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International Journal of Current Research
Vol. 5, Issue, 04, pp.1006-1011, April, 2013

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

ASYMBIOTIC SEED GERMINATION AND SEEDLING DEVELOPMENT OF *VANDA TESTACEA* (LINDL.) REICHB. F.: AN *IN VITRO* APPROACH FOR OPTIMIZATION OF CULTURAL REQUIREMENTS FOR A MEDICINALLY IMPORTANT RARE ORCHID

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ARTICLE INFO

Article History:
Received 15th January, 2012
Received in revised form
24th February, 2013
Accepted 27th March, 2013
Published online 13th April, 2013

Key words:
Asymbiotic germination,
Callus, Protocorms,
Seedling development,
Vanda testacea.

ABSTRACT

The epiphytic orchid *Vanda testacea* (Lindl.)Reichb.f. is widely known for its medicinal properties and high floristic value. Considering the poor seed germination under natural condition, seeds of *Vanda testacea* were cultured on Knudson's C medium, enriched with various concentrations of organic additives and Plant growth regulators to study asymbiotic germination, seedling development and optimization of the cultural requirements. For initiation of germination, preference for particular additives was not observed and optimal germination was recorded in basal media. Seedling survival was not affected either with organic additives or with BAP (6-benzyl amino purine), but significantly retarded in presence of NAA (α-napthalene acetic acid). 0.1% peptone stimulated seedling growth while 0.4% yeast extract and 40% coconut water were inhibitory. However, NAA was stimulatory for seedling growth while BAP retarded this process. Yeast extract stimulated organized growth of seedlings, rather than allowing unorganized growth to form callus.

INTRODUCTION

There are thousands of commercial orchids (fam: Orchidaceae) that are artificially grown for their beautiful flowers and medicinal importance. Propagation of orchids through conventional methods is not conducive to their mass scale production; they are slow growers and seed germination is rather poor under natural conditions (Rao 1977; Mukhopadhyay and Roy 1994). As a result today commercial production of orchids, as with many other species, depends heavily upon the *in vitro* techniques for rapid propagation (Rao 1977; Mukhopadhyay and Roy 1994; Suttle 1996). India represents one of the major orchid-rich regions with about 1100 species of which nearly 150 are economically important (Hedge 1996). The growth of orchids, is rather, slow mainly due to lack of propagation protocols for different native orchids of commercial importance.

Vanda testacea (Lindl.) Reichb. f. (=*V. parviflora* Lindl.) is an alkaloid rich epiphytic orchid distributed naturally from the Indian sub continent to Indochina. It is widely known for its medicinal properties. Almost all plant parts (root, leaves, flowers) in powder form or as an extract are used as herbal medicines to cure rheumatism, bronchitis, nervous disorders, piles, inflammations and also as potential anticancer drug (Chauhan 1990). This medicinally important orchid is faced with habitat destruction pressures due to extensive collections in the past. As a consequence the species has become rare and is restricted to very narrow pockets in its natural habitats (Kaur and Bhutani 2009). Thus to conserve this orchid from extinction and to increase the population size, plant tissue culture and micropropagation can play a significant role (Wochok 1981). Asymbiotic seed germination techniques which have been applied for the conservation of endangered and threatened taxa may be useful in the reintroduction of this orchid (Pedroza-Manrique *et al*. 2005; Stewart and Kane 2006). The technique of asymbiotic seed germination by *in vitro* culture, which was first introduced by Knudson (1946), has revolutionized the concept of orchid cultivation. Our study evaluates the cultural requirements for *in vitro* seed germination, protocorm growth and seedling development of this important species in presence of various organic supplements and exogenous plant growth regulators. In this study an attempt has also been made to develop efficient and reproducible methods of asymbiotic germination and *in vitro* mass propagation which might be utilized for conservation and restoration of this species.

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MATERIALS AND METHODS

Plant material, sterilization and culturing

Cultures were initiated with the seeds of *Vanda testacea* (Lindl.) Reichb. f., collected from 8-months old undehisced green pods. For surface sterilization, the freshly collected pods 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 pods were cut longitudinally and the seeds were scooped out and placed in a conical flask with 100 ml sterile distilled water and stirred for 5 min. Then 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 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.

Culture media

The germination media were based on inorganic salts of Knudson's C (Knudson 1946), with some modifications. In the media, the original iron source was replaced with iron-EDTA (Ethylene Diamine Tetra Acetate) as used in Murashige and Skoog's (MS) medium and 2% (w/v) sucrose served as carbon source. In addition, media were supplemented with organic additives like peptone (Himedia), yeast extract (Qualingens), and coconut water. The Plant growth regulator

treatments consisted of various grades of BAP (Sigma) and NAA (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 mins. The cultures were incubated at 22 ± 2 ^{o}Cfor three months and thereafter well developed seedlings were transferred to either medium with or without PGR for plantlet development.

Acclimatization of seedlings

Seedlings with well developed roots were selected for acclimatization. After taking out from flasks the seedlings were washed thoroughly to remove all traces of nutrient agar, and then transferred to clay pots containing a potting mixture. The potting mixture consisted of finely chopped dried coconut husk, small pieces of dead tree bark (mango) and broken bricks in 1:1:1 ratio. The seedlings were kept in a moist shady place in the departmental orchid culture unit, and the plants were sprinkled with water twice daily.

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. The data of all treatments were subjected to analysis of variance (ANOVA) and subsequent mean separation was performed by the Duncan's multiple range test (DMRT).

RESULTS

Asymbiotic seed germination

In each treatment, seed germination was initiated about 3 weeks after culture initiation. The effects of different organic supplements and PGRs on seed germination are shown in Table 1 and 2 respectively.

Table 1. Effects of organic additives on germination and morphogenetic responses of protocorms of *Vanda testacea* after three months of inoculation

Treatments*	Germination (%)	Necrosis (%)	MPB (%)	Callus (%)
BM	90.96 ± 0.37a	2.27 ± 0.38a	4.72 ± 0.45a	0 ± 0b
P 0.05	97.42 ± 1.5a	6.61 ± 1.7a	1.97 ± 0.72bc	9.42 ± 4.83a
P 0.1	99.59 ± 0.41a	5.17 ± 3.49a	0.43 ± 0.43c	2.15 ± 1.55b
P 0.2	100 ± 0a	2.78 ± 2.1a	0.48 ± 0.48c	4.67 ± 3.33ab
P 0.4	98.22 ± 1.78a	16.37 ± 4.5a	0.38 ± 0.38c	2.88 ± 0.6b
YE 0.05	98.22 ± 1.78a	0.96 ± 0.57a	0 ± 0c	0.67 ± 0.67b
YE 0.1	96.94 ± 3.06a	14.56 ± 11.4a	0 ± 0c	0.79 ± 0.79b
YE 0.2	92.7 ± 0.34a	22.65 ± 19.78a	0 ± 0c	0 ± 0b
YE 0.4	92.44 ± 0.34a	2.71 ± 2.71a	0 ± 0c	0 ± 0b
CW 5	100 ± 0a	14.69 ± 6.44a	2.69 ± 2.24ab	2.19 ± 1.24b
CW 10	91.77 ± 8.23a	15.37 ± 7.28a	0 ± 0c	5.72 ± 3.7ab
CW 20	95.78 ± 2.52a	22.63 ± 7.01a	0 ± 0c	2.99 ± 2.99b
CW 40	95.65 ± 4.35a	11.67 ± 11.67a	0 ± 0c	1 ± 1b

Data shown are the mean of three replicates ± SE (Standard Error). In each column, mean values followed by the same letter are not significantly different at 0.05% level (DMRT). *Treatments code: BM= Basal medium; P= Peptone (%, w/v); YE= Yeast extract (%, w/v); CW= Coconut water (%, v/v); MPB= Multiple protocorm bodies.

Table 2. Effects of PGRs on germination and morphogenetic responses of protocorms of *Vanda testacea* after three months of inoculation

Treatments (mg/L)	Germination (%)	Necrosis (%)	MPB (%)	Callus (%)
PGR-Free	99.59 ± 0.41a	5.17 ± 3.49d	0.43 ± 0.43bc	2.15 ± 1.55c
NAA 0.5	100 ± 0a	32.56 ± 18.98bc	0 ± 0c	25.98 ± 3.99a
NAA 1	100 ± 0a	5.14 ± 2.89d	0 ± 0c	0 ± 0c
NAA 2	100 ± 0a	21.92 ± 10.96cd	0 ± 0c	11.23 ± 7.33b
NAA 4	100 ± 0a	54.11 ± 8.5ab	0 ± 0c	0 ± 0c
NAA 8	100 ± 0a	75.47 ± 6.42a	0 ± 0c	0 ± 0c
BAP 0.5	95.61 ± 0.32c	0 ± 0d	0.7 ± 0.35b	4.4 ± 0.32bc
BAP 1	96.42 ± 0.5c	0 ± 0d	0 ± 0c	0 ± 0c
BAP 2	98.43 ± 0.49b	0 ± 0d	5.5 ± 0.34a	0.25 ± 0.25c
BAP 4	98.95 ± 0.6b	0 ± 0d	0 ± 0c	0 ± 0c
BAP 8	100 ± 0a	1.69 ± 0.2d	0 ± 0c	0 ± 0c

Data shown are the mean of three replicates ± SE (Standard error). In each column, mean values followed by the same letter are not significantly different at 0.05% level (DMRT).

Table 3. Effects of organic additives on seedling growth of *Vanda testacea* after three months of inoculation

Treatments*	Stage1 (%)	Stage2 (%)	Stage3 (%)	Stage4 (%)
BM	54.74 ± 0.35b	38.74 ± 0.77ab	5.61 ± 0.59bc	0.91 ± 0.45c
P 0.05	15.76 ± 11.87cde	31.13 ± 1.25abc	13.69 ± 1.56abc	30 ± 10bc
P 0.1	11.88 ± 11.37cde	10.53 ± 7.22bc	11.47 ± 6.66abc	63.97 ± 24.71a
P 0.2	0.48 ± 0.48e	12.56 ± 12.56bc	19.05 ± 6.25abc	63.24 ± 17.35ab
P 0.4	7.09 ± 7.09de	9.77 ± 9.77bc	11.5 ± 7.39abc	68.76 ± 24.49a
YE 0.05	33.51 ± 30.22bcde	24.83 ± 10.68abc	28.57 ± 13.61a	12.42 ± 6.22c
YE 0.1	36.46 ± 25.12bcd	39.31 ± 17.16ab	18.29 ± 5.44abc	5.15 ± 2.78c
YE 0.2	46.6 ± 25.39bc	49.42 ± 22.97a	3.98 ± 2.68bc	0 ± 0c
YE 0.4	100 ± 0a	0 ± 0c	0 ± 0c	0 ± 0c
CW 5	28.64 ± 15.51bcde	34.31 ± 5.92abc	9.82 ± 0.33abc	25.04 ± 8.85c
CW 10	26.2 ± 21.92bcde	23.79 ± 4.5abc	23.66 ± 9.78ab	20.63 ± 10.95c
CW 20	47.93 ± 15.6bc	20.56 ± 4.18abc	20.6 ± 10.3abc	7.92 ± 2.89c
CW 40	96.86 ± 0.38a	0.85 ± 0.85c	1.28 ± 1.28c	0 ± 0c

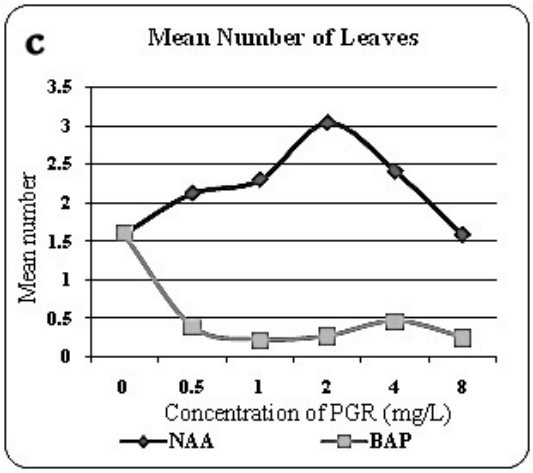
Data shown are the mean of three replicates ± SE (Standard Error). In each column, mean values followed by the same letter are not significantly different at 0.05% level (DMRT).*Treatments code: BM= Basal medium; P= Peptone (%, w/v); YE= Yeast extract (%, w/v); CW= Coconut water (%, v/v).

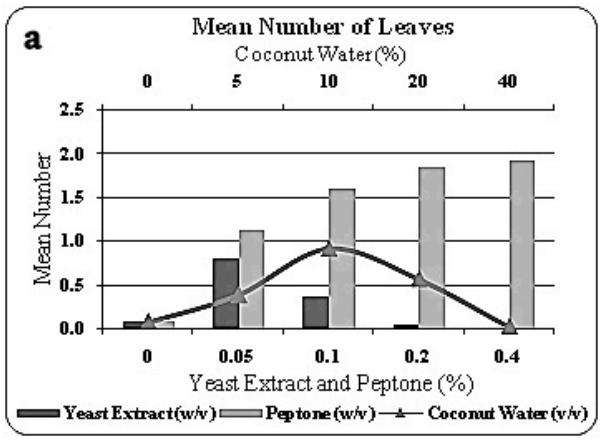
Table 4. Effects of PGRs on Seedling growth of *Vanda testacea* after three months of inoculation

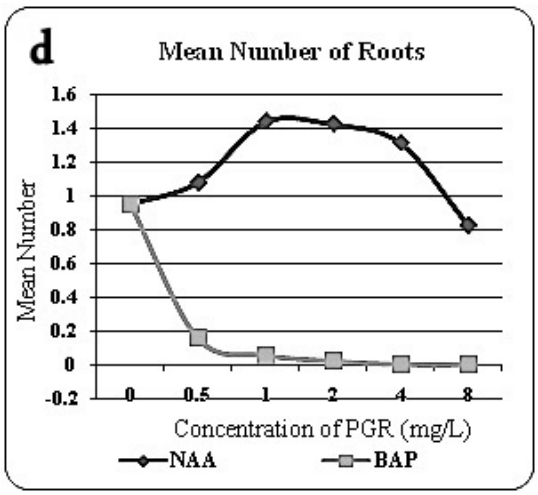
Treatments (mg/L)	Stage1 (%)	Stage2 (%)	Stage3 (%)	Stage4 (%)
PGR-Free	11.88 ± 11.37c	10.53 ± 7.22bc	11.47 ± 6.66ab	63.97 ± 24.71abc
NAA 0.5	0 ± 0d	1.31 ± 1.31c	6.54 ± 6.54b	66.18 ± 5.24abc
NAA 1	0 ± 0d	13.11 ± 8.1bc	2.68 ± 1.66b	84.21 ± 8.75ab
NAA 2	0 ± 0d	17.68 ± 11.78bc	13.91 ± 6.97ab	57.17 ± 10.11bc
NAA 4	0 ± 0d	3.7 ± 3.7c	3.03 ± 3.03b	93.27 ± 3.42a
NAA 8	0 ± 0d	17.78 ± 9.69bc	28.15 ± 14.13a	54.07 ± 23.53bc
BAP 0.5	21.48 ± 0.07c	19.28 ± 0.48bc	15.63 ± 0.51ab	39.21 ± 0.58c
BAP 1	62.58 ± 2.96a	20.63 ± 0.39abc	11.36 ± 0.32ab	5.43 ± 3.42d
BAP 2	51.24 ± 1.08b	18.1 ± 0.57bc	28.35 ± 0.17a	2.05 ± 1.25d
BAP 4	45.86 ± 1.06b	25.99 ± 1.16ab	28.15 ± 2.12a	0 ± 0d
BAP 8	42.09 ± 0.13b	37.86 ± 0.22a	20.05 ± 0.13ab	0 ± 0d

Data shown are the mean of three replicates ± SE (Standard error). In each column, mean values followed by the same letter are not significantly different at 0.05% level (DMRT).

The *in vitro* seedling development of *V. testacea* 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 (Fig.2b).By week four, some protocorms develop further to Stage 2 with slightly elongated apical regions defining the protomeristem (Fig. 2c). 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, Fig. 2d). Shoot elongation was accompanied with root development at the final stage seedlings (Stage 4, Fig. 2e).There was no significant difference in the germination percentage among the treatments, and it ranged from 91 to 100. Optimal germination was recorded in basal medium. However 0.1% peptone stimulated seedling growth while 0.4% yeast extract and 40% coconut water were found inhibitory. NAA was stimulatory for seedling growth, though no significant stimulation in rooting was observed, whereas BAP retarded this process.







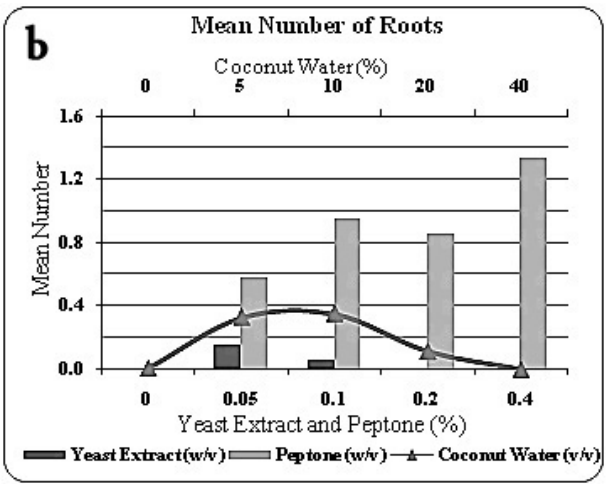


Fig. 1. Growth profile of the seedlings: (a) Mean number of leaves and (b) Root, under the influence of organic supplements; (c) Mean number of leaves and (d) Root, under the influence of Plant growth regulators.

Influence of Organic supplements

Germination and morphogenetic responses

In Knudson's C basal medium (BM), 90.96% germination was recorded. Germination could not be improved with inclusion of any of the organic additives tested (Table-1). Seedling survival was satisfactory in BM and was not affected with addition of any of the organic additives (Table-1). Of all the treatments formation of multiple protocorm body has been observed only in 5% coconut water (Table-1). Unorganised growth via callus formation occurred in 0.05% peptone.

Seedling growth

For optimum rooting of the seedlings, 0.1% peptone was essential. Upon further increase in its concentrations (0.2% - 0.4%), no further improvement in rooting was observed. However, in all media containing peptone (0.05 - 0.4%), very low frequency of the seedlings remained at globular stage while most of the seedlings attained stage 3 and 4. Frequency of seedlings at stage 3 (leafy stage) was maximum with 0.5% YE, however, seedlings in this treatment exhibited no appreciable rooting in this media. Maximum inhibition in seedling growth was observed in media supplemented with 0.4% YE or 40% CW. Mean number of leaves increased in all the treatments of peptone (Figure-1a).

Influence of Plant growth regulators

Germination and morphogenetic responses

Frequency of germination was very high (99.59%) in presence of 0.1% peptone, containing no PGR. Germination was not hindered with NAA, but decreased with BAP (0.5-4 mg/L) unless the concentration of BAP was raised to 8mg/L. Seedling survival was not affected with BAP. On the contrary, NAA was found inhibitory at 0.5 mg/L and 2-8 mg/L, and was relatively less with 1mg/L. Significant enhancement in formation of multiple protocorm bodies (MPB) was observed only with 2 mg/L BAP (Fig. 2h).Significant callusing took place in presence of 0.5 and 2 mg/L of NAA (Fig. 2g).

Seedling growth

Rooting of the seedlings was hindered with 1-8 mg/L of BAP; and growth of seedlings could not proceed beyond stage 1. On the contrary, seedlings in media containing NAA grew well beyond at least stage 1. Though the frequency of seedlings at stage 2 and stage 3 was not reduced in most of the treatments, the growth of the seedlings on media containing 4-8 mg/L of BAP was severely inhibited. Number of leaves remained unaffected in all the treatments while number of roots was significantly reduced at 4-8 mg/L of BAP (Figure-1c, d).

DISCUSSION

Asymbiotic seed germination has been advocated as suitable propagation technique for mass production and conservation of orchid species (Arditti *et al.* 1981; Kauth *et al*. 2006; Stewart and Kane 2006; Thomas and Michael 2007; Roy *et al*. 2011). Because the seeds of orchid are devoid of endosperm which means seeds have limited food reserves, hence it needs specific nutritional and environmental supplementation (Arditti *et al.* 1990). 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 (Stoutamire 1974; Harvais 1982; De Pauw *et al*. 1995). 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. The use of peptone significantly improved the survival of protocorms wherever the effect of other organic supplements had little impact in most treatments.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

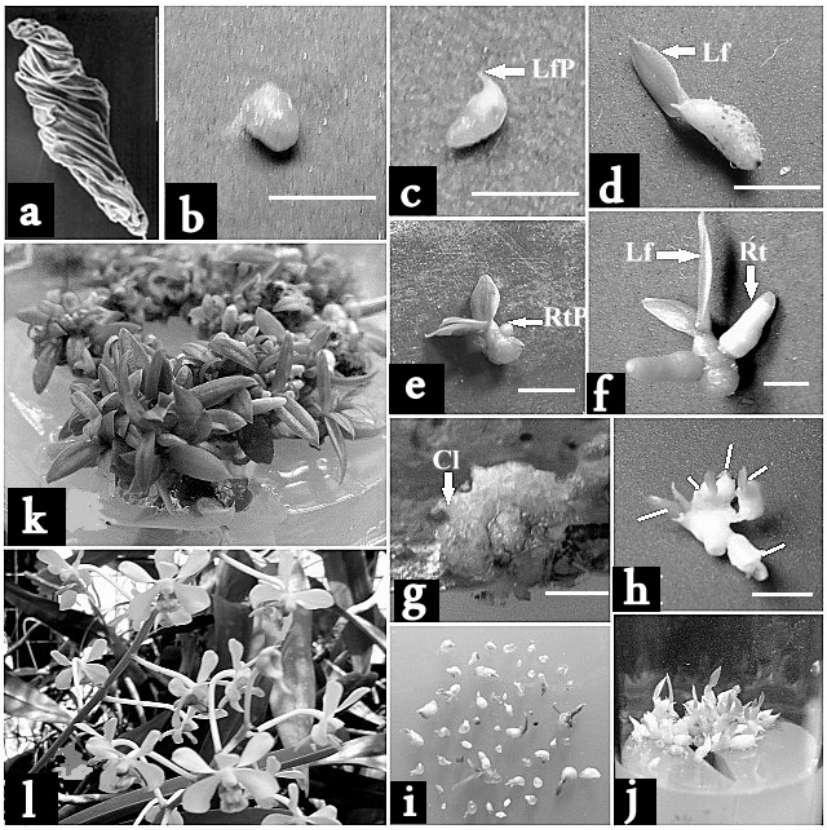


Fig. 2. Asymbiotic germination and seedling development of *Vanda testacea*: (a) Scanning electron micrograph of Seed(600x) (b) Stage 1 Early globular stage (bar=1cm), (c) Stage 2 protocorm showing initiation of leaf primordium (LfP) (bar=1cm), (d) Protocorms with distinct leaf (Lf) Stage 3 (bar=1cm), (e) Stage 4 seedling showing root primordium (RtP) development (bar=1cm), (f) Developed seedling with leaf (Lf) and root (Rt) (bar=1cm) (g) Unorganized callus (Cl) (bar=1cm), (h) Multiple protocorm bodies with many leaf primordium(▬) (bar=1cm), (i) Protocorms in various stages of growth, (j) Seedlings under aseptic conditions, (k) Complete plantlets with well developed shoots and roots, (l) Plant with flower spike.

basal solution (Kano 1965; Ichihashi and Islam1999; Roy and Banerjee 2002). In the present findings, total nitrogen content in KC medium seems to be sufficient for germination of *V. testacea*, so that application of additional organic additives (mainly peptone) did not cause any enhancement in germination. However, stimulation of seedling growth with peptone can be attributed to either the additional

nitrogen or growth substances from peptone. Peptone has also been shown to promote germination and development of *Vanda tricolor*, *Paphiopedilum*species, *Prostheciaco chleata*and *Spathoglottis plicata* (Cutris 1947). Germination frequency and protocorm mortality was not affected with yeast extract. In all concentrations of yeast extract, callus formation was either absent or negligible. Thus it can be concluded that nitrogen or other constituents of yeast extract either maintain or stimulate an organized growth of seedlings, rather than allowing unorganized growth to form callus. Coconut water failed to influence germination, protocorm mortality, seedling growth and development. But there are many evidences that seed germination and seedling development of many orchids could be improved with addition of coconut water to the medium (Lawrence and Arditti 1964; Mclntyre *et al*. 1974). However, Kotomori and Murashige (1965) observed that coconut water was not always suitable for *in vitro* seed germination of *Dendrobium*. Coconut water is generally added in orchid seed culture as a source of sugar, natural cytokinins and vitamins (Matthews and Rao 1980; Sarma 2002).

Orchid requires auxins and/or cytokinins for PLB formation and plantlet development. The type and concentration of growth regulators plays an important role during *in vitro* multiplication of many orchid species (Arditti and Ernst 1993). In *V. testacea* , NAA at its lower levels enhances unorganized growth. In presence of NAA seedling growth was faster and all seedlings at least formed leaf-primordia. But higher concentration of NAA showed inhibitory effect on PLB formation and result maximum necrosis. Kumaria and Tandon (2000) studied the effect of PGRs on peroxidase, polyphenol oxidase and IAA oxidase activities and phenolic content during protocorm initiation and development of *Dendrobiumfimbriatum*. They suggested that PGRs at low concentration in the medium might act in a manner similar to symbiotic fungi and brought about the physiological changes for protocorm development. However, higher concentrations of PGRs induced the increase of total phenolic content in the embryonic cells of seed. Polyphenolic oxidase oxidizes phenol and the oxidized products of phenol are inhibitory to plant cellular growth (Monaco *et al*. 1977). Therefore, media containing higher concentration of NAA is not conducive for PLB initiation. Parallely, with application of BAP, both frequency and number of root was restrained and majority of seedlings could not grow beyond stage 1 or stage 2.

Conclusion

The present study has described the efficient optimization of plant growth regulators and organic supplements for asymbiotic seed germination, seedling development and plant regeneration of *Vanda testacea*. Considering the declining population of this orchid species, incorporation of this simple *in vitro* technique can serve to meet the demand of pharmaceutical and floricultural industry, as well as for conservation of this ill fated taxon.

Acknowledgements

University Grants Commission, Bahadur Shah Zafar Marg, New Delhi, India, is highly acknowledged for the financial assistance [Ref. No.:39-380/2010 (SR), dated 05.01.2011].

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