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PLANT REGENERATION THROUGH SOMATIC EMBRYOGENESIS FROM LEAF AND ROOT EXPLANTS OF *Rhynchostylis retusa* (L.) Blume

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ABSTRACT

An efficient protocol for protocorm like bodies (PLBs) was development and its regeneration through direct somatic embryogenesis was standardized for *Rhynchostylis retusa* (L.). Somatic embryos were developed from leaf and roots and cultured in MS medium supplemented with different combinations of phytohormones either individually or in combination. The embryo induction was highest in leaf (75%) and root (50.2%) when a combination of 1.0 mg L^{-1} BAP + 1.5 mg L^{-1} kinetin was used. Germination of somatic embryos was highest (60%) in ½MS medium with 1.5 mg L^{-1} BAP. The effect of different PGRs was observed on multiple shoot induction. Maximum number (10.20 ± 0.58) and highest length (3.8 cm) of multiple shoots was observed in MS medium + 1.5 mg L^{-1} BAP and 1.0 mg L^{-1} NAA. Highest number (5.8) and length (4.17 cm) of adventitious roots were seen in ½MS medium + 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA. The well rooted plants were successfully hardened to the pots that contained small brick pieces, wood charcoal (1:3) and coconut husk; and eventually transferred to shade house for establishment. Plants showed 60% survival.

Key words: *Rhynchostylis retusa*, medicinal orchid; regeneration; PGRs, somatic embryogenesis

INTRODUCTION

Orchids are one of the most pampered plants and occupy top position among all the flowering plants for cut-flower production and as potted ornamentals (Parab and Krishnan, 2012). The plant is highly valued for its fantastic range of variation in colour, form and long-lasting flowers (Paudel *et al*., 2012). Many commercially important orchids are now artificially grown for their high value both in national and international markets (Kumar *et al*., 2002). However, some orchids face threat of extinction because of environmental disruption, anthropogenic activities and overexploitation for horticulture and ethno-botanical purposes (Coates *et al*., 2007). *Rhynchostylis retusa* (L.) Blume is an endangered orchid in Bangladesh. The plant is a monopodial epiphytic orchid species with beautiful flowers arranged in racemose manner. This orchid species grows in moist areas and blossoms during monsoon (Bhattacharjee and Islam, 2015a). The plant is used to treat rheumatic disease, tuberculosis, epilepsy, blood dysentery, menstrual disorders, gout, asthma, skin diseases, external inflammations (Kumar *et al*., 2012; Shanavaskhan *et al*., 2012). The plant leaf juice and aerial roots are used in ear pain and cleaning (Basumatary *et al*., 2004). In Kurigram district of Bangladesh, people use the leaves of this plant to cure rheumatic pain (Das *et al*., 2012). *R. retusa*

roots are used to cure malarial fever (Tiwari *et al*., 2012, Radhika *et al*., 2013). Juices of roots are applied to cuts and wounds while dried flowers are used as insect repellent and to induce vomiting (Subedi *et al.,* 2013). The plant has shown significant antimicrobial activity against bacteria and fungi (Bhattacharjee and Islam, 2015b). However, these species need to be protected from the danger of extermination through reforestation (Bhattacharjee and Islam, 2015a). Therefore, rapid multiplication process of these species through micro-propagation is the need of hour. The technique helps in rapid propagation of a large number of pathogen-free plants in a short time as well as helps in conservation of rare and endangered plant species (Kumar and Reddy, 2011).

Direct and indirect shoot organogenesis and somatic embryogenesis are most commonly used micro-propagation techniques (Antony *et al*., 2014). Somatic embryogenesis in recent years has shown enormous potential in providing efficient clonal propagation system. The development of synthetic seed technology, plant transformation and their conservation systems are essential for large-scale clonal propagation of elite genotypes (Bomal and Tremblay, 2000; Bandyopadhyay and Hamill, 2000). However, plant regeneration through somatic embryogenesis is one of the main pre-requisites for potential use of clonal propagation in woody plants (Park *et al*., 1998). The phenomenon of somatic embryogenesis through *in vitro* callus development in orchids is rare as the success of callus formation is constrained due to its slow growth and tendency to become necrotic (Kerbauy, 1984). Callus formation via leaf and root explants of orchids is rare and does not succeed in all cases. Even when plant regeneration from callus of orchids has been achieved, it usually occurs via protocorm like bodies (Begum *et al*., 1994; Roy and Banerjee, 2003; Huana *et al*., 2004). Plant regeneration through somatic embryogenesis has many advantages over other routes of *in vitro* plant production and appears to be the most promising area of research for large scale production, rapid plant propagation and conservation. Therefore, the success of somatic embryogenesis *via* callus culture is interesting in plant regeneration of orchids. The present study was focused on somatic embryogenesis and adventitious shoot formation from leaf and root explants of *Rhynchostylis retusa* to develop efficient propagation and regeneration method for mass multiplication.

MATERIALS AND METHODS

Plant materials, inoculation, medium and incubation of cultures

The seeds of *Rhynchostylis retusa* (L.) were collected from Rajshahi, Bangladesh. The plants were grown through *in vitro* tissue culture systems. The young cotyledonous leaves and roots were cut into small pieces (approximately 1-2 cm) and then inoculated in sterile culture bottles containing 30 mL MS semi-solid medium (Murashige and Skoog, 1962). The MS medium was fortified with 3% (w/v) sucrose, 0.7% agar and with various combination and concentrations of 6-benzylaminopurine (BAP), 2, 4-dichlorophenoxyacetic acid (2,4-D), α-naphthaleneacetic acid (NAA) and kinetin (Table 1). The pH of medium was adjusted to pH 5.8 and medium autoclaved at 121ºC with 15 lb pressure for 15 min. Culture bottles were maintained at 25±2ºC with 16/8 h photoperiod using cool white florescent light in growth chamber. Sub-culturing was done after every 6 weeks of culture initiation. Observations were recorded at weekly interval upto 3 months. Each experiment was conducted in a completely randomized design and each treatment replicated three times. Leaf and roots derived calli were multiplied and maintained on same medium and sub-cultured every month.

Shoot and root development and plant regeneration

For multiple shoot formation, the induced adventitious shoot were transferred to MS basal medium containing different concentration and combination of BAP, 2,4-D, NAA and kinetin. The cultures were maintained at 25±2ºC, with 16 h photoperiod. For root induction, single shoots were separated from multiple shoot clusters and transferred to ½ strength MS medium supplemented with various

plant growth regulators, *viz.*, i) 0.5 mg L^{-1} IAA, ii) 0.5 mg L^{-1} NAA, iii) 0.5 mg L^{-1} IBA, iv) 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} NAA, v) 0.5 mg L^{-1} NAA + 0.5 mg L^{-1} IBA and vi) 0.5 mg L^{-1} IAA + 0.5 mg L^{-1} IBA. Somatic embryos were isolated and transferred to MS and ½MS basal medium with various concentrations of BAP for germination and cultures were incubated at 16 h photoperiod.

Hardening and acclimatization

Well rooted plants were removed from the culture tubes, washed with running tap water to remove agar and transplanted into plastic cups containing small brick pieces (5-10 g each) and wood charcoal (1:3) with coconut husk. The young plants were maintained under 30-40% natural light, sprayed with water on every 2nd day and fertilized with full-strength macro-elements of MS medium after every 5th day. The acclimatized plants were eventually transferred to pot. The data were recorded with respect to per cent embryo induction, somatic embryos and multiple shoot developed after 4 weeks of culture initiation. Each experiment was repeated thrice.

Statistical analysis

The data was subjected to analysis of variance (Gomez and Gomez 1984). The mean values were compared by least significant difference at 5% significance level using SPSS (version 16.0) for Windows 7.

RESULTS AND DISCUSSION

In present study direct somatic embryogenesis was induced from leaf and root explants of *Rhynchostylis retusa*. The explants (young leaf and root) when cultured on MS medium supplemented with different plant growth regulators showed highest response for direct somatic embryo induction in leaf (75%) and root (50.2%) after 25-30 days on MS medium supplemented with 1.0 mg L^{-1} BAP and 1.5 mg L^{-1} kinetin (Table 1). The results revealed that a combination of BAP and kinetin is essential for induction of somatic embryogenesis from leaf and root explants of *R. retusa*. Chung *et al.* (2007) reported that TDZ (thidiazuron) @ 0.3, 1.0 and 3.0 mg dm^{-3} induced 5-25% leaf tip segments in *in vitro* grown plants directly from embryos after 60 d of culture. In general, leaf explants produced significantly higher embryos than root explants. Somatic embryos were induced directly at the cut ends or surfaces of leaf and root explants (Fig. 1 & 2). The embryos were green, small and globular. The findings are in agreement with earlier reports on *Nothapodytes foetida*

Table 1: Effects of PGRs on embryos induction from leaf and root of *Rhynchostylis retusa*

Plant growth regulators (PGRs)	Concentrations of PGRs (mg L^{-1})	Embryo induction (%) from targeted explants	
		Leaf	Root
BAP + 2,4-D	0.5 + 1.0	40.00 ± 0.71^{cd}	0.00^{a}
	1.0 + 1.5	50.20 ± 0.73^{f}	45.40 ± 0.51^{e}
	1.5 + 2.0	38.80 ± 0.60^{c}	2.20 ± 0.33^{b}
	2.0 + 2.5	35.40 ± 0.33^{b}	0.00^{a}
BAP + NAA	0.5 + 1.0	0.00^{a}	0.00^{a}
	1.0 + 1.5	60.00 ± 0.84^{h}	45.00 ± 0.71^{e}
	1.5 + 2.0	40.80 ± 0.73^{d}	35.00 ± 0.45^{d}
	2.0 + 2.5	0.00^{a}	0^{a}
BAP + Kinetin	0.5 + 1.0	55.40 ± 0.51^{g}	0.00^{a}
	1.0 + 1.5	75.00 ± 0.71^{i}	50.20 ± 0.80^{f}
	1.5 + 2.0	45.00 ± 0.55^{e}	30.80 ± 0.66^{c}
	2.0 + 2.5	0.00^{a}	0.00^{a}

Values represent mean ± S.E. (standard error). Each treatment was repeated thrice. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$.

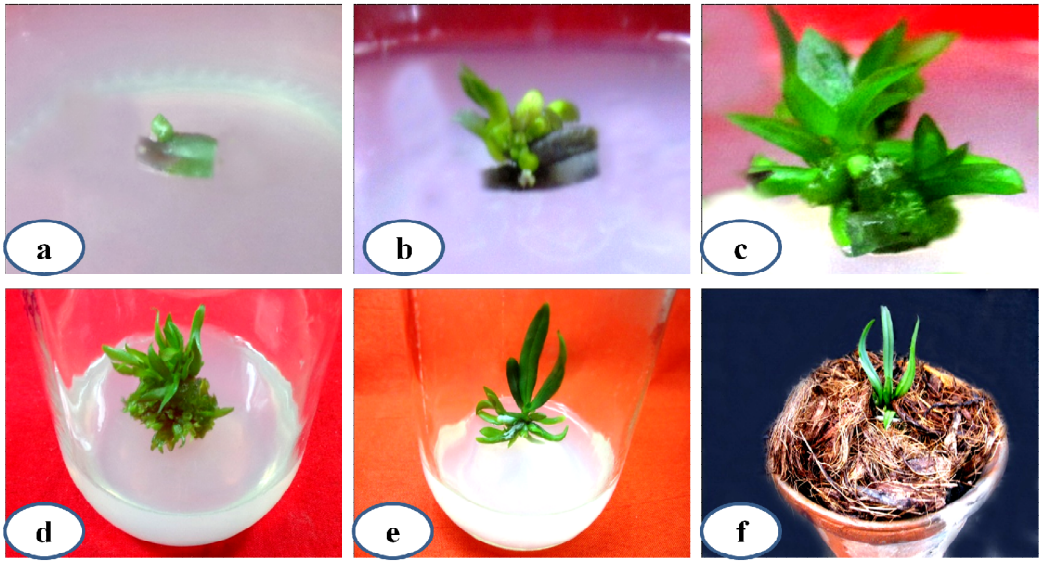

Fig. 1: Direct somatic embryogenesis from leaf explants of *Rhynchostylis retusa* and subsequent plant regeneration a) callus initiation from leaf surface; b) somatic embryos directly formed from the leaf surface; c-d) a cluster of embryos formed shoot; e) regenerated plantlet; f) acclimatized plantlet

(Khadke and Kuvalekar, 2013) and *Sapindus emarginatus* (Devaraju and Reddy, 2013). The frequency of somatic embryos was high when leaf explants was used. The somatic embryos were formed either individually or in groups directly on the cut ends or surface of explants without callus formation. Most of the globular embryos gradually developed as bipolar structures resembling heart, torpedo and cotyledon-stage embryos (Fig.1b; 2c). After 3-4 weeks of culture initiation, the cultures exhibited near-confluent growth which turned green with patches of developing embryos. The formation of somatic embryos around root segments was slower than leaf segment and more asynchronous. In another orchid *Oncidium* leaf tips reportedly showed highest ability to form embryos

Fig. 2: Direct somatic embryogenesis from root explants of *Rhynchostylis retusa* and subsequent plant regeneration; a-b) somatic embryos directly formed near the cut end of root; c) a cluster of somatic embryos directly formed vigorously from the cut end of root; d) a cluster of multiple shoot from embryo germination; e) regenerated plantlet; and f) acclimatized plantlets

Table 2: Effects of different concentration of BAP on germination of somatic embryos

Concentrations of BAP (mg L^{-1})	Germination (%) in media	
	MS medium (Mean ± S.E)	½ MS medium (Mean ± S.E)
0.5	0.00[a]	0.00[a]
1.0	35± 0.71[b]	42± 0.71[c]
1.5	50± 0.95[d]	60± 0.95[d]
2.0	30± 0.84[c]	35± 0.55[b]

'0' indicate no response. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$.

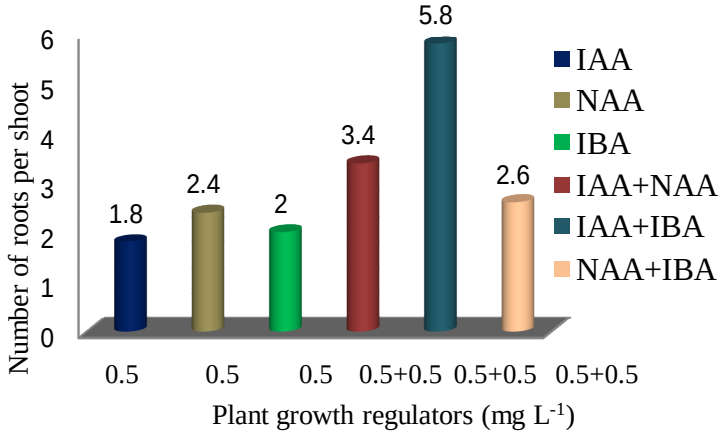

Fig. 3: Effects of different concentration and combination of PGRs with ½ MS medium on induction of roots

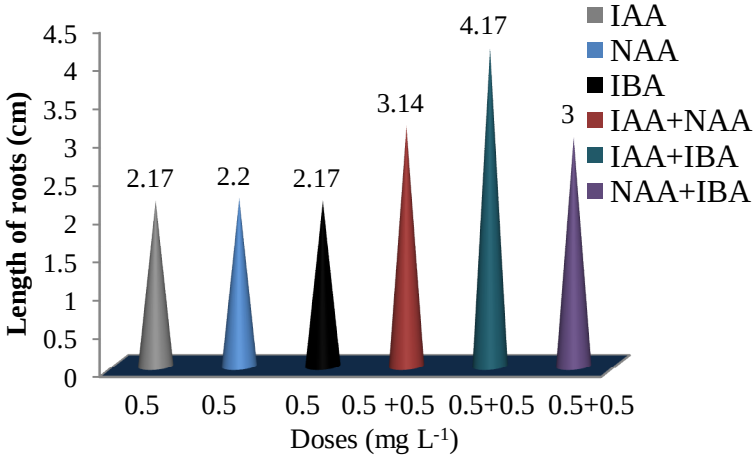

Fig. 4: Effects of different concentration and combination of PGRs with ½MS medium on root development

(Chen *et al*., 1999; Chen and Chang, 2001). Cordal *et al.* (2014) obtained somatic embryos directly from the cutting edge of blade and embryogenic callus (indirect way) from the midrib of *Phytolacca tetramera*.

The germination of embryos occurred within 3 weeks after the transfer of embryos onto MS and ½MS medium supplemented with varying concentrations of BAP (Fig. 1c, d; 2d). The highest germination frequency (60.0 ± 0.95) of somatic embryos was observed in ½MS medium supplemented with 1.5 mg L^{-1} BAP (Table 2). Adventitious shoot formation occurred 3 weeks after culture initiation. Plant regeneration with direct and indirect organogenesis from various explants sources of *N. foetida* have previously been reported (Thengane *et al*., 2001; Rai, 2002). Khadke and Kuvalekar (2013) reported almost similar results with medicinal plant *N. foetida*, a critically endangered plant species, and suggested that this system may prove beneficial for *ex situ* conservation as well as for amenability of this plant for genetic manipulation. For multiple shoot formation, the induced adventitious shoots were transferred to MS medium containing different concentration and combination of plant growth regulators. These developed into multiple shoot clumps with maximum number of multiple shoots (10.20 ± 0.58) and shoot length (3.80 ± 0.37) noticed on MS medium fortified with 1.5 mg L^{-1} BAP + 1.0 mg L^{-1} NAA as compared to all other combination and concentrations of BAP, NAA, IAA and kinetin (Table 3) .

To induce rooting, single shoots were separated from multiple shoot clusters (Fig. 1e; 2e) and transferred to rooting medium. Half strength MS medium supplemented with 3% sucrose + 0.5 mg L^{-1} IAA and 0.5 mg L^{-1} IBA showed best performance with regard to root formation (Fig. 3; 4). Well rooted plants were taken out and washed with sterile distilled water to remove agar and transplanted into plastic cups containing small brick piece (each 5-10 g) and wood charcoal (1:3) and coconut husk. The acclimatized plants were eventually transferred to their natural habitat. These

Table 3: Effects of different concentration and combination of PGRs on multiple shoot formation with shoot length of *R. retusa*

PGRs	Conc. of PGRs (mg L^{-1})	Average multiple shoots (No. explants^{-1})	Average length of shoots (cm)
BAP + NAA	0.5 + 0.5	2.80 ± 0.37^{a}	2.00 ± 0.31^{abc}
	1.0 + 0.5	4.80 ± 0.49^{b}	2.40 ± 0.51^{abc}
	1.5 + 1.0	10.20 ± 0.58^{d}	3.80 ± 0.37^{d}
	2.0 + 1.5	6.00 ± 0.55^{bc}	1.40 ± 0.24^{a}
BAP + kinetin	0.5 + 0.5	2.60 ± 0.39^{a}	1.60 ± 0.40^{a}
	1.0 + 0.5	6.20 ± 0.73^{c}	2.60 ± 0.24^{bc}
	1.5 + 1.0	5.00 ± 0.32^{bc}	2.20 ± 0.37^{abc}
	2.0 + 1.5	1.80 ± 0.37^{a}	1.40 ± 0.24^{a}
BAP + IAA	0.5 + 0.5	2.00 ± 0.31^{a}	1.40 ± 0.40^{a}
	1.0 + 0.5	5.40 ± 0.51^{bc}	2.40 ± 0.51^{abc}
	1.5 + 1.0	6.20 ± 0.58^{c}	3.00 ± 0.44^{cd}
	2.0 + 1.5	2.80 ± 0.37^{a}	1.60 ± 0.24^{a}

Values represent mean ± S.E. (Standard Error). Each treatment was repeated thrice. Values in a column with similar superscripts are not significantly different at $p \leq 0.05$.

plants depicted about 60% plants survival (Fig. 1f; 2f).

In present study we developed a simple and efficient protocol for induction of direct somatic embryogenesis of *R. retusa* 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, genetic transformation and advance biotechnological work (Chung *et al*., 2007).

Conclusion: The technique standardized may be useful in the re-establishment of *Rhynchostylis retusa* in its natural habitat and for large scale multiplication for other commercial uses. The technique is efficient, quick and highly reproducible and can be used in advance biotechnological research not only for this specie but also for the micropropagation of other orchids.

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