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In vitro Micropropagation Protocol for *Vanda* hybrid 'Dr. Anek'

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ABSTRACT

To meet the demands of good quality and true to type planting material of *Vanda* hybrid 'Dr. Anek', *in vitro* micropropagation protocol was developed at Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agriculture University. The different explants tested to initiate the cultures were leaf, root, stem and inflorescence segments. 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 $^1/_2$ 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 percentage of rooting was observed to be 72.41 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.

Introduction

Vanda orchids are one of the most sought after genus of Orchidaceae family among the five horticulturally important orchid genera in the international as well as domestic flower markets both as cut flower and potted plants. It is a monopodial orchid with vividly coloured, loosely arranged large beautiful flowers which has a long shelf life. Though tropical Asia (India) is considered as its origin, *Vanda* is widely distributed in South East Asia, Philippines, Indonesia, Southern China and northern Australia. Strap leaved *Vandas* are usually lower in plant height and are known as basket vandas. 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) 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 i*n vitro* micropropagation protocol for *Vanda* hybrid Dr. Anek" was taken up at Center for Plant Biotechnology and Molecular Biology (CPBMB), College of Horticulture, Kerala Agriculture University.

Materials and Methods

Leading *Vanda* hybrid Dr. Anek bearing dark netted pink flowers obtained from a commercial nursery was used for the present study. Different explants were used for micropropagation namely basal leaf segments, leaf tip segments, root segments, stem segments, and inflorescence segments. All explants were collected from the mother plants maintained in the net house at CPBMB, College of Horticulture. In the experiment, eight different surface sterilization treatments were tested for the different explants. Owing to limited number of explants, the surface sterilization experiment was not conducted for stem segments and hence the procedure that was 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 culture condition given was initial 48 h dark and further light conditions of 1000 lux with 26 ± 2^{0}C.

Culture establishment was tested for *Vanda* hybrids with root explants on different media compositions and the response was recorded after 21 days. Stem segments with two nodes and as well as the upper meristematic region on the stem were inoculated for culture establishment. 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^{0}C. The cultures initiated from the inflorescence segments were transferred to three different media for shoot proliferation. The cultures were repeatedly subcultured in the shoot proliferation medium of MS + 4.5 ml l^{-1} BA at 21 days interval for a period of 84 days i.e., upto S7 passages. Further, one cycle of subculture in hormone free basal MS media with charcoal led to elongation of the micro-shoots under light conditions of 1000 lux at 26 ± 2^{0}C. The elongated shoots were further subcultured into the identified rooting medium by separating them into individual shoots. Well rooted plantlets of the regenerated *Vanda* hybrids were transferred to the hardening unit for better field establishment of the *in vitro* grown plantlets. The plantlets were given 0.1 per cent carbendazim treatment for five minutes before planting them into small earthen pots filled with charcoal, coconut husk and brick pieces.

Results and Discussion

In the present study, eight different surface sterilization treatments were tested for the different explants and the results are given in Table 1 and 2. From the results obtained, it was observed that, microbial contamination was least in T_8 (0%) for both leaf and root explants but had a lower survival percentage

(0%). However in T_5 the survival per cent was highest (80%) for leaf explants and (70%) for root explants, hence T_5 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 T_5 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.. In the present study, the best treatment identified for all the explants was treating the explants with 20 minutes of 0.1per cent carbendazim followed by 5 minutes of 70 per cent ethanol and 5 minutes of 0.1per cent mercuric chloride as it recorded maximum per cent survival and minimum contamination. During the standardization of surface sterilization of inflorescence segments it was observed that the colour of the media turned brown due to the exudation of phenolic compounds. Hence, treating of the explants with 0.1per cent ascorbic acid for five minutes before treating with 70 per cent ethanol during the surface sterilization procedure could efficiently manage the problem of media browning in the present study. It has been reported by Seeni and Latha (2000) and also by (Arditti and Ernst, 1992).

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) reported that the duration of exposure to the sterilants is an important factor for successful regeneration of *in vitro* plant cultures. Chawla (2000) 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), Yu *et al.,* (2001) and Donzo (2015) the possible reason for this would the effect of antibiotic cefotaxime (250 mg l^{-1}) which was used in the nutrient media.

For establishing cultures for *Vanda* hybrids with root and leaf explants, different media compositions were tested and the response 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 Chugh *et al.,* 2009 (Table 4 and 5).

Table.1 Effect of surface sterilization treatments on leaf explants and root explants of Vanda 'Dr. Anek

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% HgCl2 1 min	100	100	0	0	Fungus	Fungus	100	100	0	0	0	0
T2.	70% ethanol 2 min + 0.1% HgCl2 2 min	100	100	0	0	Fungus	Fungus	100	100	0	0	0	0
T3.	70% ethanol 3 min+0.1% HgCl2 3 min	80	90	20	10	Fungus Bacteria	Fungus, Bacteria	60	80	20	10	0	0
T4.	70% ethanol 4 min+0.1% HgCl2 4 min	40	50	60	50	Fungus Bacteria	Fungus, Bacteria	30	30	10	20	0	0
T5.	70% ethanol 5 min+0.1% HgCl2 5 min	20	30	80	70	Fungus	Fungus	20	20	0	10	0	0
T6.	70% ethanol 6 min+0.1% HgCl2 6 min	20	20	30	20	Fungus Drying	Fungus, Bacteria, Drying	20	10	0	10	50	60
T7.	70% ethanol 8 min+0.1% HgCl2 8 min	0	10	10	20	Drying	Drying, Fungus	0	10	0	0	90	70
T8.	70% ethanol 10 min+0.1% HgCl2 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 ^{0}C

Table.2 Effect of surface sterilization treatments on inflorescence segments of Vanda 'Dr. Anek'

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 ^{0}C

Table.3 Response of leaf explants and root explants of 'Dr. Anek' 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'-(1, 2, 3-thidiazol-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 ^{0}C

Table.4 Response of stem segments and inflorescence segments of 'Dr. Anek' 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-thidiazol-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 ^{0}C

Table.5

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.	Dr. Anek	20	14.20	Bud enlargement	Bud elongation	Shoot initiation	80

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 ^{0}

Tabl.6 Response of cultures of 'Dr. Anek' 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 ^{0}C, *SPM: Shoot Proliferation Medium

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.	Dr. Anek	3	6.66	1.53	4.07

Medium: Hormone free basal MS, Culture condition: Light conditions of 1000 lux with 26 ± 2 ^{0}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.	29	Root initiation	4.46	3.30	6.63	5.93	8	72.41

Medium: MS + + 0.5 mg l^{-1} NAA + 1 mg l^{-1} IAA Culture condition: Light conditions of 1000 lux with 26 ± 2 ^{0}C

Table.10 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.	4.80	7.10	3.64	4.63

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

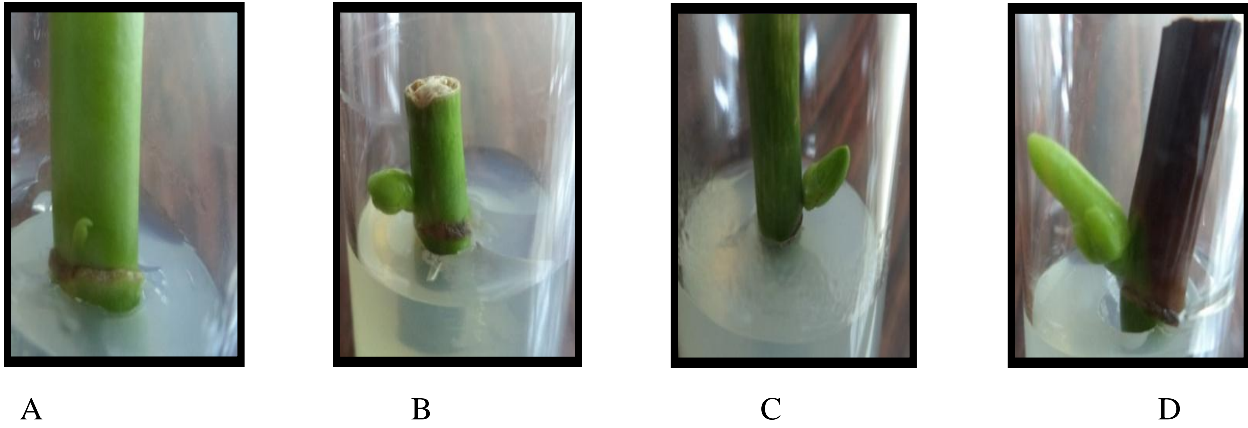

Fig.2 Cultures in shoot proliferation medium with stalk. (A. At subculture 4 passage, B. At subculture 5 passage, C. At subculture 6 passage, D. At subculture 7 passage)

Fig.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)

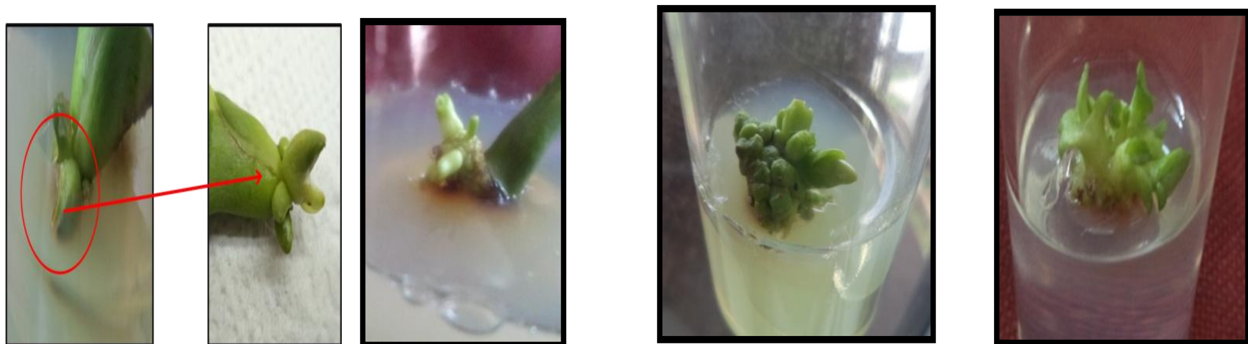

Fig.4 Cultures in elongation media

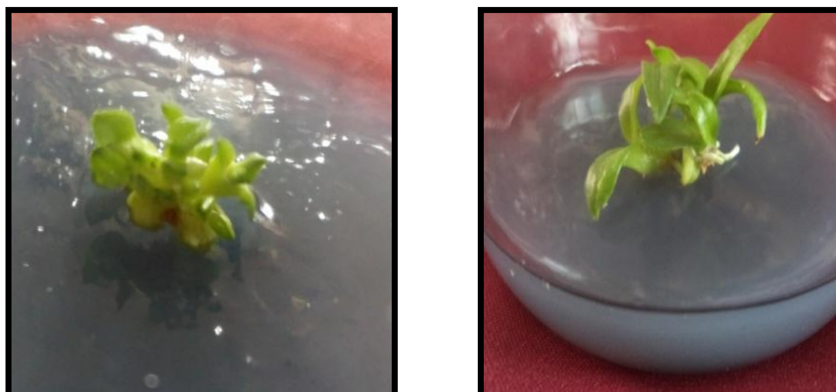

Fig.5 Rooted plantlet of *Vanda* hybrid

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

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 (Seeni and Latha, 2000), leaf segments (Mathews and Rao, 1985; Vij *et al.,* 1986), axillary buds and *in vitro* derived explants of various species of *Vanda* orchids showed positive results to different media compositions. 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* hybrids. The dormant buds present on the young immature inflorescence on the *Vanda* hybrids 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 14.20 days. The percentage of culture establishment was 80 per cent (Table 5 and 6). For cultures inoculated, the mean length of multiple shoots increased from 0.85cm to 4.20 cm and 0.25 cm to 1.53 cm in cultures with stalk and without stalk respectively 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.* 4.50 per culture as compared to cultures without stalk i.e. 6.67 per culture observed at the S7 passage (Table 7). The mean number of leaves per shoot was almost same i.e. 3.03 for cultures with stalk and 3.06 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 Latip *et al.,* (2010) and Begum *et al.,* (2002).

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 4.07 cm after one subculture passage (Table 8) (Sinha and Roy, 2004).

Bhosle *et al.,* (2005) and Thanh *et al.,* (2012) reported similar observations. The rooting percentage was calculated to be 72.41per cent. Plantlets recorded good root characters with an average 6.63 roots having a mean root length of 5.93 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 4.80 cm to 7.10 cm and. the mean number of leaves increased from 3.64 to 4.63 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.

In 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 $^{1}/_{2}$ 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.

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