mediated via unorganized callus phase. The present study has described the efficient optimization of plant growth regulators for asymbiotic seed germination and seedling development of *V. coerulea*. It offers a ready means for not only raising the large numbers of plants but also a way for *in vitro* conservation of this endangered orchid.

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