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In vitro Propagation of *Vanda testacea* (Lindl.) Reichb. F, a Medicinally Important Threatened Orchid

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Abstract

The present study was planned to enable *in vitro* conservation of *Vanda testacea*, a highly medicinal orchid species through *in vitro* asymbiotic seed germination technique in Mitra orchid medium supplemented with cytokinins (Kn - 4.65 μM, BAP - 4.44 μM), and auxin (NAA- 5.37 μM). The germination frequency and initiation of germination was higher in NAA augmented medium and seedlings developed in 12.50 ± 0.50 weeks. Coconut water (20%) proved optimum for the multiplication of protocorm like bodies. Activated charcoal successfully checked the release of brownish exudates in the cultures.

Introduction

In nature, the orchid seeds show meagre germination. The technique of *in vitro* aseptic orchid seed germination offers a good opportunity for propagating orchids (Knudson 1922). Withner (1953) suggested that orchid seeds/embryos can germinate even before reaching maturity. The asymbiotic seed germination technique ensures improved germination percentage, thus saving on the time lapse between pollination and seed sowing (Sagawa 1963). The technique guarantees optimum germination of immature seeds, due to physiologically dynamic state of their embryos and distended testa cells besides lacking dormancy factors (Yam and Weatherhead 1988). The methodology of asymbiotic seed germination is successfully tested so far in many orchid species (Arditti and Ernst 1993, Park et al. 2000, Roy and Banerjee 2001, Lo et al. 2001, Shimura and Koda 2004, Buyun et al. 2004, Yamazaki and Miyoshi 2006, Valletta et al. 2008, Sgarbi et al. 2009, Pierce et al. 2010, Kaur and Bhutani 2011, 2014).

Vanda testacea (= *Vanda parviflora* Lindl.) is an alkaloid enriched therapeutically important epiphytic species. Every part of this herb, such as root, leaf and flower is used either in powder form or as a decoction to treat ailments such as rheumatism, piles,

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bronchitis, inflammation, nervous disorder, and is also considered to be an able anti-cancer medicine (Chauhan 1990). Its populations are declining due to continuous unregulated collections and forests clearing, exceeding its natural regeneration. As a corollary, genus *Vanda* appears among other orchids in CITES Appendix II (CITES 2021). There is urgency to save *V. testacea* species from getting extinct. This communication reports one step protocol to conserve this rare species using *in vitro* tissue culture technique.

Materials and methods

The plants of *Vanda testacea* (Lindl.) Reichb. F were collected during summers from Renuka lake district Sirmaur, Himachal Pradesh (Coordinates: 30°36' 36" N 77°27'30"), repotted in epiphytic compost (charcoal pieces, pieces of brick and bark pieces in 1:1:1 ratio) covered with sphagnum moss to retain moisture. Pots were placed under natural light (70% relative humidity) and temperature (25°C/20°C day/night) in greenhouse. Seed capsules were collected at different developmental stages. Dehisced capsules (mature) contained dry powdery seeds. Green capsules (undehisced, immature) were gently cleaned with a soft brush using tap water and dish wash detergent solution. In contrast, seeds from dry, dehisced capsule were collected on thin butter paper and surface sterilized later. Inside laminar air flow hood, green capsule was surface sterilized sequentially, for 4-5 minutes with ethyl alcohol-soaked cotton swab then with mercuric chloride (0.1% (w/v) ($HgCl_2$; Qualigens, Mumbai, India) solution containing "Teepol" (1–2 drops) as a wetting agent; later rinsed 2/3 times with sterilized distilled water. Capsule was flamed for 1-2 seconds. On the other hand, dry seeds were surface sterilized with calcium hypochlorite (4%). All seeds were inoculated on Mitra orchid (Mitra et al. 1976) (M) medium fortified with 2.0% sucrose (Hi-media, Mumbai, India). In a separate set of experiment, the effect of BA, Kn and NAA-5.37μM individually was also examined. Protocorms (2.0 mm) from 16-weeks old *in vitro* cultures were inoculated into Mitra orchid medium and its combinations with coconut water (10, 20 and 30% v/v) which was obtained from immature fresh green coconuts. In cultures, the problem of phenolics was checked by using activated charcoal (2 mg/l) in medium.

The inoculations were performed under aseptic conditions. Seeds were spread as a thin layer and protocorms were cut into two halves and inoculated in Mitra orchid medium. Cultures were incubated at 25 ± 2°C, under 12-h photoperiod with 3500 lux light intensity (fluorescent tubes 40W, Philips India Ltd, Mumbai, India). For each experiment four replicates were used. Cultures were regularly observed; photographs were captured using a digital camera (Nikon Digital Sight, DS, Ri1 Nikon Corporation, Japan). Germination percentage was calculated after six weeks of inoculations using formula:-

$$\text{Seed germination Percentage} = \frac{\text{Total number of swollen seeds}}{\text{Total number of seeds inoculated}} x\ 100$$

The experiment was designed in a completely randomized manner, and germination percentage, onset of seed germination, seedling development, and time taken (in weeks) to form complete plantlets were tested by applying Tukey's multiple comparison test ($P \leq 0.05$) in a one-way ANOVA. SPSS (version 17.0) software package was used for statistical analysis. The results are expressed as a mean ± SD of four replicates. To ensure the applicability of the protocol, the experiment was repeated twice.

Results and Discussion

The age of capsule at the time of harvesting was observed to be the most crucial parameter that influenced seed germination. Nearly 88% seeds germinated from undehisced (immature) green capsules (Table 1). Whereas, the seeds harvested from dry dehisced yellow capsules, showed delayed and low germination (i.e. 30%). Earlier Naha et al. (2013) used Knudson (1946) medium to germinate *Vanda testacea* seeds. Since the,

Table 1. Effect of capsule stage on seed germination (%) of *V. testacea* in Mitra orchid medium.

Capsule stage	Germination %
Dehisced	30.00± 0.11[a]
Undehisced	88.50± 0.13[b]

Values in columns with the same superscript are not significantly different at $p \leq 0.05$ according to Tukey's test

seeds germinated in simple defined medium i.e. Mitra orchid medium, indicating their simple nutritional requirements for germination. Maturity level of the capsule played an important role. The seeds obtained from green (undehisced) capsules showed higher germination frequency than those collected from dry, dehisced-brown capsules. The higher germination in seeds have occurred due to metabolically active embryos in green capsules. The satisfactory response in immature seeds occur due to the presence of irregular layer of cuticle surrounding the embryo; these discontinuities/gaps in cuticle are caused by cellular degeneration of inner integument, and also by the absence of secondary wall thickenings in outer integument (Lee et al. 2008, Vasudevan and van Staden 2010). In context of germination ability of mature orchid seeds, earlier reports indicate a progressive decline of germination frequency due to biochemical changes that occur in mature seeds which induce dormancy by amassing repressing substances (Rasmussen 1995; Vasudevan and van staden 2010). According to Lee et al. (2007), mature embryo dehydrates due to testa lignification, growth of hydrophobic carapace sheath and increase in endogenous abscisic acid.

The germination percentage was influenced by chemical stimulus. Nearly all seeds were embryonate. Seeds germinated in 4.82 weeks in basal medium. Embryo, in seed swelled by water absorption, pierced through seed coat as globular, chlorophyllous spherules and formed pear-shaped protocorms with rhizoids at abaxial surface and a

shoot-tip at opposite end after 6 weeks. Later, they differentiated leaf primordia and roots after 19.35 weeks (Fig. 2a). Growth regulators favoured early onset of seed germination besides favouring early protocorm and seedling formation (Fig. 1, Table 2). Cytokinin (BA/Kn) lowered germination percentage, delayed morphogenetic processes but favoured healthy shoot growth. NAA favoured highest germination percentage and advanced development of seedlings. Seedlings were formed within 13.5 weeks. The findings are in confirmation with similar earlier reports of the benign effect of NAA in *Aerides odorata* (Pant and Gurung 2005), *Coelogyne suaveolens* (Sungkumlong and Deb 2008), *Dendrobium chrysotoxum* (Kaur and Bhutani 2012). Seedlings exuded brownish exudates (Fig. 2a), which was checked by addition of activated charcoal in medium (Fig. 2b).

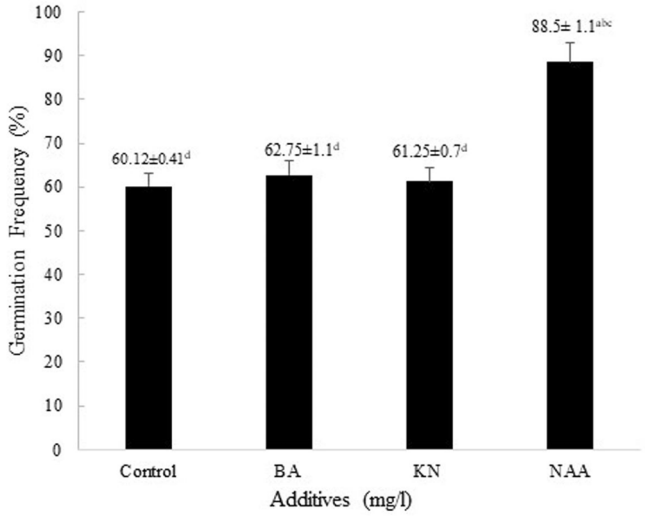


Fig. 1 Shows effect of growth additives on seed germination frequency.

Table 2. Effect of different PGRs on *in vitro* asymbiotic seed germination of *Vanda Testacea* in Mitra orchid medium.

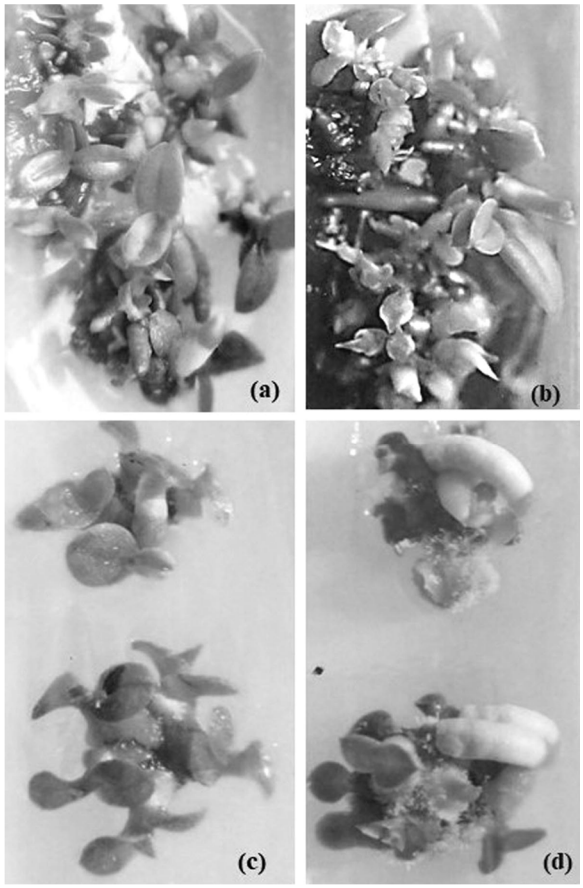
Growth adjuvants	Initiation of germination (weeks)	Protocorm Development (weeks)	Development (weeks)		Seedlings (weeks)
			Leaf	Root	
Basal	4.82±0.07^{bcd}	10.07±0.09^{d}	14.27±0.15^{bcd}	16.7±0.16^{bcd}	19.35±0.12^{abcd}
BA	3.00±0.04^{ad}	8.27±0.14^{d}	11.0±0.00^{ad}	14.02±0.09^{acd}	17.15±0.27^{ad}
KN	3.5±0.06^{bd}	8.72±0.30^{d}	10.85±0.54^{abd}	13.1±0.00^{abd}	17.05±0.36^{ad}
NAA	1.05±0.05^{abc}	6.40±0.12^{abc}	7.0±0.00^{abc}	11.06±0.0^{abc}	13.50 ± 0.50^{abc}

Concentration of growth regulator used=1mg/l; values in a column with the same superscript are not significantly different at $p \leq 0.05$ according to Tukey's test

Table 3. Effect of various concentrations of coconut water (CW; %) on *in vitro* multiplication of protocorms of *Vanda testacea* in Mitra orchid medium.

Additive	Regeneration response (%)	Number of PLBs/ explant	Number of shoots/ explant	Development of plantlets (wks)
M	20.11 ± 0.20^{bcd}	1.00 ± 0.21^{c}	1.00 ± 0.00^{bcd}	21.05 ± 0.80^{bcd}
M + $CW_{10\%}$	44.15 ± 0.40^{acd}	2.10 ± 0.50^{c}	2.00 ± 0.00^{ac}	18.25 ± 0.70^{ac}
M + $CW_{20\%}$	90.20 ± 0.40^{abd}	7.00 ± 0.40^{abd}	10.00 ± 0.00^{abd}	11.00 ± 0.40^{abd}
M + $CW_{30\%}$	61.25 ± 0.13^{abc}	2.20 ± 0.13^{c}	2.20 ± 0.20^{ac}	20.00 ± 0.30^{bc}

CW – Coconut Water [Conc. subscripted = % (v/v)]; Values in columns with similar superscripts are not significantly different at $p \leq 0.05$ according to Tukey's test.



Figs. 2(a-d). *In vitro* asymbiotic seed germination of *Vanda Testacea* in Mitra orchid (M) medium. a. Cultures in M+NAA supplemented medium and showing release of brownish exudates. b. Luxuriant growth of seedlings in M+NAA+AC supplemented medium. c. Protocorm segment derived plantlet after 21 week of culture. d. Extensive root development in CW (30%).

Protocorm segments regenerated in basal medium with low regeneration percentage. Only 20.11% explants regenerated and developed into plantlets after 21 weeks (Figure 2c). Coconut water (20%) favoured multiplication of *neo*-formations and early plantlet development within 11 weeks (Table 3) and favoured maximum and rapid regeneration response i.e. 90% in explants. CW (30%) favoured extensive root development only (Figure 2d). Earlier studies mention the constructive effect of coconut water in invoking cell divisions, thus, initiating protocorm multiplication and differentiation (Intuwong and Sagawa1973) and this could be interrelated to the presence of kinetin in its nutritive composition (cf. Kaur and Bhutani 2012). From this experiment, it is interpreted that there is adequate amount of endogenous cytokinin concentration in the seed itself. Orchid seeds do not require exogenous cytokinin, they are cytokinin autonomous (De Pauw et al. 1995).

Conclusion

The present study reports a simple one step protocol for propagation of *Vanda testacea* using auxin such as NAA which proved beneficial in promoting early seedling development. For multiplication of cultures using protocorm segments, supplementation of coconut water (20%) is quite effective. Further studies focus on the acclimatization of these seedlings raised *in vitro* and restoring them back in their natural habitat.

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