



P-ISSN: 2349-8528
 E-ISSN: 2321-4902
 IJCS 2019; 7(3): 2957-2963
 © 2019 IJCS
 Received: 15-03-2019
 Accepted: 17-04-2019

Rosemol Baby

Center for Plant Biotechnology
 and Molecular Biology,
 (CPBMB), College of
 Horticulture, Kerala Agriculture
 University, Kerala, India

Valsala PA

Center for Plant Biotechnology
 and Molecular Biology,
 (CPBMB), College of
 Horticulture, Kerala Agriculture
 University, Kerala, India

Correspondence

Rosemol Baby
 Center for Plant Biotechnology
 and Molecular Biology,
 (CPBMB), College of
 Horticulture, Kerala Agriculture
 University, Kerala, India

International Journal of Chemical Studies

Micropropagation protocol for *Vanda* hybrid ‘Sansai Blue’

Rosemol Baby and Valsala PA

Abstract

‘Sansai Blue’ (*Vanda* Crimson Glory X *Vanda* *coerulea*), a *Vanda* hybrid with large showy flower is one of the highly demanded Orchids in the domestic as well as the international Orchid market. However obtaining good quality and true to type planting material of the hybrid in sufficient quantities in limited time frame is very difficult. Hence, an *in vitro* micropropagation protocol has been developed at Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agriculture University. Explants such as leaf, root, stem and inflorescence segments were tested to initiate the cultures. Treating the explants with 0.1 per cent carbendazim for 20 minutes, followed by 70 per cent ethanol for 5 minutes and 0.1 per cent mercuric chloride for 5 min effectively reduced the microbial contamination with highest percentage of explant survival. A positive culture initiation was obtained from inflorescence segments inoculated on to $\frac{1}{2}$ MS + 10 mg l⁻¹ BA + 2 mg l⁻¹ TDZ + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime by forming direct shoots from the dormant buds. The culture establishment medium that showed positive results was MS + 4.5 ml l⁻¹ BA + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime both when inoculated with and without the stalk. Cultures established in about 100 days of inoculation of explants by producing direct shoots. The percentage of rooting was observed to be 70.37 per cent when elongated shoots of about 4 cm obtained from cultures initiated without stalk were transferred to rooting media with a composition of MS + 0.5 mg l⁻¹ NAA + 1 mg l⁻¹ IAA + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime. Hence it can be concluded that the developed micropropagation protocol can be used for commercial production.

Keywords: *Vanda* orchids, explants, *in vitro*, micropropagation

Introduction

Vanda is a monopodial orchid with a wide range of colourfull and loosely arranged large beautiful flowers which have a long shelf life. Owing to these factors, *Vanda* is one of the most sought after genus of Orchidaceae family among the five horticulturally important orchid genera both as cut flower and potted plants. Tropical Asia (India) is considered as the origin of *Vanda* orchids. *Vanda* is also widely distributed in South East Asia, Philippines, Indonesia, Southern China and northern Australia. There are mainly two types of *Vandas* such as basket vanda and the terete vandas. Basket vandas have strap leaves and are usually lower in plant height. Terete vandas are tall growing and also known as pencil vandas. Netherlands is the largest exporter of orchids. However, Thailand is the leading country in the export of tropical orchids. *Vanda* contributes around 8.9 per cent of total orchid trade and in case of cut flower *Vandas*, it is around 0.13 percentage of total orchid export from Thailand (NRC on orchids, 2015). One of the major limiting factors for its spread and large scale cultivation in India is the non-availability of good quality and true to type planting material at a reasonable price. Morel (1960) [16] was the pioneer in reporting that *in vitro* techniques could be used to produce orchids on a large scale using shoot apex cultures of *Cymbidium* species. In this context, “Development of *in vitro* micropropagation protocol for *Vanda* hybrid Sansai Blue” was taken up at Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agriculture University.

Materials and Methods

Pearl Orchids, one of the leading commercial nursery in Thrissur district of Kerala was identified to obtain the mother plants of the hybrid ‘Sansai Blue’. The plants obtained were in the flowering stage with netted blue flowers to ensure the procurement of the selected hybrid. Culture initiation experiments were done with various explants such as basal leaf segments, leaf tip segments, root segments, stem segments, and inflorescence segments for the

micropropagation experiment. The mother plants obtained from the commercial nursery was maintained in the net house at CPBMB, College of Horticulture and explants were collected from these well maintained mother plants. The first step of culture initiation was to identify the suitable surface sterilization treatment. Eight different surface sterilization treatments were tested for the various explants selected. However, the surface sterilization experiment was not conducted for stem segments due to limited number of explants. The sterilization treatment standardized for leaf and root segments was followed for the stem segments also. The culture medium in which the sterilization experiments conducted was MS + 1.5 mg l⁻¹ TDZ + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime for leaf and root segments as reported by Gantait and Sinniah, 2012 while for inflorescence segments the medium used was 1/2 MS + 10 mg l⁻¹ BA + 2 mg l⁻¹ TDZ + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime which was standardized at CPBMB. The experiments were done with initial 48 h dark and further light conditions of 1000 lux with 26 ± 2^oC.

Culture establishment was initially tested for the hybrid with root explants on different media compositions with continuous monitoring of the cultures and recording the responses after every 21 days. Further, stem segments with two nodes as well as the upper meristematic region on the stem were also tested for culture establishment with continuous monitoring. An attempt was also made to initiate cultures using inflorescence segments to develop an efficient micropropagation protocol. The culture conditions given was initial 48 h dark followed by light of 1000 lux intensity with 26 ± 2^oC for all the different explants tested. Out of all the explants tested, positive response was observed only from the inflorescence segments and these were transferred to three different media for shoot proliferation. The cultures were repeatedly subcultured in the shoot proliferation medium which showed positive response. The medium which showed positive results for shoot proliferation was MS + 4.5 ml l⁻¹ BA. Subculturing was done at 21 days interval for a period of 84 days i.e upto S7 passages. The micro shoots developed in the shoot proliferation medium was subcultured in hormone free basal MS media with charcoal for one cycle under light conditions of 1000 lux at 26 ± 2^oC for elongation. The elongated shoots were separated into individual shoots and subcultured into the identified rooting medium for inducing roots. Well rooted plantlets of the regenerated *Vanda* hybrid was treated with 0.1 per cent carbendazim for five minutes and then planted into small earthen pots filled with charcoal, coconut husk and brick pieces for hardening in the net house unit of CPMB.

Results and Discussion

The results of the eight different surface sterilization treatments tested for the different explants are given in Table 1 & 2. Treatment T₈ showed lowest microbial contamination in case of leaf and root explants. However, the survival percentage was also nill in T₈. In T₅ the survival per cent was highest (80%) for leaf explants and (70%) for root explants and at the same time, the microbial contamination was also comparatively lower. Hence T₅ was considered to be the best treatment for surface sterilization of both leaf and root explants. The fungal contamination in all treatments ranged from 0-100 per cent while bacterial contamination was in the range of 0-20 percentage in both leaf and root explants. As the time period was increased from 6-10 minutes, drying of the explants were observed and the drying percentage increased

from 50-100 percentage. This showed that T₅ i.e. treating the explants with 0.1 per cent carbendazim for 20 minutes, followed by 70 per cent ethanol for 5 minutes and 0.1 per cent mercuric chloride for 5 minutes can eliminate maximum microbial contamination with highest per cent survival of the tissues in both leaf and root explants.

The standardization of surface sterilization of inflorescence segment experiments showed that the colour of the media turned brown due to the exudation of phenolic compounds. Hence, the explants were treated with 0.1per cent ascorbic acid for five minutes before treating with 70 per cent ethanol during the surface sterilization procedure. This could efficiently manage the problem of media browning in the present study. It has been reported by Seenii and Latha (2000) [21] and also by (Arditti and Ernst, 1992) [1].

Considerable drying was observed in the tissues as the duration of exposure to both ethanol and mercuric chloride was increased from 5 minutes to 10 minutes. Increase in time of exposure to sterilants proved to be lethal for the explants. Bhadane and Patil (2016) [4] reported that the duration of exposure to the sterilants is an important factor for successful regeneration of *in vitro* plant cultures. Chawla (2000) [7] stated that it is commonly observed that the over use of chemical sterilants is lethal to plant tissues. In all the treatments, the per cent bacterial contamination was found to be lower than the fungal contamination. As reported by Silva and Fukai (2001) [22], Yu *et al.* (2001) [27] and Donzo (2015) [9] the possible reason for this would be the effect of antibiotic cefotaxime (250 mg l⁻¹) which was used in the nutrient media

Culture condition: Initial 48 h dark followed by light conditions of 1000 lux with 26 ± 2^o

The responses of culture establishment experiments for *Vanda* hybrid 'Sansai Blue' with root and leaf explants and different media compositions tested was recorded after twenty one days as detailed in Table 3. Drying was more fast and severe in root explants than in the leaf explants. Similar results were also obtained by Kerbauy, 1984; Vij, 1994 and Arditti, 2009. The possible reason would be the higher amount of phenolics present in the roots of *Vanda* hybrids than in the leaves. The stem segments remained as such without any change for two weeks. Further it started to dry from the upper part of inoculated explant. The apical meristem inoculated dried in one week. (Table 4). Since the explants were limited, it was possible to try only two media compositions to initiate the cultures using apical meristem. From the various explants tested under the study namely leaf explants, root segments, stem segments and inflorescence segments, a response in the cultures was recorded only in the case of inflorescence segments. The inflorescence segments inoculated into the media reported by Gantait and Sinniah (2012) did not respond positively. The bud on the segments turned yellow after one week of inoculation and dried in three weeks. The medium in which the response was recorded was 1/2 MS + 10 mg l⁻¹ BA + 2 mg l⁻¹ TDZ + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime as reported by Chughet *et al.*, 2009 (Table 4 & 5). Hence the study was further concentrated in developing the *in vitro* regeneration protocol for *Vanda* hybrids using inflorescence segments in the identified medium.

An observation similar to the present study was found by Korah and Shylaraj (2011). However, in contrary to the present study, shoot tips (Seenii and Latha, 2000) [21], leaf segments (Mathews and Rao, 1985; Vijet *et al.*, 1986), axillary buds and *in vitro* derived explants of various species of *Vanda* orchids showed positive results to different media compositions.

Table 1: Effect of surface sterilization treatments on leaf explants and root explants of Vanda 'Sansai Blue'

Treatment no.	Surface sterilization treatment	Per cent contamination (%)		Per cent survival (%)		Nature of contamination		Percentage fungal contamination (%)		Percentage bacterial contamination (%)		Percentage of cultures dried (%)	
		leaf explants	root explants	leaf explants	root explants	leaf explants	root explants	leaf explants	root explants	leaf explants	root explants	leaf explants	root explants
T1.	70% ethanol 1 min + 0.1% HgCl ₂ 1 min	100	100	0	0	Fungus	Fungus	100	100	0	0	0	0
T2.	70% ethanol 2 min + 0.1% HgCl ₂ 2 min	100	100	0	0	Fungus	Fungus	100	100	0	0	0	0
T3.	70% ethanol 3 min+0.1% HgCl ₂ 3 min	80	90	20	10	Fungus Bacteria	Fungus, Bacteria	60	80	20	10	0	0
T4.	70% ethanol 4 min+0.1% HgCl ₂ 4 min	40	50	60	50	Fungus Bacteria	Fungus, Bacteria	30	30	10	20	0	0
T5.	70% ethanol 5 min+0.1% HgCl ₂ 5 min	20	30	80	70	Fungus	Fungus	20	20	0	10	0	0
T6.	70% ethanol 6 min+0.1% HgCl ₂ 6 min	20	20	30	20	Fungus Drying	Fungus, Bacteria, Drying	20	10	0	10	50	60
T7.	70% ethanol 8 min+0.1% HgCl ₂ 8 min	0	10	10	20	Drying	Drying, Fungus	0	10	0	0	90	70
T8.	70% ethanol 10 min+0.1% HgCl ₂ 10 min	0	0	0	0	Drying	Drying	0	0	0	0	100	100

No. of cultures inoculated: 10 per treatment

Medium: MS + 1.5 mg l⁻¹ TDZ + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 °C

Table 2: Effect of surface sterilization treatments on inflorescence segments of Vanda 'Sansai Blue'

Treatment no.	Surface sterilization treatment	Per cent contamination (%)	Per cent survival (%)	Nature of contamination (%)	Percentage fungal contamination (%)	Percentage bacterial contamination (%)	Percentage of cultures dried (%)
T4.	70% ethanol 4 min+ 0.1% HgCl ₂ 4 min	60	40	Fungus Bacteria	50	10	0
T5.	70% ethanol 5 min+ 0.1% HgCl ₂ 5 min	20	80	Fungus	20	0	0
T6.	70% ethanol 6 min+ 0.1% HgCl ₂ 6 min	20	40	Fungus Drying	20	0	40

No. of cultures inoculated: 5 per treatment,

Medium: 1/2 MS + 10 mg l⁻¹ BA + 2 mg l⁻¹ TDZ + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 °C

Table 3: Response of leaf explants and root explants of 'Sansai Blue' for culture establishment in different media combinations

Treatment No.	Media composition	Culture establishment after 21 days of inoculation	
		leaf explants	root explants
T1.	MS + 1.5 mg l ⁻¹ N phenyl-N ² -(1, 2, 3-thiazol-5yl) urea (TDZ) + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250mg l ⁻¹ cefotaxime	Nil	Nil
T2.	1/4 MS + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Nil
T3.	Basal MS + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Nil
T4.	Mitra + 66.6 μM BA + 28.5 μM IAA + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Nil
T5.	1/2 MS + 10 mg l ⁻¹ 2,4-D + 1 mg l ⁻¹ TDZ + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Nil
T6.	MS + 1.5 mg l ⁻¹ NAA + 1 mg l ⁻¹ BAP + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Nil
T7.	1/2 MS + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Nil
T8.	1/2 MS + 10 mg l ⁻¹ BA + 2 mg l ⁻¹ TDZ + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Nil

No. cultures inoculated: 10 per medium

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 °C

Table 4: Response of stem segments and inflorescence segments of 'Sansai Blue to different media combinations for culture establishment

Treatment no.	Media composition	Culture establishment after 21 days of inoculation	
		stem segments	inflorescence segments
T1.	MS + 1.5 mg l ⁻¹ N phenyl-N ⁻ -(1, 2, 3-thiazol-5yl) urea (TDZ) + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Nil
T2.	¹ / ₂ MS + 10 mg l ⁻¹ 2,4-D + 1 mg l ⁻¹ TDZ + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	Yes
T3.	For apical meristem Mitra + 44.4 µM BA + 28.5 µM IAA + 20 g l ⁻¹ sucrose + 6 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	Nil	

No. of cultures inoculated: 3 per medium (stem segments), 5 per medium (inflorescence segments),

Culture conditions: Initial 48 h dark followed by light conditions of intensity 1000 lux with 26 ± 2 °C

Table 5: Response of inflorescence segments of Vanda hybrid in culture establishment medium

Sl. No.	Hybrid	No. of explants inoculated	Mean days for sprout initiation	Response			Percentage of culture establishment (%)
				At S1 passage (21 days)	At S2 passage (42 days)	At S3 passage (63 days)	
1.	Sansai Blue	20	17.40	Bud enlargement	Bud elongation	Shoot initiation	60

Medium: ¹/₂ MS + 10 mg l⁻¹ BA + 2 mg l⁻¹ TDZ, **Culture condition:** Initial 48 h dark followed by light conditions of 1000 lux with 26 ± 2 °C

Table 6: Response of cultures of 'Sansai Blue in shoot proliferation media

Treatment no.	Media	No. of cultures inoculated		Response after 21 days of inoculation	Multiple shoot initiation
		With stalk	Without stalk		
T1.	¹ / ₂ MS + 1 mg l ⁻¹ IAA + 1 mg l ⁻¹ BAP + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	2		Dried	-
			3	Dried	-
T2.	MS + 1.5 mg l ⁻¹ BAP + 0.5 mg l ⁻¹ NAA + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	2		Dried	-
			3	Dried	-
T3.	MS + 4.5 ml l ⁻¹ BA + 30 g l ⁻¹ sucrose + 7.5 g l ⁻¹ agar + 250 mg l ⁻¹ cefotaxime	3		Multiple initiation	Less
			3	Multiple initiation	Numerous

Table 7: Response of cultures in identified shoot proliferation medium

Sl. No.	Cultures	At inoculation to SPM*		At S4 passage (After 21 days in SPM)		At S5 passage (After 42 days in SPM)		At S6 passage (After 63 days in SPM)			At S7 passage (After 84 days in SPM)		
		Mean no. of shoot initials per culture	Mean length of shoots (cm)	Mean no. of shoot initials per culture	Mean length of shoots (cm)	Mean no. of shoot initials per culture	Mean length of shoots (cm)	Mean no. of shoots per culture	Mean length of shoots (cm)	Mean no. of leaves per shoot	No. of shoots per culture	Mean length of shoots (cm)	Mean no. of leaves per shoot
1.	With stalk	1	0.85	2.00	1.69	3.00	2.18	3.50	2.88	2.30	4.50	4.20	3.03
2.	Without stalk	1	0.25	3.33	0.61	4.00	0.85	5.00	1.34	2.22	6.67	1.53	3.06

Medium: MS + 4.5 ml l⁻¹ BA, **Culture condition:** Light conditions of 1000 lux with 26 ± 2 °C, *SPM: Shoot Proliferation Medium

The shoot initials were obtained from the buds on inflorescence segment on half strength MS medium with hormones. The combined effect of TDZ and BA would be a possible reason for the bud sprout and development in the culture establishment of *Vanda* hybrid. The dormant buds present on the young immature inflorescence on the *Vanda* hybrid produced visible shoot initials in the identified culture establishment medium by sixty days of inoculation (Figure 1). It was observed that average time taken for sprout initiation was 17.40 days. The percentage of culture establishment was 60 per cent (Table 5).

The established cultures were transferred to shoot proliferation media in two methods. One with stalk and other without stalk. Shoot proliferation was observed on MS + 4.5 ml l⁻¹ BA + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxim (Table 6). It was observed that the multiple shoots initiated were less when the cultures were inoculated with stalk and numerous shoots proliferation was observed when the cultures were inoculated without stalk.

For cultures inoculated with stalk, the mean length of multiple shoots increased from 0.98cm to 4.07 cm and for cultures inoculated without stalk, the mean length of multiple shoots increased from 0.20 cm to 1.56 cm from the day of inoculation to 84 days after inoculation in the shoot proliferation medium. The mean number of multiple shoots produced was less in cultures inoculated with stalk *i.e.* 4.00 per culture as compared to cultures without stalk *i.e.* 7.50 per culture observed at the S7 passage (Table 7). The mean number of leaves per shoot was almost same *i.e.* 3.46 for cultures with stalk and 3.14 for cultures without stalk. So it can be concluded that multiple shoot production is more in cultures without stalk and shoot elongation was more in cultures with stalk (Figure 2 & 3). Proliferation of regenerated shoots were observed on MS media fortified with 4.5 mg l⁻¹ BA. Similar observations have been reported by Latipet *al.* (2010) and Begum *et al.* (2002).

Table 8: Response of cultures in elongation medium

Sl. No.	Hybrid	No. of cultures inoculated	Mean no. of shoots per culture	Mean length at inoculation (cm)	Mean length after 21 days of inoculation (cm)
1.	Sansai Blue	2	7.50	1.55	4.10

Medium: Hormone free basal MS, **Culture condition:** Light conditions of 1000 lux with 26 ± 2 °C

Table 9: Response of cultures in rooting medium

Sl. No.	No. of cultures	Response at S9 passage	Mean no. of roots per plant at S9	Mean root length at S9 (cm)	Mean no. of roots per plant at S10	Mean root length at S10 (cm)	No. of cultures lost due to contamination and drying	Rooting percentage (%)
1.	27	Root initiation	4.11	3.50	5.83	5.61	8	70.37

Medium: MS + + 0.5 mg l⁻¹ NAA + 1 mg l⁻¹ IAA **Culture condition:** Light conditions of 1000 lux with 26 ± 2 °C

Table 8: Biometric observations of plantlets during hardening and acclimatization

Sl. No.	Mean plant height (cm)		Mean no. of leaves	
	At plant out	30 days after hardening	At plant out	30 days after hardening
1.	4.85	7.14	3.86	4.18

In the present study the cultures were proliferated both with and without stalk. The cultures with stalk were of about 4.0 cm in length but those without stalk were shorter and were about 1.5 cm at S7 passage (Table 7). Since the cultures without stalk did not elongate as compared to the cultures with stalk, these cultures were transferred to basal MS medium without any hormones for its elongation (Figure 4.). It was observed that the regenerated shoots elongated to an average length of 4.10 cm after one subculture passage (Table 8). Sinha and Roy, 2004.

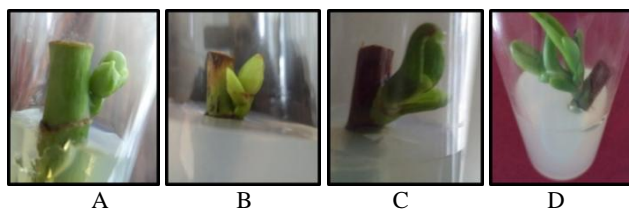


Fig. 2: Cultures in shoot proliferation medium with stalk. (A. At subculture 4 passage, B. At subculture 5 passage, C. At subculture 6 passage, D. At subculture 7 passage)

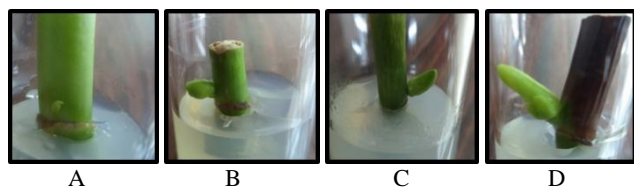


Fig. 1: Stages of culture establishment (A. On the day of inoculation, B. At S1 passage C. At S2 passage, D. At S3 passage)

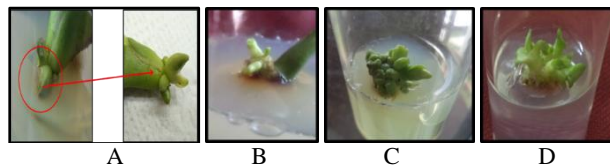


Fig 3: Cultures of in shoot proliferation medium without stalk (A. At subculture 4 passage, B. At subculture 5 passage, C. At subculture 6 passage, D. At subculture 7 passage)

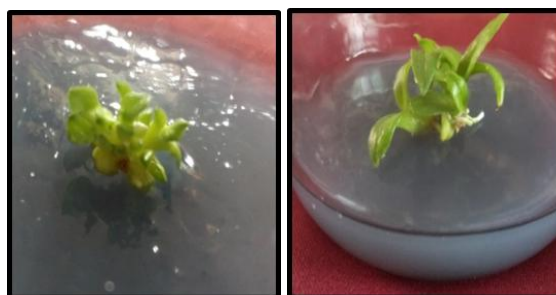


Fig 4: Cultures in elongation media



Fig 5: Rooted plantlet of *Vanda* hybrid



Fig 6: Regenerated plantlet potted in earthen pots with charcoal, coconut husk and brick pieces

Bhosle *et al.* (2005) and Thanh *et al.* (2012) reported similar observations. The rooting percentage was calculated to be 70.37 per cent. Plantlets recorded good root characters with an average 5.83 roots having a mean root length of 5.61 cm (Table 9) (Figure 5). Similar results were obtained by Paudal and Pant (2012) in rare orchid *Esmeralda clarkei*. The present study used a combination of both NAA and IAA and this was supported by the findings of Rahman *et al.* (2009). Findings by Dutta *et al.* (2011) in *Dendrobium* also support the effect of IAA on rooting when supplemented with MS media. The use of activated charcoal in the present study also had a positive influence on rooting of the cultures. A similar result showing positive influence of activated charcoal on *Phalaenopsis* rooting was observed by Bhaskar (1996) and Roy *et al.*, 2009. All the transferred plantlets could acclimatize well and 100 per cent survival was observed (Figure 6). Results of the biometric observations taken are furnished in Table 10. The mean plant height increased from 4.85 cm to 7.14 cm and the mean number of leaves increased from 3.86 to 4.18 after one month of hardening. Different potting mixtures have been reported for orchids for the hardening procedures. However the potting mixture used in this study was charcoal, coconut husk and brick pieces (1:1:1) which has been standardized at CPBMB for orchids and showed an excellent survival percentage for the regenerated seedlings of *Vanda* hybrids.

Conclusion

The results of the experiment showed that treating the explants with 0.1 per cent carbendazim for 20 minutes, followed by 70 per cent ethanol for 5 minutes and 0.1 per cent mercuric chloride for 5 min effectively reduced the microbial contamination with highest percentage of explant survival. The study showed positive results for inflorescence segments inoculated on to $\frac{1}{2}$ MS + 10 mg l⁻¹ BA + 2 mg l⁻¹ TDZ + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime as observed as direct shooting of the dormant buds. The established cultures successfully produced multiple shoots on MS + 4.5 ml l⁻¹ BA + 30 g l⁻¹ sucrose + 7.5 g l⁻¹ agar + 250 mg l⁻¹ cefotaxime both when inoculated with and without the stalk in about 100 days of inoculation of explant. The micro-shoots produced on the proliferation medium elongated on hormone free basal MS medium supplemented with 0.5 g l⁻¹ charcoal. Rooting of healthy and elongated shoots was successful on MS + 0.5 mg l⁻¹ NAA + 1 mg l⁻¹ IAA + 30 g l⁻¹ sucrose solidified on 7.5 g l⁻¹ agar and fortified with 250 mg l⁻¹ cefotaxime. Hence it can be concluded that the developed micropropagation protocol can be used for commercial production.

Acknowledgement

I am thankful to Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agriculture University, Thrissur, for providing the germplasm, laboratory and other facilities for conducting the research.

References

- Arditti J. and Ernst R. Micropropagation of Orchids. John Wiley and sons, New York, 1992, 9.
- Arditti, J. Micropropagation of orchids. John Wiley & Sons, 2009, 52.
- Begum F, Islam D, Paul RN, Mehedi M, Mondal SR. *In vitro* propagation of *Vanda* pteristhrough axillary bud derived protocorm culture. Trop. Agric. Res. Ext. 2002; 5:1-4.
- Bhadane BS, Patil RH. Data on the cost effective surface sterilization method for *C. carandas* (L.) seeds and callus induction from aseptic seedling. Data Brief. 2016; 7:1551-1555.
- Bhaskar J. Micropropagation of *Phalaenopsis*. Ph.D. thesis, Kerala Agricultural University, Thrissur, 1996; 84.
- Bhosle SV, Thengane RJ, Thengane SR. *In vitro* multiple shoot regeneration and plant production in *Alysicarpus rugosus* DC. var. heyneanus Baker. Indian J. Exp. Biol. 2005; 43(10):16-20.
- Chawla HS. Molecular markers and marker assisted selection. In: Introduction to Plant Biotechnology. Oxford and IBH Publishing, Co. Pvt. Ltd, New Delhi. 2000, 356.
- Chugh S, Guha S, Rao IU. Micropropagation of orchids: A review on the potential of different explants. Scientia Horticulturae, 2009; 122:507-520.
- Donzo K. Micropropagation of pineapple [*Ananas comosus* (L.) Merr.] with clonal fidelity analysis. M.Sc. (Ag.) thesis, Kerala Agricultural University, Thrissur, 2015; 52.
- Dutta S, Chowdhury A, Bhattacharjee B, Nath PK, Dutta BK. *In vitro* multiplication and protocorm development of *Dendrobium aphyllum* (Roxb.) CEC Fisher, Assam University J. Sci. Technol: Bio. Environ. Sci. 2011; 7:57-62.
- Gantait S, Sinniah R. Rapid micropropagation of monopodial orchid hybrid (Aranda Wan Chark Kuan 'Blue' x *Vanda coerulea* Griff. ex. Lindl.) through direct induction of protocorm-like bodies from leaf segments. Plant Growth Regul. 2012; 68:129-140.
- Kerbaux GB. Regeneration of protocorm like bodies through in vitro culture of root tips of *Catasetum* (Orchidaceae). Z. Pflanzen physiol. 1984; 113: 287-291.
- Korah PL, Shylaraj KS. Mericlone of *Phalaenopsis* and *vanda* hybrids. Kerala state council for science, technology and environment. Back to lab programme. Final technical report. Rice Research Station, Vyttila, India, 2011; 1-50.
- Latip MA, Murdad R, Aziz ZA, Ting LH, Govindasamy LM, Ripin R. Effects of N⁶-Benzyladenine and thidiazuron on proliferation of *Phalaenopsis gigantea* protocorms. As. Pac. J. Mol. Biol. Biotechnol. 2010; 18(1):217-220.
- Mathews VH, Rao PS. *In vitro* culture of *Vanda* hybrid (*Vanda* TMA X *Vanda* Miss Joaquim). Studies on seedling explants. Proc. Indian Natl. Sci. Acad. 1985; 4: 496-504.
- Morel G. Producing virus free *Cymbidiums*. Am. Orchid Soc. Bull. 1960; 29:495-497.
- NRC on orchids [National Research Center on Orchids]. *Vision 2050*. National Research Center on orchids, Pakyong, 2015; 1.
- Paudal MR, Pant B. *In vitro* micropropagation of rare orchid (*Esmeralda Clarkei* Rehb. f.) from shoot tip section. Int. J. Biology Pharmacy Allied Sci. 2012; 1(11):1587-1597.
- Rahman MS, Hasan MF, Das R, Hossain MS, Rahman M. *In vitro* micropropagation of orchid (*Vanda tessellata*) from shoot tip explants. J. Biosci. 2009; 17:139-144.
- Roy AR, Patel RS, Patel VV, Sajeev S, Deka BC. Asymbiotic seed germination, mass propagation and seedling development of *Vanda coerulea* Griff ex. Lindl.

- (Blue Vanda): an *in vitro* protocol for an endangered orchid. *Scientia Horticulturae*, 2009; 128: 325-331.
21. Seeni S, Latha PG. *In vitro* multiplication and ecorehabilitation of the endangered Blue Vanda. *Plant Cell Tissue Organ Cult.* 2000; 61:1-8.
 22. Silva JAT, Fukai S. The impact of carbenicillin, cefotaxime and vancomycin on chrysanthemum and tobacco TCL morphogenesis and *Agrobacterium* growth. *J. Appl. Hort.* 2001; 3(1):3-12.
 23. Sinha P, Roy SK. Regeneration of an indigenous orchid, *Vanda teres* (Roxb.) Lindl. Through *in vitro* culture. *Plant Tissue Cult.* 2004; 14(1):55-61.
 24. Thanh NT, Hang PL, Ket NV, Anh TTL, Phe PV, Gam NTH *et al.* The role of different medium and plant hormones on multiple shoots of Jewel orchids (*Anoectochilus setaceus* Blume). *VNU J. Sci. Natural Sci. Technol.* 2012; 28:47-53.
 25. Vij SP. Micropropagation of orchids through root segments. In: *Proceedings of Nagoya International Orchid Conference*, Nagoya, Japan, 1994; 72-80.
 26. Vij SP, Sood A, Sharma M. Micropropagation of *Aerides Maculosa* Lindl. (Orchidaceae). *Curr. Sci.* 1986; 55:1100-1101.
 27. Yu T, Yeh S, Yang J. Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. *Bot. Bull. Acad. Sin.* 2001; 42:281-286.