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EFFECTS OF PLANT GROWTH REGULATORS AND EXPLANTS ON PROPAGATION IN THE MONOPODIAL AND SYMPODIAL ORCHID: A STUDY *IN VITRO*

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Abstract

In vitro culture response of different plant growth regulators was assessed in two indigenous orchid species namely, *Dendrobium aphyllum* and *Rhynchostylis retusa* of two different growth groups *i.e.*, sympodial (*D. aphyllum*) and monopodial (*R. retusa*) orchid for optimum callus induction and plantlet regeneration. Leaf, nodal and inter-nodal segments of *in vitro* grown seedlings of both the species were cultured on MS (Murashige and Skoog, 1962) and PM (Phytamax Sigma Chemical Co., USA) media supplemented with different concentrations and combinations of plant growth regulators (PGRs). The inter-nodal segments of both the species and leaf segments of *D. aphyllum* failed to response to any of the PGR combinations, whereas, leaf and nodal segments of *R. retusa* and nodal segments of *D. aphyllum* gave positive response producing different types of calli as well as multiple shoot buds depending on nutritional stimulus. In subsequent subcultures, the callus tissues underwent differentiation *via* PLB formation on a broad spectrum of PGR supplemented media. The nodal explants of both the species produced multiple shoot buds. Thus both embryogenesis and organogenesis took place and PGR combinations played an important role in the differentiation of the tissues. High concentration of auxins and low concentration of cytokinins was proved to be effective for differentiation in *R. retusa,* such effects, however, could not be observed in *D. aphyllum*. The well-rooted *in vitro* plantlets were transferred to pots containing a potting mixture composed of saw dust, coconut coir, and coal pieces with ~90% survival in outside environment.

Introduction

DUE TO ruthless collection by increasing orchid lovers, over-exploitation for medicinal purposes, deforestation for urbanization, destruction of habitats by reclamation, shifting cultivation, killing of pollinators, and unauthorized trade has led to reduction in natural populations of the members of the family Orchidaceae, which is one of the largest and most diverse of all flowering plant families (Dressler, 1993; Hágsater and Dumont, 1996; Koopowitz and Hawkins, 2012; Seaton *et al.*, 2013; Swarts and Dixon, 2009). Meanwhile many orchid species have become extinct and many others are on the verge of becoming rare and endangered. Considering the present status of orchids, the family Orchidaceae as a whole was included in the CITES (Convention on International Trade in Endangered Species) Appendix II, hence, mass propagation, eco-rehabilitation and conservation of orchids are utmost necessary. Application of *in vitro* techniques might be the best solution for mass propagation and conservation of this versatile group of plants. Although some techniques have already been devised for propagation and conservation of orchids, further perfection of the protocols is still required for specific orchids. The major obstacles for mass propagation of orchids for commercial purposes as well as conservation are: 1) non availability of efficient and reliable protocols for seed germination; 2) poor understanding of early seedling growth and development; 3) obligate mycorrhizal association for natural seed germination; 4) selection of suitable explants for micropropagation, scaling-up and automation of the techniques; 5) very slow and laborious vegetative propagation; 6) species specificity to culture medium; 7) limited germination success under controlled laboratory conditions of many rare and endangered orchids species, and 8) high mortality of *in vitro* seedlings during transplantation. Modern biotechnological approaches such as tissue culture, production of synthetic seeds, cryopreservation are routinely used for mass propagation, genetic improvement as well as conservation of plant germplasm. Establishment of simple, reliable, economical, rapidly multiplying and highly reproducible protocol is very important for commercial cultivation and conservation of orchids. Development and deployment of new technologies is very important for improving rapid and mass propagation and conservation of orchids. The modification of traditional tissue culture medium by adding specific plant growth regulators (PGRs), different complex additives (peptone, yeast extract, banana pulp *etc.*), automation of plant production through adapting bioreactor system and culture conditions are required for development of efficient germination and micropropagation protocols. Recently, orchids have become the center of attention of new areas of research, including genetic

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engineering, functional genomics, proteomics, and metabolomics, all of which require standardized micropropagation techniques. The successful application of the new approaches will help in further improvement of orchids and orchid products. Such research is very important in the context of conservation of plant biodiversity.

Based on the growth pattern, orchids have been classified into two major groups *viz.*, i) Monopodial, and ii) Sympodial. The first group is characterized by a single unbranched axis of growth. On the other hand, multibranching rhizome or stem with axillary shoots exhibit the latter group. These two different growth patterns may be the result of differences in inherent endogenous hormonal levels and their functions. The application of exogenous plant growth regulators plays an important role on tissue differentiation and it depends on the nature of species, explant and, concentration and combinations of PGRs. Considering these, the present investigation was undertaken with a view to studying 1) the responses of a monopodial and a sympodial orchid to tissue culture; 2) explant-PGR interaction in the process of organogenesis and embryogenesis, and 3) establishment of simple, reliable, economical, rapidly multiplying and highly reproducible protocol for *Dendrobium aphyllum* and *Rhynchostylis retusa.*

Materials and Methods

Explants, Nutrient Media and Culture Conditions

Four months old undehisced green capsules of *Dendrobium aphyllum* and *Rhynchostylis retusa* were collected from the hilly forest of Cox-S-Bazar district (200 m above mean sea level) of Bangladesh. Two different nutrient media namely, MS (Murashige and Skoog, 1962) and PM (Phytamax®, Catalog No. P-6793, Sigma Chemical Co., USA) supplemented with 2–3% (w/v) sucrose and with or without peptone (2.0 gl^{-1}) were used for seed culture. Nodal, inter nodal and leaf segments (0.5–1.0 cm in size) of *in vitro* raised seedlings were used for further experiments. MS or PM medium fortified with different concentrations and combinations of auxins *i.e.*, IAA (0.5 – 2.5 mgl^{-1}), NAA (1.0–2.5 mgl^{-1}), Picloram (pic; 0.5–2.0 mgl^{-1}) and Cytokinins *i.e.*, Kinetin (0.5 – 2.5 mgl^{-1}), and Zeatin (ZN; 1.0 – 1.5 mgl^{-1}). The pH of the medium was adjusted at 5.8 before autoclaving at 121°C at 117 kPa for 20 min. Different types of glass vessels including test tubes (1.5 × 15 cm), culture bottles, conical flasks (100–150 cc) were used. Culture vessels with inoculated explants were maintained in a culture room where a cycle of 14/10 h light-dark at 60 $mmolm^{-2}s^{-1}$ provided by cool white fluorescent lamps (Philips Truelight 36w/86 65001 K B7, Philips, India), and 60% RH at 25 ± 2°C. Regular subculturing was done at 20–25 days interval.

Establishment of Axenic Culture

The capsules were rubbed with a hair brush under running tap water to remove dust particles and then surface sterilized by 0.1% (w/v) $HgCl_2$ solution for 10 min with occasional agitation and washed thoroughly with sterile distilled water. Finally, the capsules were dipped in 70% ethanol for 1 min. followed by flaming for 1–2 sec. The surface sterilized capsules were placed on a sterile filter paper and cut longitudinally with a sterile surgical blade and the seeds were cultured on the surface of the agar-gelled medium. All the operations were performed in a laminar air-flow cabinet. When seeds germinated and protocorms came out, these were taken out aseptically from the culture vessels and the masses of protocorms were sub-cultured to fresh culture media for further growth. The nodal, inter nodal and leaf segments of *in vitro* grown seedlings (5-6 cm in size) were used for micropropagation as well as study the effects of different PGRs on *in vitro* morphogenesis of the two different growth groups *i.e.*, monopodial and sympodial orchids. The callus, protocorm-like bodies (PLBs) or shoot buds developed from the cultured explants were subcultured regularly to fresh nutrient media.

Rooting and Transplantation of Seedlings

Seedlings grown in *in vitro* culture conditions exhibited fewer roots, which may not support successful acclimatization on their transfer to *ex vitro* conditions. On the other hand, shoot buds that produced from nodal explants did not produce any roots. Thus, for induction of stout root system these were grown on different rooting media made up of half strength PM medium supplemented with IAA (0.5–1.0 mgl^{-1}). The well-rooted plantlets were taken out from the culture vessels and washed thoroughly under running tap water for removal of agar medium attached to the root surface and transferred to pots containing a potting mixture of saw dust, coconut coir, and coal pieces at 1:1:2 (w/w).

Data Collection and Statistical Analysis

The experiments were designed following Complete Randomize Block Design (CRD). Five replicates were taken per treatment. The effects of different PGRs in induction of callus, shoot buds, PLBs and roots in the *in vitro* experiments were tested applying Duncan's multiple range test ($P > 0.5$) in one way ANOVA. The

statistical analyses were performed using the Statistica ver. 7 (Statsoft, Tulsa, USA). The experiments were repeated thrice.

Results and Discussion

Germination of Seeds

The seeds of *D. aphyllum* and *R. retusa* germinated on both MS and PM media. Maximum seed germination (97%) of *D. aphyllum* was recorded in PM medium (Fig. 1A) whereas seeds of *R. retusa* preferred MS medium for best seed germination (95%) (Fig. 1B). Species-specific media for seed germination have been reported in orchids (cf. Arditti and Ernst, 1984; Pathak *et al.,* 2001). Different nutritional recipes have been suggested by workers in various orchid species such as *Acampe papillosa* (Piri *et al.,* 2013), *Aerides multiflora* (Pathak *et al.,* 2005), *Aerides multiflora*, *Rhynchostylis retusa*, *Saccolabium calceolare* and *Vanda testacea* (Vij *et al.*, 1981), *Cymbidium aloifolium* (Hossain *et al.*, 2009), *Cymbidium elegans* (Sharma and Tandon, 1990), *Cymbidium giganteum* (Hossain *et al.*, 2010), *Cymbidium iridioides* (Jamir *et al.*, 2002), *Cymbidium macrorhizon* (Vij and Pathak, 1988), *C. pendulum* (Pathak and Vij, 2007), *Dactylorhiza hatagirea* (Vij *et al.,* 1995), *Dendrobium aphyllum* (Hossain *et al.,* 2013) *Dendrobium chrysanthum* (Anuprapha and Pathak, 2012), *Dendrobium farmeri*, *D. primulium*, *D. moschatum*, and *D. fimbriatum* var. *oculatum* (Devi *et al.*, 1990), and *Gastrochilus calceolaris* (Pathak *et al.*, 2011), *Goodyera biflora* (Pathak *et al.,* 1992), *Satyrium nepalense* (Chauhan *et al.,* 2010), and *Vanda coerulea* (Aggarwal *et al.,* 2008). Seed germination occurred within 4–5 wks and developed seedlings in subsequent subculture on the same medium (Fig. 1C-D).

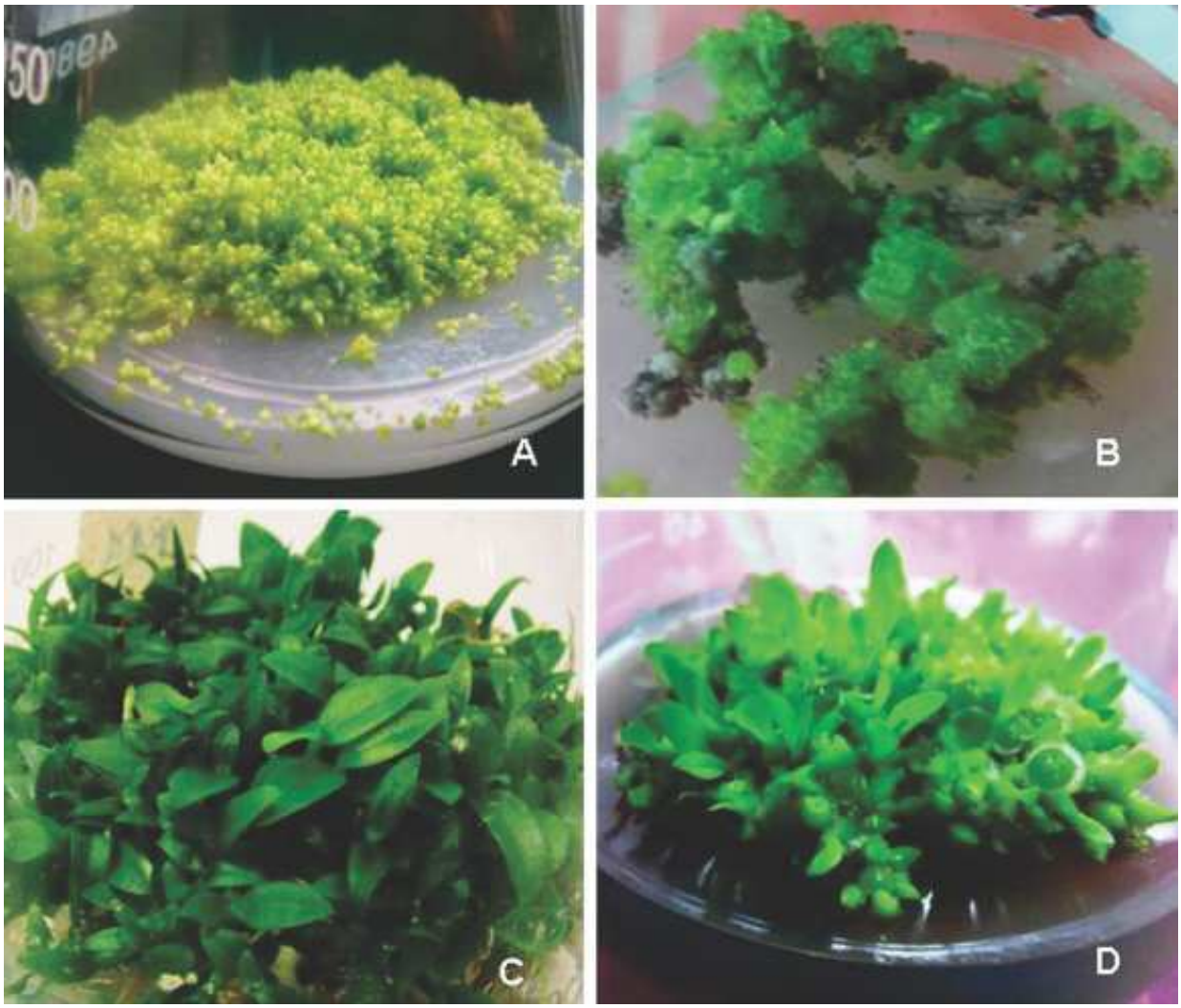

Fig. 1. A-D. Seed germination in *D. aphyllum* and *R. retusa*: A, Germination of seeds of *D. aphyllum* on PM medium; B, Germination of seeds of *R. retusa* on MS medium; C, Development of seedlings of *D. aphyllum* and *R. retusa,* respectively.

Table1. Response of different explants of *R. retusa* and *D. aphyllum* to different PGRs and their combinations.

Species	Basal Medium	Explants	IAA (1.5 mgl^{-1})	NAA (1.5 mgl^{-1})	pic (1.5 mgl^{-1})	BAP (1.5 mgl^{-1})	ZN (1.5 mgl^{-1})	KN (1.5 mgl^{-1})	BAP (1.5 mgl^{-1}) + IAA (2.5 mgl^{-1})	IAA (2.5 mgl^{-1}) + ZN (0.5 mgl^{-1})	IAA (1.5 mgl^{-1}) + KN (2.5 mgl^{-1})	NAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1})	NAA (2.5 mgl^{-1}) + ZN (1.0 mgl^{-1})	NAA (1.5 mgl^{-1}) + KN (1.5 mgl^{-1})	pic (1.5 mgl^{-1}) + BAP (1.5 mgl^{-1})	pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1})	Pic (1.5 mgl^{-1}) + KN (1.5 mgl^{-1})
Rhynchostylis retusa -	MS	LS	C	C	C	-	C	-	-	-	-	-	PLBs	-	-	PLBs
	NS	C	-	C	-	-	-	-	MSB	-	MSB	MSB	-	-	MSB	-
	IS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PM	LS	C	C	C	-	C	-	-	-	-	-	PLBs	-	-	PLBs	-
		NS	C	-	C	-	-	-	-	MSB	-	MSB	MSB	-		MSB	-
		IS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dendrobium aphyllum -	MS	LS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	NS	-	-	C	C	-	-	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB
	IS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	PM	LS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
		NS	-	-	C	C	-	-	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB	MSB
		IS	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Results are based on observations recorded from 15 culture vessels; LS , Leaf segment; NS, Nodal segment; IS, Inter-nodal segment; C,Callus; MSB, Multiple shoot buds; PLBs, Protocorm like bodies.

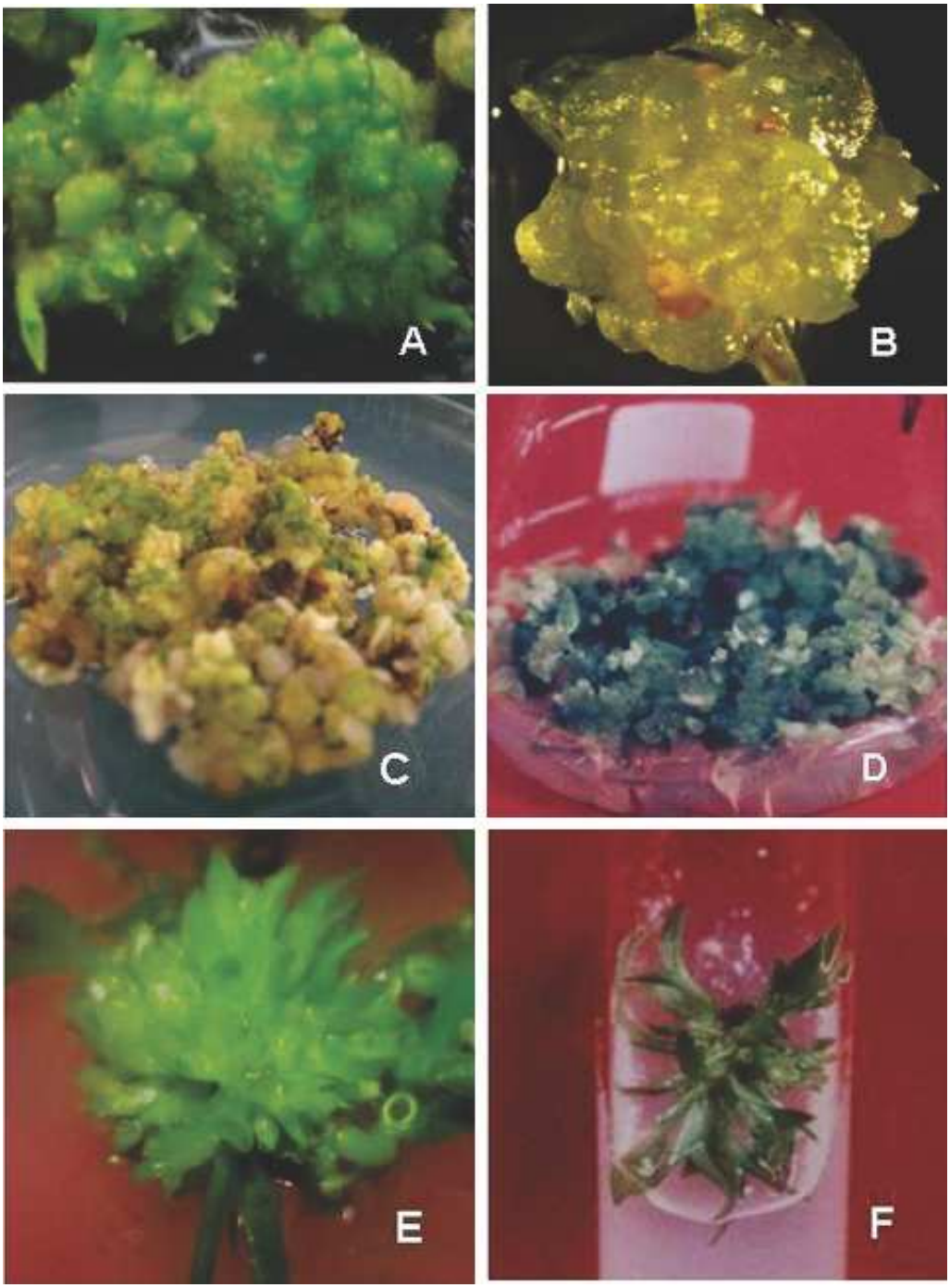

Fig. 2. A-F. *In vitro* propagation of *D. aphyllum* and *R. retusa* using leaf and nodal segments: A, Induction of PLBs in leaf segments of *R. retusa*; B-C, Induction of green, compact callus and loose friable callus in leaf segments of *R. retusa*; D, Green and compact callus differentiated into PLBs; E-F, Induction of multiple shoot buds in nodal segments of *R. retusa,* and *D. aphyllum* respectively.

Effects of PGRs on In Vitro Differentiation

Different explants *i.e.*, leaf, nodal and inter-nodal segments of *in vitro* grown seedlings of the two species of different growth types gave differential response to the different PGR concentrations and combinations (Table1). The effects of PGRs on different explants are briefly described below:

Effects of PGRs on Leaf Explants

The response of leaf explants of the two species was quite different in same PGRs supplemented media. In case of *R. retusa,* the leaf segments produced different

Table 2. Effects of subculturing of loose and friable callus tissues on broad spectrum of different PGRs supplemented media.

Basal medium	PGRs	Nature of response		Time (days) required for induction of shoot buds	
		R. retusa	*D. aphyllum*	*R. retusa*	*D. aphyllum*
MS	NAA (2.0 mgl^{-1}) + BAP (2.5 mgl^{-1})	Callus multiplied without differentiation	Shoot bud formation	_	22-25
	IAA(2.5 mgl^{-1}) + BAP (1.5 mgl^{-1})	Shoot bud formation	Shoot bud formation	42-46	32-35
	pic (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1})	Callus multiplied without differentiation	Shoot bud formation	-	2325
	IAA (2.0 mgl^{-1}) + ZN (0.5 mgl^{-1})	Shoot bud formation	Callus multiplied without differentiation	40-42	-
	NAA (2.0 mgl^{-1}) + ZN (1.0 mgl^{-1})	PLB formation	Callus multiplied without differentiation	45-50	-
	pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1})	PLB formation	Shoot bud formation	45-48	30-35
PM	NAA (2.0 mgl^{-1}) + BAP (2.5 mgl^{-1})	Callus multiplied without differentiation	Shoot bud formation	_	20-22
	IAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1})	Shoot bud formation	Shoot bud formation	42-46	30-32
	pic (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1})	Callus multiplied without differentiation	Shoot bud formation	_	25-27
	IAA (2.0 mgl^{-1}) + (ZN 0.5 mgl^{-1})	Shoot bud formation	Callus multiplied without differentiation	35-38	-
	NAA(2.0 mgl^{-1} + ZN (1.0 mgl^{-1})	PLB formation	Callus multiplied without differentiation	40-42	-
	pic (2.0 mgl^{-1}) + (ZN 1.5 mgl^{-1})	PLB formation	Shoot bud formation	45-48	30-34

types of callus tissue as well as PLBs depending on the PGRs. While the leaf segments of *D. aphyllum* did not give any response to any of the media combinations used. Moreover, it became brown within 3-4 wks of culture and finally died. Induction of PLBs *i.e.*, direct embryogenesis took place in *R. retusa* when the leaf segments were grown on MS or PM medium fortified with i) NAA (2.5 mgl^{-1}) + ZN (1.0 mgl^{-1}) and ii) pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1}) (Fig. 2A). Based on the study of the effects of different PGRs *i.e.*, auxins (NAA, IAA, IBA, 2, 4-D) and cytokinins (ZN, KN, BAP, TDZ) *in vitro*, it was documented that PGRs activate the proliferative loci of the leaf segments and regulate subsequent development into plantlets (Arditti, 2008; Vij and Pathak, 1990). Cytokinins have been found to be essential for regeneration from leaf explants in *Acampe praemorsa* (Nayak *et al.*, 1997) and *Aerides maculosum* (Murthy and Pyati, 2001). The embryo formation on leaf explants was retarded by auxins IAA, IBA, NAA, and 2, 4-D but promoted by cytokinins like 2iP, ZN, KN, BAP and TDZ (Chen and Chang, 2001). Chen and Chang (2004) tested the effect of auxins

Table 3. Rooting response in shoot buds of *D. aphyllum and R. retusa.*

Culture medium	Number of roots/shoot buds (mean ± S. E.)		Length of roots (cm) after 30days of culture (mean ± S. E.)	
	D. aphyllum	*R.retusa*	*D. aphyllum*	*R. retusa*
PM	2.60 ± 0.16^{c}	2.70 ± 0.15^{c}	2.52 ± 0.12^{e}	2.50 ± 0.12^{e}
½PM	3.00 ± 0.26^{c}	2.90 ± 0.10^{c}	2.80 ± 0.15^{de}	2.88 ± 0.16^{cde}
½PM + IAA(0.5 mgl^{-1})	5.12 ± 0.29^{a}	5.10 ± 0.25^{a}	4.44 ± 0.16^{a}	4.33 ± 0.10^{a}
½PM + IAA(1.0 mgl^{-1})	4.20 ± 0.25^{b}	4.10 ± 0.31^{b}	4.57 ± 0.26^{a}	4.79 ± 0.23^{a}

Mean values within a column followed by the same letters are not significantly different at $P=0.05$ according to Duncan's multiple range test.

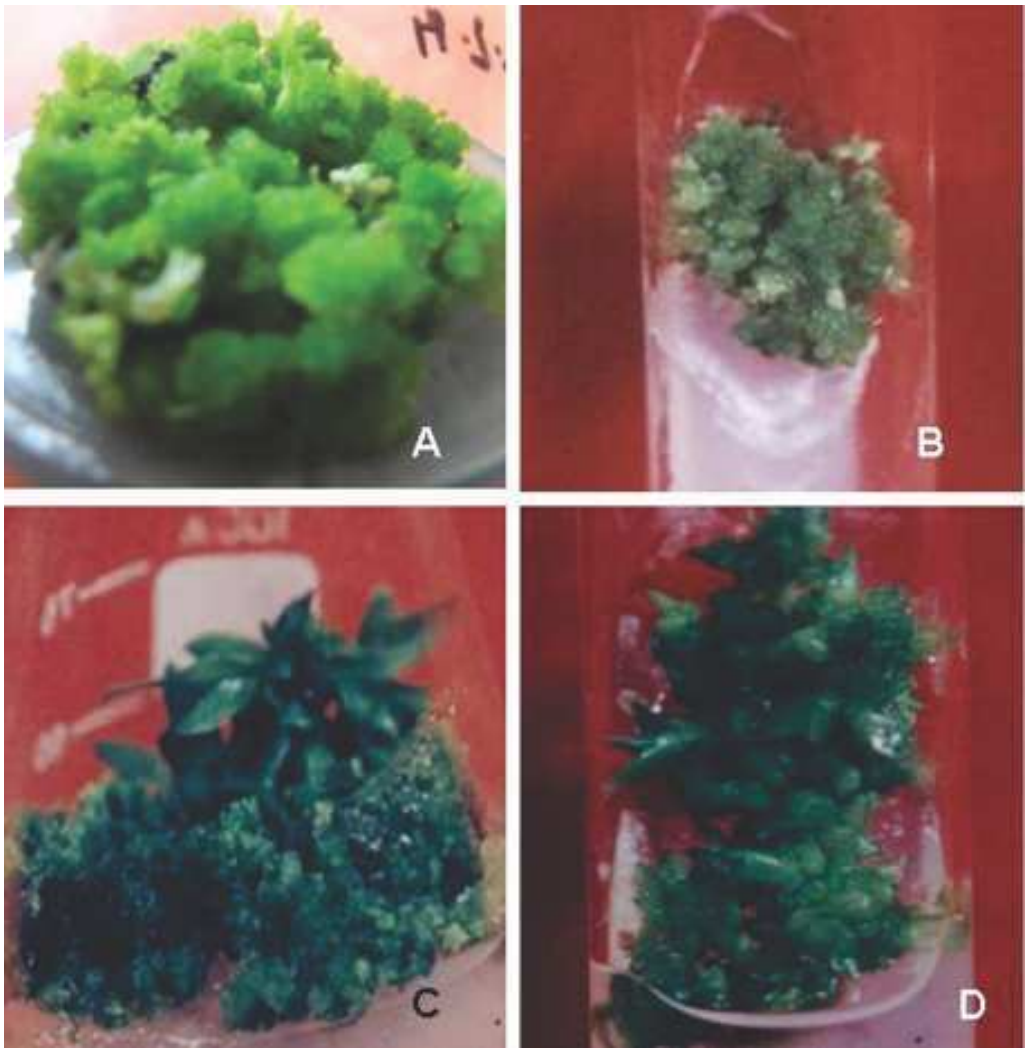

Fig. 3. A-D. *In vitro* propagation of *D. aphyllum* and *R. retusa* using nodal segments: A- B, Induction of green, compact callus in nodal segments of *R. retusa* and *D. aphyllum* respectively; C-D, Lose and friable callus differentiated into PLBs when grown in broad spectrum of PGRs in *R. retusa* and *D. aphyllum.* respectively.

(IAA, 2, 4-D), two auxin transport inhibitors (TIBA and quercetin) and an auxin antagonist (PCIB) on direct somatic embryogenesis from leaf tip region. Except for TIBA, all the other growth regulators retarded embryo formation. Beneficial effect of using combination of auxins and cytokinins has been demonstrated in *Oncidium* (Chen and Chang, 2000), *Renanthera imschootiana* (Seeni and Latha, 1992), *Rhynchostylis retusa* (Vij *et al.*, 1984), *Vanda coerulea* (Seeni and Latha, 2000), *Vanda* hybrid (Mathews and Rao, 1985), and *Vanda spathulata* (Decruse *et al.*, 2003).

The leaf explants also produced different types of callus tissues depending on the PGRs (Table 1). Green and compact callus were produced in medium containing i) IAA (1.5 mgl^{-1}) and ii) NAA (1.5 mgl^{-1}) (Fig. 2B), while loose and friable callus were produced in medium containing i) pic (1.5 mgl^{-1}) and ii) Zn (1.5 mgl^{-1}) (Fig. 2C). After three subsequent subcultures, the green and compact callus differentiated into PLBs (Fig. 2D). On the other hand, loose and friable callus was, however, failed to undergo either organogenesis or embryogenesis but proliferated without differentiation. Along with the PGRs, orientation of explants on the media, physiological age of leaf and source of leaf are crucial factors for regeneration *in vitro*. The available reports of the physiological age of explants indicated that young leaves respond better than the older ones with respect to the number of regenerants developed upon inoculation in a suitable medium (Chugh *et al.*, 2009; Chung *et al.*, 2005; Pathak and Vij, 2001; Vij and Pathak, 1990; Vij *et al.*, 1986). Available reports affirmed that young leaves show better response in *Vanda* Kasem's Delight Tom Boykin (Vij *et al.*, 1994) and *Vanda coerulea* (Vij and

Agarwal, 2003). Tenjensangba and Deb (2005) reported that young leaves (15 weeks old) of *Cleisostoma racimeferum* develop PLBs *in vitro* while older leaves were unable to regenerate.

Effects of PGRs on Nodal Explants

The response of the nodal segments of the two different species to different PGRs and their combinations also differed highly as leaf segments. The nodal segments of *R. retusa* produced multiple shoot buds on i) IAA (2.5 mgl^{-1}) + ZN (0.5 mgl^{-1}), ii) NAA (2.5 mgl^{-1}) + ZN (1.0 mgl^{-1}), iii) NAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1}), and iv) pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1}). But the average number of multiple shoot buds induced per explant varied in different PGR combinations. The maximum number of multiple shoot buds per explant was recorded on MS + sucrose [2% (w/v)] + IAA (2.5 mgl^{-1}) + ZN (0.5 mgl^{-1}) (Fig. 2E). This finding indicates that high concentration of auxin and low concentration of cytokinin enhanced multiple shoot bud formation in nodal segments of *R. retusa*. On the other hand, the nodal segments of *D. aphyllum* also underwent direct organogenesis producing multiple shoot buds on a number of media compositions used (Table 1) and the maximum number of multiple shoot buds were produced in PM + IAA (1.5 mgl^{-1}) + BAP (2.5 mgl^{-1}) (Fig. 2F). This finding indicates that low concentration of auxin and high concentration of cytokinin enhanced multiple shoot bud formation in nodal segments of *D. aphyllum*. The above findings clearly indicated that the nature and magnitude of the requirement of PGRs is different for monopodial and sympodial orchids. A number of earlier reports demonstrated that, the combinations, concentrations,

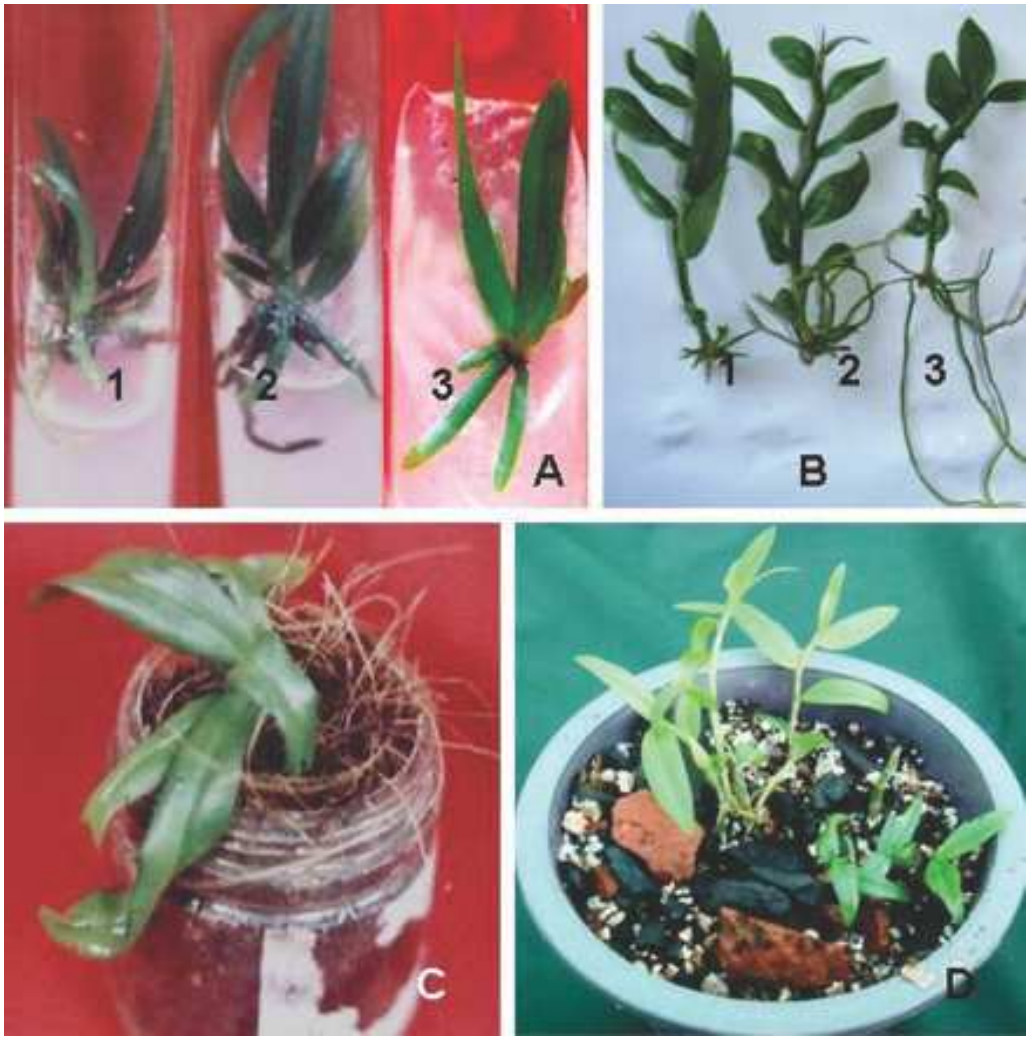

Fig.4. A-D. Root induction and seedling development in *D. aphyllum* and *R. retusa*: A-B, Induction of stout root system in the multiple shoot buds as well as PLB derived seedlings of *R. retusa* and *D. aphyllum* respectively (1 = ½PM medium, 2 = ½PM + 0.5 mgl^{-1} IAA and 3 = ½PM + 1.0 mgl^{-1} IAA); C-D, Establishment of *in vitro* grown seedlings of *R. retusa* and *D. aphyllum* in outside pots, respectively.

and the ratio of exogenous PGRs supplements are critically important for morphogenetic response in orchids (Begum *et al.*, 1994; Chang and Chang, 1998; Deb and Pongener, 2012; Hossain *et al.*, 2013a; Huan *et al.*, 2004; Mahendran and Bai, 2012; Malabadi *et al.*, 2008; Teixeira da Silva *et al.*, 2006, 2007a, b ; Teng *et al.*, 1997; Vij *et al.,* 1994)

The nodal segments of both the species also produced different types of callus tissues in some of the PGRs combinations (Table. 2). In case of *R. retusa* green and compact callus were induced in i) IAA (1.5 mgl^{-1}) and ii) Pic (1.5 mgl^{-1}) (Fig. 3A). While, in case of *D. aphyllum* green and compact callus were induced in i) BAP (1.5 mgl^{-1}) and ii) pic (1.5 mgl^{-1}) (Fig. 3B). These findings demonstrated that for induction of green callus in *R. retusa* needs exogenous supply of both auxin / cytokinin while *D. aphyllum* needs only cytokinins. After three subsequent subcultures, the green and compact callus differentiated into PLBs. Thus, indirect embryogenesis was observed. This type of response was not only due to exogenous supply of hormones but also dependent on the endogenous level of hormones. The inter-nodal segments of both the species did not give any response to any one of the media used for leaf and node culture. Thus the overall results indicated that different explants of the same species and the same explants of the two species gave different response depending on various PGRs and their combinations. Appropriate combination of cytokinins with auxins was critically important in induction of somatic embryos or PLBs in orchids as also reported earlier by some workers (Huan *et al.*, 2004; Malabadi *et al.*, 2008; Roy and Banerjee, 2003; Teixeira da Silva *et al.*, 2005, 2006, 2007a, b; Teng *et al.*, 1997). PLB production is comparatively more efficient than organogenesis, easy to carry out, and can provide large number of propagules for mass propagation within a short period of time (Hossain *et al.*, 2010).

Culture of Loose and Friable Callus

As mentioned earlier, the explants in both the species produced loose and friable callus in some of the PGR combinations and those failed to undergo differentiation, proliferated profusely. For induction of organogenesis or embryogenesis, these callus tissues were further grown in broad spectrum of PGRs supplemented media (Table 2). The callus of *R. retusa* differentiated into PLBs when grown in i) IAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1}) and ii) IAA (2.0 mgl^{-1}) + ZN (0.5 mgl^{-1}) iii) NAA (2.0 mgl^{-1}) + ZN (1.0 mgl^{-1}) and iv) pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1}) (Fig. 3C). On the other hand, the callus of *D. aphyllum* produced multiple shoot buds in i) NAA (2.0 mgl^{-1}) +BAP (2.5 mgl^{-1}), ii) IAA (2.5 mgl^{-1}) + BAP (1.5 mgl^{-1}), iii) pic (1.0 mgl^{-1}) + BAP (2.0 mgl^{-1}), and iv) pic (2.0 mgl^{-1}) + ZN (1.5 mgl^{-1}) (Fig. 3D). These findings indicated that the concentrations and combinations of PGRs switched the process of differentiation. The comparative results of *in vitro* culture based on the growth pattern of the two species showed remarkable differences. High concentration of auxins and low concentration of cytokinins proved to be effective for differentiation in monopodial orchid, *R. retusa* but such observations, however, could not be made in sympodial orchid *D. aphyllum*.

Rooting and Acclimatization of Plantlets

For induction of stout root system, the multiple shoot buds as well as PLB derived seedlings were grown on different rooting media. Half strength agar solidified PM medium fortified with IAA (0.5-1.0 mgl^{-1}) were used for this purpose. Medium fortified with IAA (0.5 mg l^{-1}) proved to be most effective for induction of well developed root system for both MSBs (>4/MSB) and seedlings (>5/seedling) (Table 3; Fig. 4A, B). The shoot buds or seedlings also produced roots in IAA (1.0 mgl^{-1}) containing combination but those roots were very thin and long, making them fragile and prone to damage during *ex vitro* transfer. PM medium without any PGRs produced a few stunted roots per MSB or seedling. It is pertinent to mention here that roots developed in PLB sourced seedlings were stronger and healthier than those developed in MSBs. Well-rooted plantlets were then transferred to the greenhouse with 90% and 92% survival in *D. aphyllum* and *R. retusa* respectively (Fig. 4C, D). Induction of healthy root system in *in vitro* plantlets is very important for their survival in outside environment. Root development is an innate nature of plants which is controlled by endogenous level of hormones (Jarvis, 1986). Hossain *et al*. (2013 a, b) reported that scarcity of nutrition ions in the culture medium could enhance root induction *in vitro*, most probably to explore nutrient ions and water from the medium. In *in vitro* conditions, addition of exogenous hormone (auxins) to the medium enhances rooting response (Hossain *et al.*, 2013 a, b). Stimulatory effects of IAA on rooting were also reported in some orchids (Das *et al*. 2007; Hossain *et al.*, 2010). The present study suggested that combined effects of deprived nutrition and additional presence IAA enhanced the development of stout root system in *D. aphyllum*.

Conclusion

The results indicated that the two species of the two different growth groups *i.e.,* monopodial and sympodial, differed highly in terms of their response in tissue

culture. The type of explants and the PGR supplements were found to be equally important for regeneration purpose. Both embryogenesis and organogenesis were induced but the kind of differentiation was species, PGR and explant dependent.

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