Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(4): 179-184
Received: 25-05-2020
Accepted: 27-06-2020

Tapash Kumar Bhowmik
Assistant Professor
Department of Botany
University of Chittagong
Chittagong, Bangladesh

Md. Mahbubur Rahman
Professor, Department of Botany
University of Chittagong
Chittagong, Bangladesh

Corresponding Author:
Tapash Kumar Bhowmik
Assistant Professor
Department of Botany
University of Chittagong
Chittagong, Bangladesh

In vitro study of medicinally important orchid *Aerides multiflora* Roxb. from nodal and Leaf explants

Tapash Kumar Bhowmik and Md. Mahbubur Rahman

Abstract
An efficient procedure described for mass propagation of *Aerides multiflora* Roxb. using *in vitro* sourced nodal and leaf explants. Regeneration response varied with the explants, nutrient compositions and PGRs. Nodal segments responded the best than leaf segments. *In vitro* grown nodal segments directly produced highest average number of multiple shoot buds (8.83 ± 0.45/segment) *via* organogenesis sprouted on MS medium with 1.0 mg/l NAA + 2.0 mg/l BAP followed by (8.65 ± 0.54/segment) MS + 0.5 mg/l NAA + 1.0 mg/l BAP. Leaf segments underwent proliferation *via* embryogenesis and produced maximum per cent of greenish PLBs on MS medium with 1.0 mg/l IAA + 2.0 mg/l BAP followed by MS + 1.0 mg/l NAA + 2.0 mg/l BAP. Highest increased individual shoot bud length was achieved on agar solidified MS with 1.0 mg/l NAA + 1.0 mg/l BAP (2.62 ± 0.14 cm) followed by liquid MS + 1.0 mg/l IAA + 1.0 mg/l BAP (2.53 ± 0.11 cm) medium. Elongation of shoot bud was better in solidified media than liquid condition and MS was superior than PM. Increase in length as well as the number of roots developed MSBs derived seedlings were best responses on agar solidified MS with 1.0 mg/l IBA (4.32 ± 0.24 cm/shoot bud and 3.19 ± 0.22 no/shoot bud) followed by MS + 0.5 mg/l IAA (3.91 ± 0.23 cm/shoot bud and 3.01 ± 0.16 no/shoot bud). The plantlets were hardened *in vitro* for 3-4 wks prior to transfer in green house.

Keywords: *Aerides multiflora*, medicinal orchid, MSBs, PGRs, PLBs

1. Introduction
The Orchids are known for the complexity and captivating beauty of their highly colourful and long lasting flowers of diverse shapes and sizes and are considered as doyens among the ornamentally significant plants. Application of tissue culture techniques has added new possibilities in plant breeding and propagation. Mass multiplication of superior plants for cut flower production and establishment of plants in a compressed time frame are now within the realms of reality [1]. Orchids represent the first horticultural crop propagated through tissue culture technique [2]. Orchidaceae is the second largest family among the flowering plants consisting of 35000 species [3]. In Bangladesh, orchids are naturally grown in Chittagong, Chittagong Hill Tracts, Cox's Bazar, Greater Sylhet, Gazipur and Sundarbans mangrove forest [4] and 187 orchid species in Bangladesh [5].

Aerides multiflora Roxb. is monopodial aromatic orchid grown as a tree trunk epiphyte of Bangladesh. Flowering time May-June [6]; distributed in Bangladesh, India, Malaysia, Philippines and Thailand [7]. This species has horticultural potential for its attractive colourfull lip [8]. *A. multiflora* is used to treat wounds in India [9] and leaf paste is also applied to cuts and wounds in Nepal [10-11], whereas powdered leaf constitutes a tonic [12]. Epiphytic Pseudobulb paste is used to treat fractured and dislocated bones [10] and tubers exhibit an antibacterial effect *in vitro* [13-14].

At present, our scientists are successfully grown various orchids for commercial purposes by *in vitro* micropropagation technique [15]. Micropropagation protocols have been developed for several species and hybrids [16]. Tissue culture technique has been widely used for the *in vitro* mass propagation of several commercially and medicinally important orchids [17-18]. Natural populations are progressively on decline because of habitat destruction pressures due to indiscriminate collections by orchid lovers, habitat destruction, over exploitation by orchid lovers and medicinal purposes are the main factors [8]. *A. multiflora* is a high demanding orchid species in the world cut flowers market and need to be conservation by *in vitro* micropropagation techniques. The aim has been to develop an efficient and reproducible micropropagation protocol from *in vitro* developed nodal and leaf explants.

2. Materials and Methods

MS [19] with different concentrations and combinations of PGRs were used for prepared sixteen types of micropropagation media. Eighteen types of solid & liquid elongation media were prepared using with different concentrations and combinations of PGRs based on full strength MS [19] and PM [20] medium. 0.8% (w/v) agar was also used in solid media but in liquid media no agar was added. Half strength MS0 and nine types of full strength auxin supplemented MS media were prepared for well-developed root system. The pH of the medium was adjusted to 5.8 with 0.1N NaOH or before mixing agar. Agar (0.8%, w/v) was added as gelling agent. Agar was dissolved by boiling the mixture and about 50-100 ml media was dispensed into different sizes each culture vessel and autoclaved at 121ºC for 15 min/15 lb inch for 20-25 min. The cultures were maintained in a growth room at 14/10h continuous light and dark conditions illuminated with fluorescent tube of 2000-3000 lux at 25 ± 2 ºC.

The *in vitro* developed seedlings of *A. multiflora* were used for micropropagation. *In vitro* grown seedlings were placed on sterile tile in the laminar air flow cabinet and cut 0.5 to 1.0 cm size nodal and leaf segments using sterilized surgical blade and forceps. Then the cutting were put into the culture vessel containing 0.8% (w/v) agar solidified MS and PM based micropropagation media supplemented with different concentration and combinations of auxins and cytokinins.

Nodal segments produced directly multiple shoot buds (MSBs) *via* organogenesis those were subcultured in the elongation media and thereafter in rooting media for induction of well-developed root system. But leaf segments proliferated and produced Protocorm Like Bodies (PLBs) which were subcultured on the elongation media and thereafter in rooting media for induction of well-developed root system. Half strength MS0 with 1.5% (w/v) sucrose and nine different types of 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose and three kinds of auxins *viz*. IAA, IBA, NAA were used for induction of strong and stout root system. The efficiency of the media in terms of enhancing the development of root system was assessed based on the increase in number and length of roots that developed within 30d of culture in rooting media. In rooting medium the plantlets grew further and produced well developed root system. Rooted seedlings transferred to outside environment by the successive phases of acclimatization. Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well.

3. Results and Discussions

In vitro developed *Aerides multiflora* plantlets were used as a source of node and leaf for rapid micropropagation [21-24] and summarized in Table-1. The nodal explants were cultured on 0.8% (w/v) agar solidified MS media supplemented with various combinations and concentrations of PGRs and produced multiple shoot buds (MSBs) *via* direct organogenesis. The nodal segments maximum number of shoot buds (8.83 ± 0.45/segment) were produced when cultured on 0.8% (w/v) agar solidified MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l NAA + 2.0 mg/l BAP (Fig.1a) followed by 8.65 ± 0.54/segment on MS + 3% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP [22]. Cytokines, like BAP and Kn; auxins like IAA, IBA, NAA and Picloram are implicated in the development [24]. Leaf segments underwent proliferation and produced seedlings *via* PLBs and maximum percent of greenish PLBs were produced on MS medium fortified with 3% (w/v) sucrose + 1.0 mg/l IAA + 2.0 mg/l BAP (Fig. 1b) followed on MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 2.0 mg/l BAP.

Table 1: Development of multiple shoot buds/ PLBs from nodal and leaf explants of *Aerides multiflora* when grown on 0.8% (w/v) agar solidified MS media supplemented with different PGRs.

Combinations and concentrations of PGRs	Explants	% of induced multiple shoot buds/ PLBs per segment	Time (d) required for sprouting of multiple shoot buds/ PLBs	Number of multiple shoot buds/ PLBs produced per segment (Mean ± S.E.)
0.5 mg/l IAA + 1.0 mg/l BAP	NS**	45	40 - 44	4.56 ± 0.29
	LS***	45	50 - 58	Green PLBs
0.5 mg/l IAA + 1.0 mg/l Kn	NS**	50	34 - 38	4.85 ± 0.37
	LS***	—	—	—
1.0 mg/l IAA + 2.0 mg/l BAP	NS**	50	35 - 38	5.03 ± 0.35
	LS***	55	45 - 52	Green PLBs
1.0 mg/l IAA + 2.0 mg/l Kn	NS**	55	32 - 35	5.34 ± 0.31
	LS***	30	58 - 62	Greenish PLBs
0.5 mg/l IBA + 1.0 mg/l BAP	NS**	35	33 - 36	4.17 ± 0.26
	LS***	35	56 - 60	Greenish PLBs
0.5 mg/l IBA + 1.0 mg/l Kn	NS**	45	32 - 35	4.35 ± 0.29
	LS***	—	—	—
1.0 mg/l IBA + 2.0 mg/l BAP	NS**	55	32 - 36	5.42 ± 0.33
	LS***	30	55 - 60	Green PLBs
1.0 mg/l IBA + 2.0 mg/l Kn	NS**	50	34 - 38	5.27 ± 0.30
	LS***	25	60 - 64	Green PLBs
0.5 mg/l NAA + 1.0 mg/l BAP	NS**	75	30 - 35	8.65 ± 0.54
	LS***	35	52 - 58	Green PLBs
0.5 mg/l NAA + 1.0 mg/l Kn	NS**	65	32 - 35	7.12 ± 0.42
	LS***	45	50 - 55	Green PLBs
1.0 mg/l NAA + 2.0 mg/l BAP	NS**	75	30 - 34	8.83 ± 0.45
	LS***	50	50 - 56	Green PLBs
1.0 mg/l NAA + 2.0 mg/l Kn	NS**	65	32 - 36	7.18 ± 0.38
	LS***	45	48 - 55	Green PLBs
0.5 mg/l Pic + 1.0 mg/l BAP	NS**	70	33 - 38	8.06 ± 0.32
	LS***	—	—	—
0.5 mg/l Pic + 1.0 mg/l Kn	NS**	65	35 - 38	7.09 ± 0.41

	LS***	20	60 - 65	Yellowish PLBs
1.0 mg/l Pic + 2.0 mg/l BAP	NS**	65	35 – 40	7.01 ± 0.36
	LS***	35	55 - 58	Green PLBs
1.0 mg/l Pic + 2.0 mg/l Kn	NS**	60	30 - 35	6.87 ± 0.40
	LS***	25	58 - 62	Greenish PLBs

NS = Nodal Segment, LS= Leaf Segment, '-' indicates no response;
Based on observations recorded from 10 cultured segments in each medium.

The requisite of auxins and cytokinins for regeneration of multiple shoot buds and seedlings development has been find out of many orchid species [25-27]. The ratio of auxin and cytokinins for shoot bud formation varies from species to species [28]. BAP and NAA were best for shoot bud formation in *Spathoglottis plicata*, *Cymbidium giganteum*, *Geodorum densiflorum*, *Vanda spathulata* and *Dendrobium bensoniae*; respectively [28-32].

Different concentrations and combinations of PGRs (BAP, Kinetin, NAA, IAA, IBA and Picloram) supplemented 0.8% (w/v) agar solidified & liquid MS and PM elongation media were used for enhancing elongation of MSBs or PLBs. The efficiency of a medium in terms of enhancing shoot elongation was determined based on the increase in length of shoot system within 30d of culture (Table-2). Highest increased mean of individual shoot bud length (2.62 ± 0.14 cm) was achieved on agar solidified MS with 1.0 mg/l NAA + 1.0 mg/l BAP (Fig. 1c) followed by liquid MS + 1.0 mg/l IAA + 1.0 mg/l BAP (2.53 ± 0.11 cm) medium (Fig. 1d). It is marked that elongation of shoot bud was better in solidified media than liquid condition and MS was superior to PM [33-36]. The elongation rate was different depending on PGR supplements liquid and solid media and solid culture was best for elongation [37-38].

In elongation media, shoot buds produced few weak roots. Half strength MS0 and nine different types of PGRs (IAA, IBA, NAA) supplemented MS media were used for induction of strong and stout root system (Table-3). The efficiency of the rooting media was evaluated based on the increase in length and number of roots developed per seedling within 30d of culture. Increase in length as well as the number of roots developed MSBs derived seedlings were best responses on agar solidified MS medium supplemented with 3% (w/v) sucrose + 1.0 mg/l IBA (4.32 ± 0.24 cm/shoot bud; 3.19 ± 0.22 no/shoot bud and Fig.1e) followed by MS medium with 3% (w/v) sucrose + 0.5 mg/l IAA (3.91 ± 0.23 cm/shoot bud and 3.01 ± 0.16 no/shoot bud) [39-41]. Auxin supplemented medium was more efficient for induction of strong and stout root system. It is noted that low concentration of auxin is more suitable than high concentration for induction of well developed root system. IAA was effective for rooting in *Dendrobium thyrsiflorum* [42], whereas, NAA was best in *Coelogyne fuscescens* [43]. Combined effect of auxines was more effective of induced rooting in *Dendrobium* hybrid orchid, *Rhyncostylis retusa* respectively [44-45].

Table 2: Elongation of multiple shoot buds/ PLBs developed from nodal and leaf explants of *A. multiflora* on 0.8% (w/v) agar solidified and liquid media with different kinds of PGRs.

Culture medium with different combinations and concentrations of PGRs	Average initial length (cm) of individual shoot bud	Average length (cm) of individual shoot bud after 30d of culture on elongation medium	Increase in length (cm) of shoot bud within 30d of culture on elongation medium	Average initial length (cm) of individual shoot bud	Average length (cm) of individual shoot bud after 30d of culture on elongation medium	Increase in length (cm) of shoot bud within 30d of culture on elongation medium
	Solid media			Liquid media		
MS + 3% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.45±0.10	2.91±0.11	1.46±0.09	1.45±0.07	3.26±0.09	1.81±0.06
MS + 3% (w/v) sucrose + 0.5 mg/l IAA + 1.0 mg/l BAP	1.65±0.12	3.54±0.13	1.89±0.15	1.42±0.10	3.76±0.12	2.34±0.09
MS + 3% (w/v) sucrose +1.0 mg/l IAA + 1.0 mg/l BAP	1.50±0.12	3.58±0.16	2.08±0.17	1.52±0.15	4.05±0.13	2.53±0.11
MS + 3% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.52±0.07	3.53±0.07	2.01±0.11	1.40±0.13	3.34±0.12	1.94±0.10
MS+3% (w/v) sucrose+0.5 mg/l NAA+1.0 mg/l BAP	1.65±0.14	3.99±0.17	2.34±0.12	1.35±0.11	3.70±0.16	2.35±0.14
MS + 3% (w/v) sucrose +1.0 mg/l NAA + 1.0 mg/l BAP	1.50±0.13	4.12±0.18	2.62±0.14	1.48±0.09	3.99±0.11	2.51±0.13
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.42±0.08	3.20±0.09	1.78±0.12	1.40±0.12	3.12±0.08	1.72±0.09
MS+3% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.58±0.09	3.76±0.11	2.18±0.13	1.55±0.15	3.87±0.10	2.32±0.12
MS + 3% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.40±0.12	3.79±0.15	2.39±0.17	1.45±0.16	3.94±0.13	2.49±0.14
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 0.5 mg/l BAP	1.55±0.10	3.56±0.07	2.01±0.08	1.40±0.12	3.37±0.09	1.97±0.13
PM + 2% (w/v) sucrose +	1.60±0.16	3.94±0.14	2.34±0.12	1.45±0.14	3.73±0.16	2.28±0.10

0.5 mg/l IAA + 1.0 mg/l BAP						
PM + 2% (w/v) sucrose + 1.0 mg/l IAA + 1.0 mg/l BAP	1.68±0.14	3.81±0.11	2.13±0.17	1.40±0.07	3.87±0.13	2.47±0.11
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 0.5 mg/l BAP	1.64±0.09	3.75±0.12	2.11±0.11	1.45±0.09	3.48±0.10	2.03±0.13
PM + 2% (w/v) sucrose + 0.5 mg/l NAA + 1.0 mg/l BAP	1.60±0.17	3.98±0.16	2.38±0.14	1.42±0.11	3.63±0.14	2.21±0.16
PM + 2% (w/v) sucrose + 1.0 mg/l NAA + 1.0 mg/l BAP	1.65±0.15	4.17±0.13	2.52±0.16	1.40±0.15	3.84±0.12	2.44±0.11
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 0.5 mg/l BAP	1.70±0.13	3.53±0.10	1.83±0.09	1.38±0.17	3.50±0.14	2.12±0.15
PM + 2% (w/v) sucrose + 0.5 mg/l Pic + 1.0 mg/l BAP	1.62±0.14	3.88±0.12	2.26±0.10	1.32±0.13	3.67±0.09	2.35±0.12
PM + 2% (w/v) sucrose + 1.0 mg/l Pic + 1.0 mg/l BAP	1.75±0.11	4.12±0.14	2.37±0.09	1.35±0.12	3.83±0.10	2.48±0.15

All the values are mean ± SE, shoot length of each treatment contains 10 replicates.

Fig 1a: Multiple shoot buds sprouted from nodal segment on agar solidified MS + 1.0 mg/l NAA + 2.0 mg/l BAP

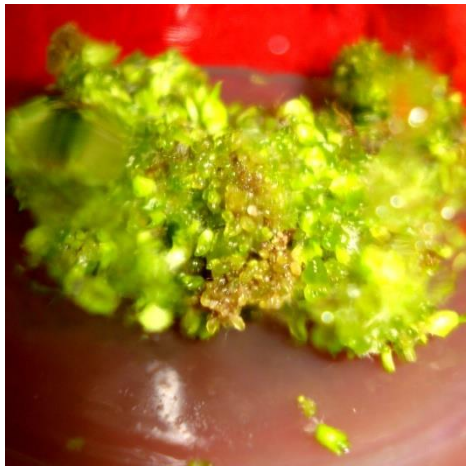

Fig 1b: Development of PLBs from leaf segment on agar solidified MS + 1.0 mg/l IAA + 2.0 mg/l BAP

Fig 1c: Elongated seedlings on agar solidified MS + 1.0 mg/l NAA + 1.0 mg/l BAP

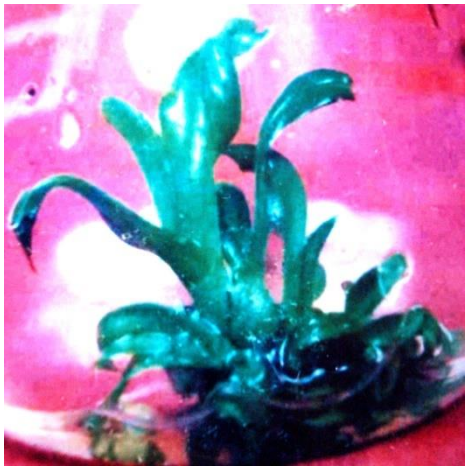

Fig 1d: Elongated seedlings on liquid MS + 1.0 mg/l IAA + 1.0 mg/l BAP

Fig 1e: Strong and stout root system on agar solidified MS + 1.0 mg/l IBA

Fig 1f: *In vitro* developed plantlets growing in pot outside of the culture room

Fig 1(1a-1f): *In vitro* micropropagation, Shoot bud elongation, rooting and hardening of *Aerides multiflora*.

Table 3: Mean increase in length (cm) and number of roots shoot bud originated seedlings in ½ MS0 and auxin supplemented MS rooting media

Culture medium			Average increased length and number of roots per shoot bud	
			Mean length (cm) ± S.E.	Mean no. of roots/ shoot bud ± S.E.
½ MS0			3.57 ± 0.19	2.83 ± 0.20
Auxin (mg/l)	IAA	0.5	3.91 ± 0.23	3.01 ± 0.16
		1.0	3.62 ± 0.22	2.78 ± 0.17
		1.5	3.14 ± 0.20	2.26 ± 0.15
	IBA	0.5	3.72 ± 0.26	2.39 ± 0.15
		1.0	4.32 ± 0.24	3.19± 0.22
		1.5	3.93 ± 0.23	2.54 ± 0.19
	NAA	0.5	2.06 ± 0.12	2.03 ± 0.14
		1.0	2.48± 0.15	2.25 ± 0.15
		1.5	1.85 ± 0.12	1.83 ± 0.11

Root length and number of roots of each treatment contains 10 replicates.

The well developed plantlets were transferred from culture room to the outside environment through successive phase of acclimatization. For the purpose, the mouth of the culture vessels was kept open for one day in the culture room and then kept outside of the culture room for 6h in the next day. Later on, those were kept outside of the culture room for 12h. Finally the seedlings were taken out of the culture vessels and rinsed with running tap water for removal of agar attached to the roots. Then the seedlings of *A. multiflora* were transferred to plastic pots containing a potting mixture of sterilized small brick, charcoal pieces and peat moss at a ratio of 1 : 1 : 0.5 and kept in the green house (at 25-30 ^{0}C and RH 60-70%). Transplanted seedlings were watered regularly for about 2-3 months where the seedlings established and grew well. Then, they were finally established in Orchidarium of the Botanical Garden of Chittagong University (Fig. 1f).

4. Conclusions

Combine effect of PGRs is very much effective for development of MSBs and elongation of individual shoot bud in *A. multiflora*. BAP, NAA, and IAA have a great role for *in vitro* micropropagation. Nodal segment produce directly few MSBs whereas, leaf segment produce huge number of PLBs *via* embryogenesis which are used in cell or protoplast culture and helps to huge number of seedlings production. Agar solidify culture was better in terms of promoting shoot elongation than its liquid counterpart. IBA supplemented MS was better than IAA, NAA and half strength MS0 media for induced well developed rooting, respectively. This micropropagation technique has a great role for *ex situ* conservation of this highly medicinally important orchid.

5. References

1. Kitto SL. Commercial micropropagation. Hort. Science. 1997; 32(6):1012-1014.
2. Prakash L, Lee L, Goh CJ. *In vitro* propagation of commercial orchid: An assessment of current methodologies and development of novel approach-thin section culture. J Orchid Soc. India. 1996; 10:31-41.
3. Dressler RL. Phylogeny and classification of the orchid family, Cambridge University Press. Cambridge, 1993.
4. Huda MK. An updated enumeration of the family orchidaceae from Bangladesh. The Journal of the Orchid Soc. of India. 2007; 21(1, 2):35-49.
5. Rahman MA, Huda MK, Rashid ME. Orchid species diversity in Bangladesh and their revised nomenclatural updates. Biod. Bull. Bangladesh. 2017; 10:1-63.
6. Ahmed M, Pasha MK, Khan MA. A taxonomic account of *Aerides* Lour. (Orchidaceae) from Bangladesh. Bangladesh J Bot. 1989; 18(2):147-155.
7. Huda MK. Diversity, Ecology, Reproductive biology and Conservation of Orchids of South East Bangladesh. Ph. D. Thesis. Department of Ecology and Soil Science. Aberdeen University, UK. 2000, 266.
8. Encyclopedia of Flora and Fauna of Bangladesh, Angiosperms, Asiatic Society of Bangladesh, Published. 2008; 12:125-125.
9. Rao AN. Medicinal orchid wealth of Arunachal Pradesh. News Letter ENVIS NODE. Indian Med. Plant. 2004; 1(2):1-7.
10. Pant B. Medicinal orchids and their uses: Tissue culture a potential alternative for conservation. African Journal of Plant Science. 2013; 7(10):448-467.

11. Pant B, Raskoti BB. Medicinal orchids of Nepal. Kathmandu, Himalayan Map House (P) Ltd, 2013.
12. Subedi A, Kunwar B, Choi Y. Collection and trade of wild harvested orchids in Nepal. J Ethnobiol. Ethno. Med. 2013; 9:64-73.
13. Singh A, Duggal S. Medicinal orchids: an overview. Ethnobot. Leaflets. 2009; 13:351-363.
14. Hossain MM. Therapeutic orchids: traditional uses and recent advances-An overview. Fitoterapia. 2011; 82:102–140.
15. Tim WY, Arditti J. Orchid Propagation: From Laboratories to Greenhouses-Methods and Protocols. 2018, 151-178.
16. Teo CKH, Kunisaka JT, Sagawa Y. Clonal propagation of strap-leaved Vanda by shoot tips culture. Am. Orchid Soc. Bull. 1973; 42:402-405
17. Malabadi RB, Mulgund SG, Kallappa N. Micropropagation of *Dendrobium nobile* from shoot tip sections. Journal of Plant Physiology. 2005; 162:473-478.
18. Bhowmik TK, Rahman MM. Effect of Different Basal Media and PGRs on *In Vitro* Seed Germination and Seedling Development of Medicinally Important Orchid *Cymbidium aloifolium* (L.) Sw. Journal of Pharmacognosy and Photochemistry. 2017; 6(1):167-172.
19. Murashige T, Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum. 1962; 15:473-497.
20. Arditti J. Clonal propagation of orchids by means of tissue culture: A manual In: J Arditti (ed.), *Orchid Biology:* Reviews and Perspectives, I. University Press, Ithaca, New York. 1977, 114-1255.
21. Bhadra SK, Bhowmik TK. Axenic germination of seeds and rhizome-based micropropagation of an orchid *Arundina graminifolia* (D. Don) Hochr. Bangladesh J of Botany. 2005; 34(2):59-64.
22. Bhadra SK, Hossain MM. *In vitro* germination and micropropagation of *Geodorum densiflorum* (Lam.) Scheltr., an endangered orchid species. Plant Tissue Culture. 2003; 13(2):165-171.
23. Sheelavantmath SS, Murthy HN, Pyati AN, Kumer HGA, Ravishankar BV. *In vitro* propagation of the endangered orchid, *Geodorum densiflorum* (Lam). Schltr. through rhizome section culture. Plant Cell, Tissue and Organ Culture. 2000; 60(2):151-154.
24. Kauth PJ, Vendrame WA, Kane ME. Micropropagation of *Calopogon tuberosus*, a native terrestrial orchid, by shoot production from corm explants. In vitro, Cell Development Biology. 2006; 42:23-23.
25. Arditti J, Ernst R. Micropropagation of orchids, Wiley & Sons Inc., New York, 1993.
26. Hossain MM, Sharma M, Pathak P. *In vitro* propagation of *Dendrobium aphyllum* (Orchidaceae) seed germination to flowering. J Plant Biochem. Biotechnol. 2012; 22:157-167.
27. Bhadra SK, Hossain MM, Nandy P. Establishment of *in vitro* mass propagation protocol in 'Rasna' *Vanda tessellate* (Roxb.) Hook. ex. D. Don. J Orchid Soc. India. 2005; 19(1, 2):35-40.
28. Teng WL, Nicholson L, Teng MC. Micropropagation of *Spathoglottis plicata*. Plant Cell Rep. 1997; 16:831-8 35.
29. Hossain MM, Sharma M, Teixeira da SJA, Pathak P. Seed germination and tissue culture of *Cymbidium giganteum* Wall. Ex Lindl. Sci. Hortic. 2010; 123:479-487.
30. Sheelavantmath SS, Murthy HN, Pyati AN, Kumar HGA, Ravishankar BV. *In vitro* propagation of the endangered orchid, *Geodorum densiflorum* (Lam.) Schltr. Through rhizome section culture. Plant Cell, Tissue & Organ Cult. 2000; 60:151-154.
31. Decruse SW, Gangaprasad A, Seeni S, Menon VS. Micropropagation and eco-restoration of *Vanda spathulata*, an exquisite orchid. Plant Cell, Tissue & Organ Cult. 2003; 72:199-202.
32. Riva SS, Islam A, Hoque ME. *In vitro* Regeneration and Rapid Multiplication of *Dendrobium bensoniae*, an Indigenous Ornamental Orchid. The Agriculturists. 2016; 14(2):24-31.
33. Bhowmik TK, Rahman MM. *In vitro* seed germination and rhizome based micropropagation of *Calanthe densiflora* Lindl: An indigenous terristerial orchid of Bangladesh. International J of Botany Studies. 2017; 2(1):110-116.
34. Bhadra SK, Hossain MM. *In vitro* germination and micropropagation of *Geodorum densiflorum* (Lam.) Scheltr., an endangered orchid species. Plant Tissue Culture. 2003; 13(2):165-171.
35. Bhadra SK, Bhowmik TK. *In vitro* seed germination of *Phaius tankervilleae* (Banks ex L'herit) Blume. The Chittagong University J of Science. 2005; 29(1):123-127.
36. Clayton D, Cribb P. The Genus *Calanthe*. *Natural History Publications* (Borneo), Kota Kinabalu, in Association with Royal Botanic Gardens Kew, London, 2013.
37. Hoque MI, Roy AR, Sarker RH, Haque MM. Micropropagation of *Cymbidium bicolor* through *in vitro* culture. Plant Tissue Culture. 1994; 4(1):45-51.
38. Watad AA, Gaba V, Alper Y, Levin R. Liquid culture as a route for high efficiency micropropagation. In Vitro Cell Development Biology. 1995; 31(3):30A Abstract.
39. Aktar S, Nasiruddin KM, HuQ H. *In vitro* root formation in *Dendrobium* orchid plantlets with IBA. J Agric. Rural Dev. 2007; 5(1, 2):48-51.
40. Dang JC, Kumaria S, Kumar S, Tandon P. Micropropagation of *Ilex khasiana*, a critically endangered and endemic holly of Northeast India. 2011; plr 012 doi:10.1093/aobpla/plr012.
41. Islam SMS, Islam T, Bhattacharjee B, Mondal TK, Subramaniam S. *In vitro* pseudobulb based micropropagation for mass development of *Cymbidium finlaysonianum* Lindl. Emirates Journal of Food and Agriculture. 2015; 27(6):469-474.
42. Tikendra L, Amom T, Nongdam P. Effect of Phytohormones on Rapid *In vitro* Propagation of *Dendrobium thyrsiflorum* Rchb.f.: An Endangered Medicinal Orchid. Pharmacognosy Magazine. 2018; 14(58):495-500.
43. Koirala D, Pradhan S, Pant B. Asymbiotic seed germination and plantlet development *Coelogyne fuscescens* Lindl., A medicinal orchid of Nepal. Scientific World. 2013; 11(11):97-100.
44. Khatun MM, Khatun H, Khanam D, Al-Ami M. *In vitro* root formation and plantlet development in *Dendrobium* orchid. Bangladesh J Agril. Res. 2010; 35(2):257-265.
45. Islam SMS, Bhattacharjee B. Plant regeneration through somatic embryogenesis from leaf and root explants of *Rhyncostylis retusa* (L.) Blume. Applied Biological Research. 2015; 17(2):158-165.