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MASS PROPAGATION OF PLBs DERIVED FROM LEAF AND SHOOTS OF *VANDA TESSELLATA* (ROXB.) HOOK. *EX* G. DON AN ENDANGERED MEDICINAL ORCHID IN BANGLADESH

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Abstract

Somatic embryos were induced from leaf and shoot explants of *Vanda tessellata* (Roxb.) Hook. *ex* G. Don cultured on MS medium supplemented with BAP, NAA and Kn. BAP (0.5 mg/l) and Kn (1.0 mg/l) showed better performance on somatic embryogenesis with embryos forming mostly near the cut ends of stem and leaf surfaces. Maturation of somatic embryos were produced on the same induction medium. Germination and regeneration efficiency of somatic embryos were recorded as highest (60.83%) in half strength of MS medium supplemented with 1.0 mg/l BAP. Maximum number of multiple shoots (10.80 ± 0.22) was obtained in MS medium with 1.5 mg/l BAP and 1.0 mg/l NAA. Individual shoots produced highest number of adventitious roots (5.0 ± 0.37) on half strength of MS medium supplemented with 0.5 mg/l IAA and 0.5 mg/l NAA. This protocol provides a rapid and simple way to regenerate plants through somatic embryogenesis which can improve the mass propagation, advanced biotechnological work and conservation of orchids in future.

Introduction

Orchidaceae is one of the largest angiosperm families comprising 800 genera and 25000 to 30000 species spread all over the world (Chugh *et al*. 2009). Almost 800 species are identified and adding to the family list in every year. Many of them have floricultural importance grown for their beautiful flowers (Bektas *et al*. 2013). Some orchids are threatened by extinction because of environmental disruption, human succession of natural habitats, medicinal properties and over exploitation for horticulture and ethnobotanical reasons (Nongdam and Chongtham 2011). 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 in the world (Coates and Dixon 2007). *Vanda tessellata* is one of them listed as an endangered orchid species in Bangladesh. It is an epiphytic orchid and the demand of this orchid is increasing day by day for its medicinal and floricultural importance (Bhattacharjee and Islam 2014). The roots of this orchid have anti-inflammatory activity, antipyretic; useful in dyspepsia, bronchitis, inflammations, piles and hiccup. Ghani *et al*. (2003) reported that the roots of orchids containing tetracosyl ferrulate and β-sitosterol-D-glucoside and possess significant anti-inflammatory activity. They also mentioned that the plant contained an alkaloid, a glucoside, tannins, β-sitosterol, γ-sitosterol and a long chain aliphatic compound, fatty oils, resins and colouring matters. One of the special features of this family is the production of a large number of tiny seeds with only minimal reserves of nutrients (Arditti and Ghani 2000). Orchid produced a large number of seeds per pod but only 0.2 - 0.3% or less produced plants in nature. So the concept of *in situ* conservation faces many difficulties to overcome the problem. In this regard *in vitro* seed

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propagation technique is very helpful for orchid conservation (Singh 1992, Bhattacharjee *et al.* 2015a,b). Thus *in vitro* cultural techniques are now adopted for quick propagation of commercially important orchid species to develop rapid propagation of economically important and specially endangered orchids for their proper conservation. Orchid seeds are artificially germinated for commercial purpose and seedlings are raised in large scale in many countries. The most convenient techniques for orchids are using by *in vitro* cell culture, somatic embryogenesis, direct and indirect oragnogenesis (Antony *et al*. 2014, Islam and Bhattacharjee *et al*. 2015, Islam *et al*. 2015). The phenomenon of somatic embryogenesis via callus is rather rare as success of callus formation in orchids has limitations due to slow growth and a tendency to become necrotic (Kerbauy 1984). There are not enough reports on *in vitro* somatic embryogenesis by leaf and stem in *V. tessellata*. So the present investigation has been undertaken to study the *in vitro* mass multiplication.

Materials and Methods

Vanda tessellata (Roxb.) Hook. *ex* G. Don species was grown through *in vitro* culture in the laboratory. The leaves and stems were cut into small pieces (1 - 2 cm) and were used as explants. Semi-solid MS medium fortified with 3% (w/v) sucrose, 0.8% agar PT (RM7695, Himedia) and various combinations and concentrations of BAP, 2,4-D, NAA and Kn were used. Protocorm-like bodies (PLBs) were transferred to both MS and half strength of MS supplemented with various concentrations of BAP and cultures were incubated under a 16 hrs photoperiod. Plant regeneration rate was calculated from number of plantlets obtained from three replicates of each treatment. For multiple shoot formation, the induced adventitious shoot were transferred to MS basal medium containing with different combinations and concentrations of PGRs (BAP, NAA, Kn and IAA) and sub-cultured every month. Then single shoots were separated carefully and transferred to half strength of MS medium. On the basis of growth regulators either single or in combination the medium was divided into six groups: T_1 = IAA 0.5 mg/l, T_2 = NAA 0.5 mg/l, T_3 = IBA 0.5 mg/l, T_4 = IAA 0.5 mg/l + NAA 0.5 mg/l, T_5 = NAA 0.5 mg/l + IBA 0.5 mg/l and T_6 = IAA 0.5 mg/l + IBA 0.5 mg/l.

Embryos induced from leaf and stem were multiplied and maintained on the same medium and sub-cultured monthly. Each experiment was repeated thrice and data were recorded on the basis of embryo induction (%), number and length of shoot formation and root development. The data were subjected to analysis of variance (ANOVA). Mean values of treatments were compared by LSD test calculated at $p < 0.05$ using by SPSS program (Gomez and Gomez 1984).

Results and Discussion

Young leaf and stem explants of *in vitro* grown *V. tessellata* were cultured on MS medium supplemented with 0.5 - 2.0 mg/l BAP, 1.0 - 2.5 mg/l 2,4-D, 1.0 - 2.5 mg/l NAA and 1.0 - 2.5 mg/l Kn. At the beginning of the culture the leaf and stem explants showed swelling and then embryos like structure were formed within 6 - 7 weeks of culture initiation. Those embryos showed greenish, small and globular type arising individually or in a group. In some cases visible PLBs were induced directly at the cut surfaces of leaf explants (Fig. 1b, c). Reciprocally, similar types of results were obtained in different plant species previously reported (Devaraju and Reddy 2013). Somatic embryos were obtained directly from the cutting edge of the blade and embryogenic callus (indirect way) from the midrib (Cordal *et al*. 2014). Embryo formation was observed after 5 weeks of culture initiation. After 6 weeks, embryos derived from stem tissue had grown vigorously and formed a cluster of embryos (Fig. 2c). Both stem and leaf explants showed better and faster embryos formation and subsequent PLBs development in MS + 0.5 mg/l BAP +

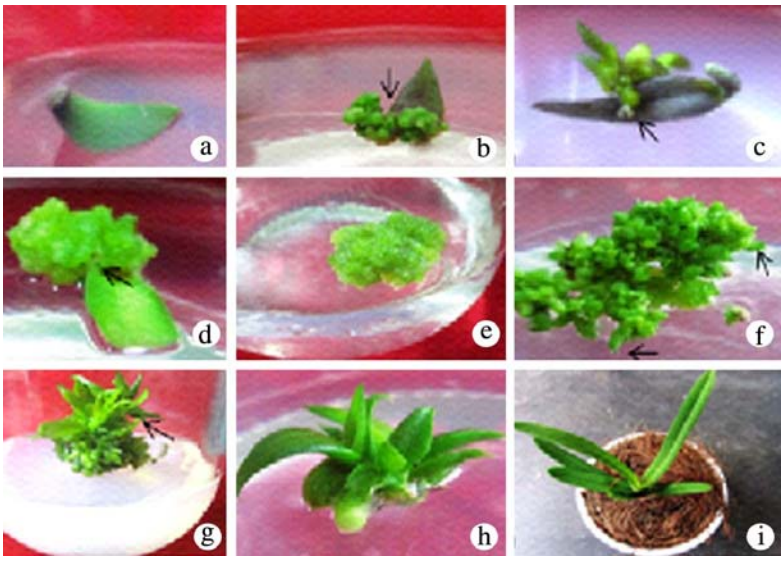
1.0 mg/l Kn than other concentrations and combinations of plant growth regulators (Table 1). The induction frequency of embryos was significantly higher when lower concentration of BAP and kinetin were added in the medium. The frequency of direct embryo induction was highest with leaf explants (25.0%) as compared to stem (16.20%) with 0.5 mg/l BAP and 1.0 mg/l kinetin (Table 1). On the other hand, thidiazuron (TDZ) at 0.3, 1.0 and 3.0 mg/l induced 5 - 25% of leaf tip segments of *in vitro* grown plants directly formed embryos after 60 days of culture, and 1.0 mg/l TDZ was the best treatment (Chung *et al*. 2007). In another orchid, *Oncidium*, leaf tips generally had the highest ability to form embryos (Chen *et al*. 1999). These embryos formed directly without callus formation. Most of the globular embryos developed gradually as bipolar structures resembling heart, torpedo and cotyledon-stage embryos (Fig. 1e, 2d). After 6 - 7 weeks of culture initiation the cultures exhibited near-confluent growth, which turned green with patches of developing embryos.

Table 1. Effects of different concentration and combination of PGRs with MS medium on induction of embryos from leaf and stem explants of *V. tessellata*.

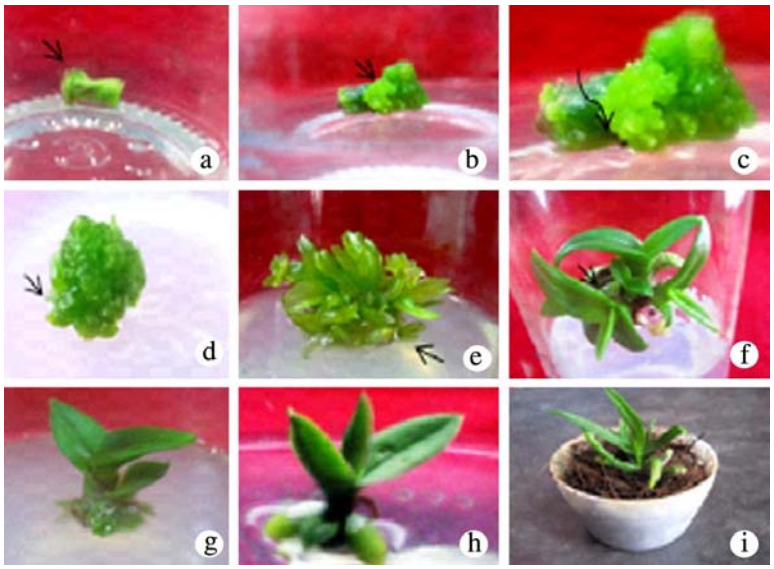
Growth regulators (mg/l)	% of embryo induction	
	Leaf	Stem
BAP + 2,4-D		
0.5 + 1.0	$10.00 \pm 0.33^{\mathbf{b}}$	7.33 ± 0.45^{cd}
1.0 + 1.5	10.67 ± 0.48^{bc}	6.67 ± 0.42^{abc}
1.5 + 2.0	11.83 ± 0.68^{bc}	6.83 ± 0.41^{bc}
2.0 + 2.0	6.83 ± 0.38^{a}	5.33 ± 0.45^{ab}
BAP + NAA		
0.5 + 1.0	15.60 ± 0.23^{d}	12.40 ± 0.43^{e}
1.0 + 1.5	$13.60 \pm 0.47^{\mathbf{cd}}$	11.80 ± 0.34^{e}
1.5 + 2.0	10.20 ± 0.38^{b}	7.40 ± 0.39^{cd}
2.0 + 2.0	6.00 ± 0.26^{a}	5.00 ± 0.26^{a}
BAP + Kn		
0.5 + 1.0	25.00 ± 0.15^{f}	16.20 ± 0.39^{f}
1.0 + 1.5	18.80 ± 0.15^{e}	12.00 ± 0.30^{e}
1.5 + 2.0	13.00 ± 0.26^{bcd}	11.80 ± 0.39^{e}
2.0 + 2.0	10.40 ± 0.75^{b}	8.80 ± 0.52^{d}

Means in a column with the different letter/s (superscript) are significantly different according to least significant difference (LSD) at $p < 0.05$ levels.

After two weeks of culture initiated embryos were isolated and transferred to MS and half strength of MS basal medium with various concentrations (0.5 - 2.0 mg/l) of BAP. The highest regeneration frequency (60.83%) of the somatic embryos was recorded from leaf explants in half strength of MS medium supplemented with 3% sucrose and 1.0 mg/l BAP (Fig. 3). Similarly embryos obtained from stem explants showed 53.67% regeneration response in the same medium that subsequently formed adventitious shoots (Fig. 4). Plant regeneration with direct and indirect organogenesis from various explants sources of *N. foetida* has already been reported (Rai 2002).



Figs. 1a - i: Direct somatic embryogenesis from leaf explants of *Vanda tessellata* and subsequent plant regeneration. (a) Inoculation of leaf explants on the medium, (b) somatic embryos directly formed from near the cut end region of leaf, (c)) somatic embryos directly formed from the leaf surface, (d) a cluster of embryos directly formed from the leaf base region, (e) a cluster of leaf derived embryos, (f-g) embryos formed shoots; (h) regenerated plantlet with well developed root and (i) acclimatized plantlets.



Figs. 2a - i: Direct somatic embryogenesis from stem explants of *Vanda tessellata* and subsequent plant regeneration. (a) Swelling of stem explants on the medium, (b) somatic embryos directly formed near the cut end region of stem, (c)) somatic embryos directly formed vigorously from the stem surface, (d) a cluster of stem derived embryos, (e) regeneration of stem derived embryos, (f) a cluster of multiple shoots, (g) individual shoot isolated from multiple shoot clusters, (h) root induction in individual shoot and (i) acclimatized plantlets.

For multiple shoot formation adventitious shoots were transferred to MS medium that contained different combinations and concentrations of cytokinin and auxin. A range of cytokinins (Kn, BAP, 2-iP and zeatin) has been used for micropropagation studies (Bektas *et al*. 2013). In this case, maximum number of shoots/explants (10.80 ± 0.22) and the highest response in shoot length (3.10 cm) was recorded when 1.5 mg/l BAP and 1.0 mg/l NAA were added with MS medium (Table 2).

For rooting the best performance (5.20, 3.38 cm) were obtained when shoots were transferred to half strength of MS supplemented with 3% (w/v) sucrose + 0.5 mg/l IAA + 0.5 mg/l NAA (Table 3, Figs. 1h, 2h). On the other hand, the rooting parameters were changed significantly with different concentrations of IBA. The best rooting was achieved in MS medium containing 0.5 ppm IBA (Akin *et al*. 2014). Similarly, IBA, as a synthetic auxin, was found to be the most favorable root inducer and was preferred based on the results of prior studies (Islam *et al*. 2015, Siddique and Islam 2015). Well rooted plants were hardened successfully in the potting mixture containing coconut husk, charcoal, brick pieces in the ratio of 2 : 1 : 1. However, survival rate of *in vitro* grown plants depends using a suitable hardening process.

Table 2. Effects of different concentrations and combinations of PGRs in MS medium on multiple shoot formation and individual shoot length of *V. tessellata*.

Growth regulators (mg/l)	Shoot development	
	Average no. of multiple shoots/explants ± S.E.	Average length of shoots (cm) ± S.E.
BAP + NAA		
0.5 + 0.5	4.80 ± 0.34^{b}	1.60 ± 0.23^{b}
1.0 + 0.5	8.40 ± 0.14^{f}	2.05 ± 0.03^{d}
1.5 + 1.0	10.80 ± 0.22^{g}	3.10 ± 0.14^{f}
2.0 + 1.5	6.40 ± 0.39^{d}	1.90 ± 0.23^{cd}
BAP + Kn		
0.5 + 0.5	3.80 ± 0.28^{a}	1.30 ± 0.15^{a}
1.0 + 0.5	7.80 ± 0.22^{e}	1.63 ± 0.13^{b}
1.5 + 1.0	8.80 ± 0.22^{f}	2.05 ± 0.10^{d}
2.0 + 1.5	4.80 ± 0.28^{b}	1.25 ± 0.11^{a}
BAP + IAA		
0.5 + 0.5	3.60 ± 0.30^{a}	1.20 ± 0.20^{a}
1.0 + 0.5	5.60 ± 0.39^{c}	1.80 ± 0.26^{c}
1.5 + 1.0	7.80 ± 0.26^{e}	2.30 ± 0.13^{e}
2.0 + 1.5	4.40 ± 0.15^{b}	1.60 ± 0.07^{b}

Each value represents mean ± S.E. Means in a column with the different letter/s (superscript) are significantly different according to least significant difference (LSD) at $p < 0.05$ levels.

Table 3. Effects of different combination and concentration of PGRs with half strength of MS on induction of roots and its lengths in individual shoot from multiple shoot clusters of *V. tessellata*.

Treatments	PGRs			Number of roots/plants (M ± S.E.)	Length (cm) of roots (M ± S.E.)
	IAA	NAA	IBA		
T_1	0.5	-	-	2.40 ± 0.14^{a}	2.00 ± 0.17^{a}
T_2	-	0.5	-	3.60 ± 0.29^{b}	2.40 ± 0.11^{a}
T_3	-	-	0.5	2.42 ± 0.38^{a}	2.30 ± 0.12^{a}
T_4	0.5	0.5	-	5.20 ± 0.38^{c}	3.38 ± 0.11^{b}
T_5	-	0.5	0.5	3.80 ± 0.22^{b}	2.76 ± 0.21^{ab}
T_6	0.5	-	0.5	4.00 ± 0.42^{b}	2.60 ± 0.25^{ab}

M = Mean, PGRs = Plant growth regulators. Values represent mean ± SE (Standard error). Means in a column with the different letter/s (superscript) are significantly different according to LSD at $p < 0.05\%$ levels.

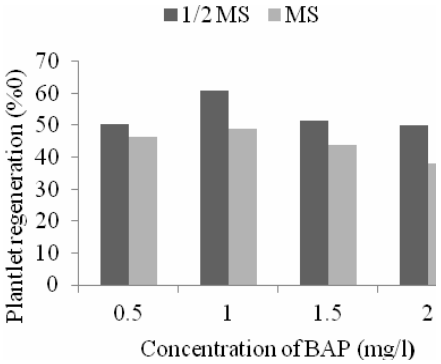


Fig. 3. Effect of BAP on regeneration of somatic embryos into plantlets derived from leaf explants.

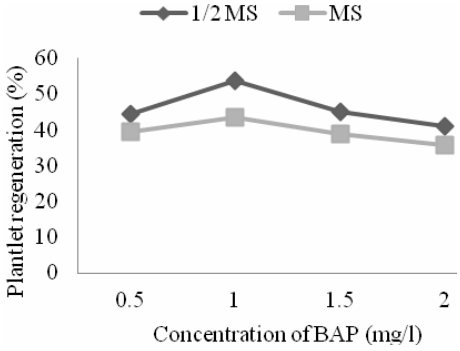


Fig. 4. Effects of BAP on regeneration of somatic embryos into plantlets derived from stem explants.

Many workers used different matrices or substrates with manipulation in salt solution for hardening of orchids and other crops. They used mixture of charcoal pieces, brick pieces, soaked cotton and chopped moss for *Saccharum officinarum* (Gill *et al*., 2004) and for *Cymbidium* (Deb and Pongener 2013). In this case, we used coconut husk, charcoal, brick pieces in the ratio of 2 : 1 : 1 and eventually established under natural condition. It was observed that around 60% of the plants were survived. In the present investigation, we developed a simple and efficient protocol for induction of direct somatic embryogenesis of *V. tessellata* and successfully obtained plantlets from regenerated embryos. Regeneration system through direct somatic embryogenesis is suitable for further studying on physiology and morphology status of embryo development, mass propagation and genetic transformation (Cordal *et al*. 2014).

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