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EFFECTS OF PLANT GROWTH REGULATORS ON MULTIPLE SHOOT INDUCTION IN *Vanda tessellata* (Roxb.) Hook. Ex G.Don AN ENDANGERED MEDICINAL ORCHID

Bakul Bhattacharjee & * S. M. Shahinul Islam,
Plant Genetic Engineering Lab., Institute of Biological Sciences, University of Rajshahi, Rajshahi-6205, Bangladesh.
*Corresponding author: shahin_ibsc@ru.ac.bd

ABSTRACT
An efficient protocol for regeneration of endangered medicinal orchid of *Vanda tessellata* via multiple shoot has been established using shoot segments as explants. Under this study four culture media *viz.* PM, MS, ½MS and B_5 were used. The most effective medium for seed germination where 80.0% germination was recorded is MS. Twenty different in combinations of plant growth regulators (PGRs) were tested for multiple shoot induction. It was revealed that the combination of 1.0 mgl^{-1} NAA and 1.0 mgl^{-1} BAP was proved to be the best medium formulation for multiple shoot development and elongation. In this case the average number of induced shoots per explants was 7.52. The single shoots were isolated from the multiple shoot and sub-cultured in ½MS medium supplemented with NAA (0.5-2.0 mgl^{-1}), IAA (0.5-2.0 mgl^{-1}) and IBA (0.5-2.0 mgl^{-1}) for root induction. Maximum root induction (6.1) and its growth (4.0 cm) found in ½MS medium supplemented with 1.0 mgl^{-1} IAA. The well rooted plantlets were hardened successfully in the potting mixture containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition.

KEY WORDS: *Vanda tessellata*, threatened, PLBs, multiple shoot, plant regeneration.

INTRODUCTION

Orchids, the doyens among ornamentals, are one of the most important global cut flower and pot plants and their sheer beauty has enchanted and fascinated people since early times (Teixeira da Silva JA, 2013). Orchid family Orchidaceae is a morphologically diverse and widespread family of Monocots. It includes 800 genera and between 25,000 to 30,000 species spread all over the world (Singh *et al*., 2007; Chugh *et al*., 2009, Nongdam and Chongtham, 2011). Many of the commercially important orchids are now artificially grown for beautiful flowers. Some orchids are also threatened by extinction because of environmental disruption, human succession of natural habitats, medicinal properties and over exploitation for horticulture and ethno botanical reasons (Coates *et al.*, 2007). The agro-climatic conditions of Bangladesh are congenial for natural orchid vegetation in different regions especially in Chittagong Hills tracks, Rangamati, Sylhet and Sundarbans. Taxonomical studies revealed that there are about 115 different varieties of local orchids in Bangladesh (Bhadra *et al.*, 2002). But habitat destruction, deforestation, climate change, indiscriminate collection by hobbyists and traders are some reasons for the extinction of local orchids. Therefore, it is very important to collect and conserve the local orchid varieties that would be useful for the improvement of orchids in Bangladesh. However, due to lack of proper cultivation practices, destruction of plant habitats and illegal and indiscriminate collection of plants from natural habitats, many medicinal plants are severely threatened (Devendra *et al*., 2011). *Vanda tessellata* (Roxb.) Hook. is an epiphytic orchid and it is now listed as endangered in Bangladesh . It has a lot of economic importance such as medicinal, glycosidal, alkaloidal *etc*. The roots of *Vanda tessellata* are antipyretic; useful in dyspepsia, bronchitis, inflammations, piles and hiccup. Externally the root is used in rheumatism and allied disorders and diseases of the nervous system. It is also remedy for secondary syphilis and scorpion-sting. Juice of the leaves is given in otitis and the paste as febrifuge. The roots possess significant anti-inflammatory activity and the plant contains an alkaloid, a glucoside, tannins, β-sitosterol, γ-sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. Roots contain tetracosyl ferrulate and β-sitosterol-D-glucoside (Ghani *et al*., 1990-1994). A novel aphrodisiac compound isolated from the flowers of *Vanda tessellata* (Roxb.) Ex Don, which activates neuronal and endothelial (Subramoniam *et al*., 2013). One of the special features of this family is the production of a large number of minute seeds with only minimal reserves of nutrients (Arditti and Ghani, 2000). In spite of a very large number of seeds produced, only 0.2-0.3% germinates in nature (Singh, 1992). Because of this feature, orchids depend upon mycorrhizal fungi for the carbon resources necessary for the germination and subsequent growth (Rasmussen, 1995). This necessitates the development of rapid propagation techniques for conservation of orchids. *In vivo* vegetative propagation is undesirable due to the heterozygosity of seed, minute seed size, presence of reduced endosperm and the requirement of an association with mycorrhizal fungi (Saiprasad and Polisetty, 2003). The concept of *in situ* conservation of orchids is wrought with many difficulties. This necessitates the application of *in vitro* seed propagation technique for orchid

conservation (Stenberg *et al.*, 1998, Gangaprasad *et al.*, 1999). Thus *in vitro* cultural techniques are now adopted for quick propagation of commercially important orchid species. Orchid seeds are artificially germinated for commercial purpose and seedlings are raised in large scale. Micropropagation of orchid seedlings can also be done with the use of the aseptically grown seedlings. In this context, it is important to develop rapid propagation of economically important orchids. Development of such propagation technique is also important for conservation of this group of endangered plant species. The present investigation was undertaken to establish a suitable protocol for *in vitro* germination, micropropagation and root induction. The effects of plant growth regulators on the development of multiple shoot formation and elongation have been investigated for this species also. In the present studies we have developed a very successful and efficient micropropagation method that can be successfully employed in breeding of *Vanda tessellata* for commercial purposes.

MATERIALS & METHODS

Plant materials

The immature capsule of *Vanda tessellata* was used as seed source which was collected from Rajshahi, Bangladesh. The plantlets of *Vanda tessellata* were grown through *in vitro* condition for further used as the source of explants for experiment purpose.

Seed sterilization and *in vitro* seed germination

At first, the capsules were washed by running tap water and surface sterilized by detergent. Then capsules were treated with 0.2% (w/v) $HgCl_2$ solution for 10 minutes and finally dip in 70% ethanol for 10-12 seconds. Then washed them with sterile distilled water and sterilized capsules were cut longitudinally by a sterile surgical blade. Around 100 mg seeds were cultured per vessel (Fig. 1a). Various media *viz.* MS (Murashige and Skoog, 1962), B_5 (Gamborg *et al.*, 1968) and PM (Phytamax™, Sigma, USA) were used under this study. The basal media (MS and B_5) amended with 3% (w/v) sucrose and the PM was amended with 2% (w/v) sucrose. The pH for all medium was adjusted at 5.6- 5.8. Inoculated vessels were maintained in the culture room under a period of 16 hrs light and dark for 8 hrs at 25±2°C. After two weeks of inoculation, some of the seeds were taken out and dispersed in one drop of water on a glass slide and examined its developments under a light microscope. Once the spherules were formed, then protocorm like bodies (PLBs) developmental stages were recorded in every week up to 8-10 weeks. Germination percentages were calculated employing the following formula:

$$\frac{\text{No. of seed swelling of the embryo}}{\text{No. of total seeds}} x\ 100$$

PLBs development and seedlings elongation

To evaluate the regeneration efficiency of protocorms, the PLBs were transferred to half strength MS medium supplemented with different concentration and combination of auxins and cytokinins. The protocorms, which developed from the germinated orchid seeds were isolated aseptically and transferred into fresh culture vessels containing the same germinating medium. Further subculture of the protocorm was done at an interval of 15-20 days. Prior to each subculture the density of seedlings per vessel was reduced. For estimating rapid elongation of shoots, germinating seedlings were transferred into different types of shoot elongation media. The elongation media were based on agar solidified MS medium supplemented with BAP (0.5-2.0 mgl^{-1}), NAA (0.5 mgl^{-1}), Kin (0.5 mgl^{-1}) and IAA (1.0 mgl^{-1}).

Multiple shoot induction

For multiple shoot development the *in vitro* growing seedlings were used when it raised up to 4-5 cm in height. These shoot segments were transferred into shoot induction and elongation media for development of adventitious shoots. Subculture of these explants was done at an interval of 4 weeks.

Rooting and acclimatization

Newly developed adventitious shoots when reached a height of 2-3 cm were transferred into rooting medium for root development. For efficient root induction we used three plant growth regulators *viz.* IAA, IBA and NAA in ½MS medium. The well rooted *in vitro* grown plantlets were hardened successfully in the potting mixture containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition following by the methods of Rahman *et al.* (2009).

RESULTS

In vitro seed germination and seedling elongation

Seed germination occurred after four weeks of culture. We have investigated four different agar solidified media. The highest percentage of seed germination (80.0%) obtained with MS medium, whereas the lowest (45.0%) was recorded in B_5 medium (Fig. 1b and Table 1).

TABLE 1: Effect of different media on *in vitro* germination of *V. tessellata* seeds

Media	Amount of seeds per culture vessel	Seed germination		Mean time of germination
		Days	% of germination	Days
MS	100 mg	28 - 35	80.0	31.5
½ MS	100 mg	34 - 40	65.0	37.0
PM	100 mg	50 - 55	50.0	52.5
B_5	100 mg	45 - 50	45.0	47.5

MS = Murashige and Skoog (1962); PM = Phytamax™, P-1056, Sigma, USA; B_5 = Gamborg *et al.* (1968).

It was also reveals that the period of germination on MS medium was comparatively shorter (average germination time 31.5 days) than that observed on ½ MS, PM and B_5 media. On the other hand, the highest rate of shoot elongation (5.0 cm) was achieved in MS medium fortified with 1.5 mgl^{-1} BAP and 0.5 mgl^{-1} NAA (Table 2).

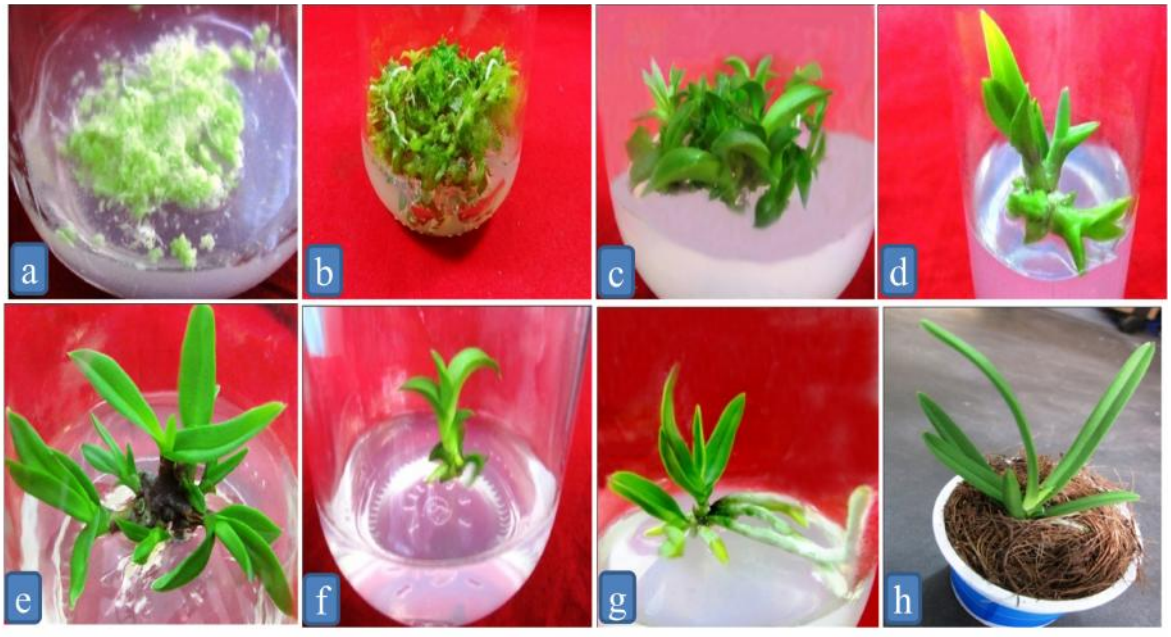
TABLE 2: Effect of different concentrations and combinations of PGRs on seedlings development with MS medium.

PGRs (mgl^{-1})	Initial length (cm) Mean	Length (cm) after 30 days of culture initiation (Mean)	Seedling growth (cm) (M ± S.E)
BAP + NAA			
0.5 + 0.5	3.10	4.00	0.95^{c} ± 0.03
1.0 + 0.5	3.20	4.32	1.12^{c} ± 0.09
1.5 + 0.5	3.00	8.00	5.00^{a} ± 0.54
2.0 + 0.5	3.00	3.85	0.89^{c} ± 0.04
BAP + Kinetin			
0.5 + 0.5	2.75	3.50	0.90^{c} ± 0.04
1.0 + 0.5	2.80	3.80	1.02^{c} ± 0.12
1.5 + 0.5	3.42	6.50	3.12^{b} ± 0.24
2.0 + 0.5	3.00	3.95	0.95^{c} ± 0.03
BAP + IAA			
0.5 + 1.0	2.00	2.65	0.68^{c} ± 0.10
1.0 + 1.0	2.70	3.65	0.95^{c} ± 0.12
1.5 + 1.0	2.85	3.90	1.07^{c} ± 0.14
2.0 + 1.0	2.40	3.20	0.88^{c} ± 0.08

PGRs = Plant growth regulators (PGRs), values represent mean ± S.E (standard error). Each treatment was repeated thrice. Means in a column with the different letter (superscript) are significantly different according to least significant difference (LSD) at $P < 0.05$ levels.

Multiple shoot induction and individual shoot elongation

Differentiation of shoot buds was observed to the shoot segments which was isolated from the shoots elongated *in vitro* from the germinated seedlings (Fig. 1c). Shoot differentiation was first observed after 28 days of culture on MS media fortified with BAP, NAA, IAA and Kin at the concentration of (0.5-1.5) mgl^{-1}. The highest rate of shoot induction (7.52) per explants was observed in MS medium fortified with1.0 mgl^{-1} BAP and 1.0 mgl^{-1} NAA (Fig. 1e and Table 3). The lowest multiple shoot (2.81) was also observed in MS with 0.5 mgl^{-1}BAP and 0.5 mgl^{-1} Kinetin. Results indicated that out of 20 treatment combinations, 1.0 mgl^{-1} BAP + 1.0 mgl^{-1} NAA is found suitable for multiple shoot induction (Table 3).



FIGURES 1(a-h): Developmental stages of multiple shoot buds in *V. tessellata*. (a) *In vitro* seed germination; (b-c) Seedlings is undergoing for elongation; (d-e) multiple shoot induction from single shoot segment; (f) single shoot buds; (g) Elongated shoot bud is going to develop roots on agar solidified medium; (h) plantlets acclimatized in a pot .

TABLE 3: Effect of different concentrations and combinations of plant growth regulators (PGRs) with MS medium on the development of multiple shoot buds from shoot segments.

PGRs	Concentrations (mgl^{-1})	Average no. of shoot buds sprouting in each shoot segments	Time required (days)
BAP + NAA	0.5 + 0.5	$3.07^{abc} \pm 0.26$	
	1.0 + 0.5	$3.28^{abcd} \pm 0.24$	
	0.5 + 1.0	$4.38^{efgh} \pm 0.19$	
	1.0 + 1.0	$7.52^{i} \pm 0.28$	28-35
	1.0 + 1.5	$5.06^{h} \pm 0.82$	
	1.5 + 1.0	$4.86^{fgh} \pm 0.24$	
	1.5 + 1.5	$4.00^{cdefg} \pm 0.54$	
BAP + IAA	0.5 + 0.5	$2.87^{ab} \pm 0.24$	
	1.0 + 0.5	$3.87^{bcdef} \pm 0.09$	
	0.5 + 1.0	$4.28^{defgh} \pm 0.35$	
	1.0 + 1.0	$4.37^{efgh} \pm 0.29$	35-42
	1.0 + 1.5	$3.00^{abc} \pm 0.54$	
	1.5 + 1.0	$4.00^{cdefg} \pm 0.35$	
	1.5 + 1.5	$2.86^{a} \pm 0.09$	
BAP + Kinetin	0.5 + 0.5	$2.81^{a} \pm 0.28$	
	1.0 + 0.5	$5.00^{gh} \pm 0.54$	
	0.5 + 1.0	$3.20^{abc} \pm 0.20$	42-49
	1.0 + 1.0	$5.12^{h} \pm 0.24$	
	1.5 + 1.0	$4.24^{defgh} \pm 0.25$	
	1.5 + 1.5	$3.76^{abcde} \pm 0.37$	

Values represent mean ± S.E. (standard error). Each treatment was repeated thrice. Means in a column with the different letter (superscript) are significantly different according to least significant difference (LSD Test) ($P < 0.05$).

On the other hand, the individual shoots were separated and sub-cultured them into the fresh elongation medium. After 30 days of culture the highest shoot elongation (3.75 cm) was observed in MS medium fortified with 1.5 mgl^{-1} BAP and 0.5 mgl^{-1} NAA. The minimum elongation (0.75cm) was recorded from MS with 2.0 mgl^{-1} BAP + 0.5 mgl^{-1} kinetin (Table 4).

TABLE 4: Effect of different concentrations and combinations of PGRs with MS medium on the elongation of individual shoot

PGRs (mgl^{-1})	Initial length (cm) (Mean)	Length (cm) of shoot after 30 days of culture (Mean)	Increased length (cm) (M ± S.E)
BAP + NAA			
0.5 + 0.5	2.00	3.05	$1.04^{c} \pm 0.22$
1.0 + 0.5	2.75	4.05	$1.36^{c} \pm 0.29$
1.5 + 0.5	3.25	7.00	$3.75^{a} \pm 0.37$
2.0 + 0.5	2.05	4.25	$2.26^{b} \pm 0.39$
BAP + IAA			
0.5 + 0.5	1.95	2.90	$0.99^{c} \pm 0.15$
1.0 + 0.5	2.80	4.32	$1.50^{bc} \pm 0.22$
1.5 + 0.5	2.85	5.55	$2.68^{a} \pm 0.09$
2.0 + 0.5	3.00	4.85	$1.87^{b} \pm 0.09$
BAP + Kinetin			
0.5 + 0.5	2.50	4.06	$1.55^{b} \pm 0.20$
1.0 + 0.5	2.70	4.70	$2.00^{ab} \pm 0.27$
1.5 + 0.5	2.30	4.50	$2.28^{a} \pm 0.18$
2.0 + 0.5	2.25	3.00	$0.75^{c} \pm 0.07$

Values represent mean ± S.E. (standard error). Each treatment was repeated thrice and each treatment counted of 5 replicate culture vessels. Means in a column with the different letter (superscript) are significantly different according to least significant difference (LSD Test) ($P < 0.05$ levels).

Root induction, elongation and acclimatization

All elongated shoots were transferred into rooting media for induction of roots. Results indicate that the average length of induced roots reached highest (4.0 cm) when the shoots were cultured on half strength MS medium supplemented with 1 mg/l IAA (Fig. 2). In addition, this combination of medium was also effective for developing adventitious roots. In this medium the average number of root per shoot was 6.10 (Fig. 3). The well rooted micro propagated plantlets were hardened successfully in the

potting mixture containing coconut husk, charcoal, brick pieces in the ratio of 2:1:1 and eventually established under natural condition following by the methods of Rahman *et al*. (2009). In our investigation 80% plantlets survived acclimatization process and grew to a normal flowering plant under field condition (Fig. 1h).

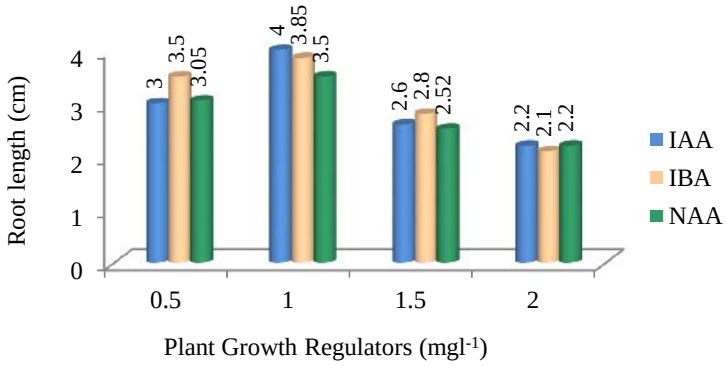


FIGURE 2: Effects of different auxins on root development with ½ MS media.

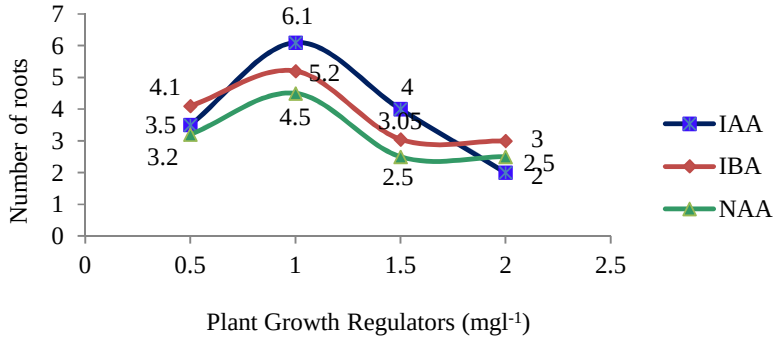


FIGURE 3: Effects of different auxins with ½ MS on number of root per plant.

DISCUSSION

We have developed necessary parameters that are involved in the tissue culture processes. For germination of seeds among the four investigated basal media we found MS medium was the most suitable one. In MS medium the exposed seeds (80.0%) started to germinate (Table.1). In addition to germination rate, the time of germination was found to be the shortest also in this MS medium. Considering these two features, rate and time of germination we suggest that MS medium can be effectively used for *in vitro* germination of orchid seeds. Mohanty *et al*. (2012) reported that MS medium was equally effective also for germination of *Cymbidium mastersii* species. One of the key steps of *in vitro* propagation is the elongation of the donor plant materials that can be used as the source for regenerating multiple shoots. To investigate this feature we have exposed the germinated orchid seedlings to various elongation media containing different plant growth regulators in different combinations and concentrations. We found that the rate of elongation reached highest when the germinated seedlings were exposed to MS containing 1.5 mgl^{-1} BAP and 0.5 mgl^{-1} NAA. In this medium the average length of germinated seedlings resulted about 5.00 cm (Table 2). Such type of positive effect of this culture condition for elongation of germinated seedlings was reported previously by Pant and Pradhan (2010). Analyzing the results obtained in mcropragation of the induced shoots we found that the media containing BAP and NAA developed the highest number of adventitious shoots (in average 7.52 shoots per explants). Furthermore, in this medium the time of shoot initiation was the shortest (in average 28-35 days). It was observed that in addition of NAA and BAP in the MS medium lead to optimum micropropagation of *Vanda tessellata* species and this combination can be successfully employed by the orchid breeders. Multiple shoots were produced in combination of BAP and NAA medium reported previously in various orchid species by different researcher (Nhat and Dung, 2006; Rahman *et al*., 2009; Long *et al*., 2010; Pant and Shresta, 2011). One of the major problems for orchid breeders is to generate a well developed root system facilitating adaptation of *in vitro* grown plantlets to field conditions. For investigating this problem we tested three potential plant growth regulators of IBA, IAA and NAA. Shoots when exposed to half strength MS medium supplemented with 1 mgl^{-1} IAA developed the highest number of adventitious roots (Fig. 3). The length of these roots was also highest in this

medium (Fig. 2). The effect of IAA or IBA on induction of roots in other orchid species like *Vanda coerulea* was also reported by several authors (Malabadi *et al*., 2004, William *et al*., 2003, Mitra *et al*., 1976). These results are in complete agreement with those we obtained in half strength MS supplemented with 1 mgl^{-1} of IAA.

CONCLUSION

The results suggest that combined effect of BAP and NAA is more effective for multiple shoot induction from shoot segments of *Vanda tessellata*. On the other hand, single effect of IAA with ½MS medium was proved the best for root induction from the individual elongated shoot. Under this study an efficient regeneration protocol for *in vitro* micropropagation in *V. tessellata* through shoot segment has been established. For advance level of biotechnological research and orchid conservation those experimental findings can be helped to plant breeder and scientist.

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