Micropropagation of *Phalaenopsis circus* via direct organogenesis using protocorm-like bodies explant

Hasan Kiaheirati, Davood Hashemabadi* and Behzad Kaviani

Department of Horticultural Science, Rasht Branch, Islamic Azad University, Rasht, Iran

Abstract

Phalaenopsis orchid has high economic value in the floriculture industry and is one of the most popular orchids in the world. Tissue culture techniques make it possible to propagate and conserve this species. The aim of the present study was to evaluate the effect of different concentrations of two plant growth regulators (PGRs), namely N-Phenyl-N'-1,2,3-thiadiazol-5-yl-urea (TDZ) and 2,4-dichlorophenoxyacetic acid (2,4-D), alone and in combination with each other, on the number of protocorm-like bodies (PLBs), leaves, and roots, along with leaf and root lengths of micropropagated *Phalaenopsis circus* using leaf explants through organogenesis method. Also, correlations between these traits in the *Phalaenopsis circus* plantlets under study were calculated. Plantlets produced from PLBs were cultured on MS (Murashige and Skoog) basal media enriched with various levels and combinations of TDZ and 2,4-D. The optimal concentrations of the PGRs for micropropagation of *Phalaenopsis circus* are reported and discussed. The maximum number of PLBs (75.00) was obtained on the medium enriched with 1.00 mg l^{-1} 2,4-D. The highest length of leaf (5.23 cm) was calculated on the medium enriched with 2.00 mg l^{-1} 2,4-D. Concentration of 1.00 mg l^{-1} TDZ induced the maximum number (6.07) of leaf. The longest root length (4.15 cm) and the largest number of root (4.93) was obtained in the medium augmented with 2.00 mg l^{-1} 2,4-D.

Keywords: *In vitro* culture, orchid, Orchidaceae, plant growth regulators, protocorm

Kiaheirati, H., D. Hashemabad, and B. Kaviani. 2023. 'Micropropagation of *Phalaenopsis circus* via direct organogenesis using protocorm-like bodies explant'*. Iranian Journal of Plant Physiology 13* (1), 4417-4424**.**

Introduction

Orchids belonging to Orchidaceae family are among the most diverse flowering plant families (Chugh et al., 2009), with more than 800 genera and 25,000 species. *Phalaenopsis circus* orchid has been developed through many artificial hybrids and is one of the most popular orchids in the world with high economic value in the floriculture industry as both a cut and pot flowering plant. *Phalaenopsis circus* comprises approximately 60 species native to tropical rainforests of South and South-East Asia, Australia, and New Guinea (Winkelmann et al., 2006).

Many orchid species are vulnerable, rare, and/or threatened, and their propagation is highly critical. Sexual propagation of orchids, i.e. through seeds, leads to the production of heterozygous plants. Also, natural clonal propagation of orchids is a slow process and results in traits segregation and is not possible for *Phalaenopsis circus*. One of the most important approaches for conservation of these plants is *in vitro* propagation. Through micropropagation, a large number of plants are

* *Corresponding Author*
E-mail Address: davoodhashemabadi@yahoo.com
Received: January, 2022
Accepted: August, 2022

produced from a small plant material (explant or an intervening callus stage). On the other hand, *in vitro* propagation of orchids has a few problems like low rate of shoot multiplication, high cost of production, poor rooting frequency, exudation of phenolic compounds from explants, transplantation to fields, and somaclonal variations (Chugh et al., 2009; Bhattacharyya et al., 2016). In fact, it is an ongoing research to establish protocols for cost-effective yet efficient *in vitro* propagation of orchids as a proper alternative procedure for high frequency regeneration of these plants.

Organogenesis is an important method for micropropagation of the plant by developing organs like roots, shoots, and flowers, either directly from an explant or from the callus culture. Several techniques for tissue culture of *Phalaenopsis* spp. through cell suspension culture and callus induction have been developed and reported in the literature (Sinha et al., 2010).

Various explants have been commonly used for tissue culture of the genus from the Orchidaceae family including seeds (Roy et al., 2011; Zeng et al., 2012; Mahendran, 2014), nodal segments (Bhattacharyya et al., 2016), leaf segments (foliar explants), rhizome segments, root segments, shoot tips (Chugh et al., 2009), flower buds, tubers (Panwar et al., 2012), protocorms (produced by the very small zygotic embryos of orchids) (Kaviani et al., 2017), PLBs (produced by explants, e.g. leaf) (Mohammadi et al., 2019; Asa et al., 2019), and inflorescence axes (Sinha et al., 2010). For example, Park et al. (2002) introduced an *in vitro* propagation method for *Phalaenopsis* sp. using PLBs derived from leaf explants. Studies suggest that among all these explants, protocorms and PLBs are more efficient because of rapid multiplication on solid or liquid culture media and maximum production in a short period of time (Luo et al., 2003a; Roy et al., 2011; Zeng et al., 2012).

For tissue culture, plant cells are initially grown in an artificial medium. Composition of the medium for organogenesis of orchids is species-specific and depends on several factors including type and concentration of plant growth regulators (PGRs), vitamins, and other additives (Luo et al., 2009). Various plant growth regulators (PGRs) such as α-naphthaleneacetic acid (NAA), indole-3-butyric acid (IBA), 1-phenyl-3-(1,2,3-thiadiazol-5-yl)-urea (TDZ), 6-benzyladenine (BA), and 6-furfurylaminopurine or kinetin (Kin) have been applied to *in vitro* propagation of *Phalaenopsis* orchids through organogenesis and embryogenesis (Kuo et al., 2005; Sinha et al., 2007; Kaviani et al., 2017; Mohammadi et al., 2019; Asa et al., 2019).

In sum, research on the protocols for *in vitro* propagation of orchids has centered on a number of factors including the species of Orchidaceae family, employing explants, types and concentrations of plant growth regulators (PGRs) in the culture media, and the organ used as explant to nest PLBs (Sinha et al., 2010; Baker et al., 2014; Kaviani et al., 2017; Zakizadeh et al., 2019; Mohammadi et al., 2019; Asa et al., 2019). Studies recommend *in vitro* propagation of Orchidaceae family through PLB explants to achieve genetically stable plants with improved quality. However, the protocols for micropropagation are species-specific in terms of the type and optimal concentration of PGRs used in the culture medium. The aim of the present study was to evaluate the effect of different concentrations of two PGRs, namely TDZ and 2,4-D, alone and in combination with each other, on the number of PLBs, leaves, and roots, along with leaf and root lengths of micropropagated *Phalaenopsis circus* using PLB explants through organogenesis method. Also, correlation coefficients between these traits in the *Phalaenopsis circus* plantlets under study were calculated.

Materials and Methods

Capsules of *Phalaenopsis circus* were isolated from the flowers of plants grown in the greenhouse of the Hyrcan Agricultural Sciences and Biotechnology Research Institute, Amol, Iran. The approximate 150-day-old capsules were first washed with tap water along with a few drops of dishwashing liquid for 30 min before they were rinsed thoroughly with distilled water. Then, they were surface sterilized in 50% sodium hypochlorite solution containing 5% active chloride for 20 min with a drop of Tween 20,

rinsed completely in distilled water, and finally kept in 70% alcohol for 1 min. The disinfected capsules were longitudinally incised using a sterilized surgical blade to remove seeds. The seeds were then cultured on MS (Murashige and Skoog, 1962) medium for production of PLBs. The media were supplemented with 3% sucrose and solidified with 0.8% agar. Media pH was adjusted to 5.6-5.8 with 1 N HCl or NaOH before autoclaving at 121 ℃, 105 kPa for 20 min. Following establishment, all cultures were incubated at 24 ± 2 ℃, 70-80% RH, and 16-h photoperiod of 50-60 µmol m^{-2} s^{-1} irradiance from cool-white fluorescent tubes.

The PLB explants were cultured in MS media containing 3% sucrose and 0.8% agar. The media pH was adjusted to 5.6-5.8 with 0.1 N NaOH or HCl prior to autoclaving. All media contained in culture bottles were autoclaved at 105 kPa and 121 ℃ for 20 min. The media were enriched with different concentrations of TDZ (0.00, 0.10, 1.00, 2.00, and 5.00 mg l^{-1}) and 2,4-D (0.00, 0.01, 0.10, 1.00, and 2.00 mg l^{-1}), either individually or in combination for organogenesis.

After 90 days, the number of PLBs, leaves, and roots along with leaf and root lengths were recorded. Well-rooted *in vitro* plantlets were taken out from culture vessels and washed thoroughly with sterilized distilled water to remove adherent nutrient from the plantlet body before they were transplanted in plastic pots (18 cm height × 12 cm diameter) filled with a potting mixture of LECA (Light Expanded Clay Aggregate), peat moss, coco peat, charcoal soil, coco chips, and perlite by the proportion of 15:10:20:5:30:20%. All pots were then transferred to the greenhouse with the temperature set at 20±2 ℃ / 4±2 ℃ day/night under light intensity of 3500 Lux, RH of 80-90%, and 14-h photoperiod for acclimatization. The pots were covered with polyethylene bags to retain moisture inside and the covers were removed gradually during two weeks. The plantlets were initially covered with a polythene sheet to maintain relative humidity (90%). The number of surviving plants was recorded three months after transfer.

The experiment was carried out in a completely randomized design. Treatments involved the media enriched with 25 different concentrations of two PGRs alone and combined. For each treatment, three replicates and for each replicate, three specimens (explants) were considered. PGR-free MS medium was used as control. Data were subjected to analysis of variance (ANOVA) and means were compared by the LSD test at P≤0.05 using the SPSS ver. 17 (SPSS Inc., USA). Also, coefficients of correlation were obtained between the plantlet parameters.

Results

There were statistically significant differences (p≤0.01) among different concentrations of 2,4-D in combination with TDZ and PLBs number, as well leaf length (Table 1). Tables 2 and 3 show the effect of different concentrations of TDZ and 2,4-D, individually and in combination, on measured parameters. The maximum number of PLBs (75.00 per explant) in *P. circus* was obtained in explants treated with 1.00 mg l^{-1} 2,4-D without TDZ (Table 2, Fig. I). Combined treatments of the explants with 1.00 mg l^{-1} 2,4-D + 0.10 mg l^{-1} TDZ and 0.10 mg l^{-1} 2,4-D + 1.00 mg l^{-1} TDZ resulted in the highest number of PLBs more than 50.00 per explant. There were no positive relationships

Table 1
Analysis of variance of the effect of different concentrations of 2,4-D, NAA, IBA, Kin, and TDZ on measured parameters of *Phalaenopsis circus* grown *in vitro* condition during organogenesis and embryogenesis

		Mean squares				
Source of variations	df	PLBs number	Leaf length	Leaf number	Root length	Root number
2,4-D	4	2482.51**	6.12**	1.61^{ns}	6.82**	21.49**
TDZ	4	123.95**	0.38^{ns}	27.81**	0.17^{ns}	0.45^{ns}
2,4-D × TDZ	16	186.55**	0.69**	0.36^{ns}	0.23^{ns}	0.67^{ns}
Error	48	22.91	0.24	0.66	0.15	0.90
CV (%)	-	12.65	17.08	20.67	13.21	32.85

* and **: Significant at the 0.05 and 0.01 probability levels, respectively; ^{ns}: not significant at p≤0.05

Table 2
Mean comparison of the effect of different concentrations of 2,4-D and TDZ, individually, on measured parameters of *Phalaenopsis circus* grown *in vitro* condition during organogenesis

Treatments	PLBs Number per Explant	Leaf Length (cm)	Leaf Number	Root Length (cm)	Root Number
2,4-D (mg l^{-1})					
0.00	16.47d	2.21d	-	2.51c	2.07c
0.01	38.67c	2.59c	-	2.72bc	2.20c
0.10	44.40b	2.63bc	-	2.60bc	2.27c
1.00	50.47a	2.95b	-	2.87b	3.00b
2.00	39.27c	3.89a	-	4.15a	4.93a
TDZ (mg l^{-1})					
0.00	42.20a	-	2.40d	-	-
0.10	36.93bc	-	4.20b	-	-
1.00	39.07ab	-	6.07a	-	-
2.00	34.80c	-	3.53c	-	-
5.00	36.27bc	-	3.40c	-	-

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

between the increased levels of 2,4-D and TDZ concentrations and the number of BLBs (Table 2). The minimum number of PLBs (14.67 per explant) was observed in explants treated with 5.00 mg l^{-1} TDZ without 2,4-D (Table 2, Fig. I). All treatments in the absence of 2,4-D induced less than 18 PLBs per explant (Table 2). The maximum leaf length (5.23 cm per explant) was obtained in the treatment with 2.00 mg l^{-1} 2,4-D without TDZ followed by the mean leaf length 4.40 cm recorded in the medium enriched with 2.00 mg l^{-1} 2,4-D + 0.10 mg l^{-1} TDZ. On the other hand, the lowest mean root length (2 cm per explant) was observed in control plantlets.

Finally, Table 4 shows the results of correlational analysis of the plantlet parameters under study. Coefficient of correlations revealed a positive correlation between leaf length and leaf number, root length and root number, and also between root length and root number. The number of surviving plants was recorded 3 months after transfer, showing 100% establishment rate (Fig. II).

Discussion

The direct shoot regeneration and multiplication from PLBs and protocorms explants is a successful approach for *in vitro* propagation of orchids. Shoot multiplication from orchid protocorms and PLBs has been reported in the literature (Chugh et al., 2009; Lee and Yeung, 2018), and many researchers have used this method in propagation of various species of Orchidaceae family including *Phalaenopsis amabilis* (Mohammadi et al., 2019),

Table 3
Mean comparison of the effect of different concentrations of 2,4-D and TDZ, individually and in combination, on measured parameters of *Phalaenopsis circus* grown *in vitro* condition during organogenesis

2,4-D + TDZ (mg l^{-1})	Mean Comparison	
	PLBs Number	Leaf Length
0.00 + 0.00	18.00h	2.00h
0.00 + 0.10	16.67h	2.40e-h
0.00 + 1.00	17.00h	2.07gh
0.00 + 2.00	16.00h	2.33fgh
0.00 + 5.00	14.67h	2.23fgh
0.01 + 0.00	33.00g	2.27fgh
0.01 + 0.10	37.00efg	2.50d-h
0.01 + 1.00	40.00d-g	2.77d-h
0.01 + 2.00	39.00d-g	2.53d-h
0.01 + 5.00	44.33cde	2.87d-g
0.10 + 0.00	45.33bcd	2.57d-h
0.10 + 0.10	42.33c-f	2.80d-h
0.10 + 1.00	52.67b	2.70d-h
0.10 + 2.00	40.00d-g	2.63d-h
0.10 + 5.00	41.67c-f	2.43d-h
1.00 + 0.00	75.00a	2.83d-g
1.00 + 0.10	52.67b	3.17cde
1.00 + 1.00	49.33bc	2.63d-h
1.00 + 2.00	39.00d-g	3.23cd
1.00 + 5.00	36.33fg	2.90def
2.00 + 0.00	39.67d-g	5.23a
2.00 + 0.10	36.00fg	4.40b
2.00 + 1.00	36.33fg	3.73bc
2.00 + 2.00	40.00d-g	3.20cde
2.00 + 5.00	44.33cde	2.90def

Means with different letters on the same column are significantly different ($p<0.05$) based on LSD test.

Table 4
Correlation between measured parameters

Parameters	PLBs Number	Leaf Length	Leaf Number	Root Length	Root Number
PLBs number	1				
Leaf length	-0.148	1			
Leaf number	-0.064	0.237*	1		
Root length	-0.187	0.668**	0.209	1	
Root number	-0.196	0.559**	0.157	0.559**	1

*, **: Significant at the 0.05 and 0.01 probability levels, respectively

Fig. I. Micropropagation process of *Phalaenopsis circus* using different concentrations of 2,4-D and TDZ

Fig. II. Acclimatization process of plantlets produced *in vitro*

Catasetum pileatum cv. Alba (Zakizadeh et al., 2019), *Orchis catasetum* (Baker et al., 2014), *Eulophia nuda* Lindl (Panwar et al., 2012), *Vanda coerulea* (Roy et al., 2011), *Habeneria marginata* (Sheelavanthmath and Murthy, 2001), *Dendrobium aphyllum* (Talukdar, 2001), *Cymbidium aloifolium* (Bujarbarua and Sharma, 1997; Kaur and Sharma, 1997), and *Renanthera imschootiana* (Seeni and Latha, 1992).

In this study, the largest number of PLBs in *Phalaenopsis circus* was recorded under 2,4-D alone treatment. PLBs production is influenced by concentration and combination of auxins and cytokinins (Arditti and Ernst, 1993). The combination, type, concentration, and the relation between PGRs plays a critically important role in the formation of shoots, protocorms, and PLBs in many orchids (Arditti and Ernst, 1993; Bhattacharyya et al., 2016; Kaviani et al., 2017). The most commonly used auxins in orchid culture media are indole acetic acid (IAA), NAA, IBA, and 2,4-D. Similarly, Kin, BA, BAP, TDZ, and Zt are also commonly used in orchid culture media (Yam and Arditi, 2018). Maximum PLB regeneration and the highest root length in *Orchis catasetum* were achieved on the media supplemented with BA and NAA (Baker et al., 2014). Bhattacharyya et al. (2016) found that the exogenous application of PGRs in appropriate concentrations promoted shoot multiplication from PLBs. Also, a combination of 1.00 mg l^{-1} Kin and 1.00 mg l^{-1} IBA was reported to induce maximum PLB regeneration and the largest number of leaves in *Catasetum pileatum* cv. Alba (Zakizadeh et al., 2019). In *Vanda coerulea* species, Roy et al. (2011)

found that a combination of NAA and BAP resulted in maximum PLB regeneration.

Findings of the current study showed that the control medium (without PGRs) or the medium with low and high concentrations of PGRs resulted in low number of PLBs. Unlike our findings, some researchers reported that the proliferation of shoots is closely related to the type and concentration of cytokinins used (Lee and Yeung, 2018; Bhattacharyya et al., 2016; Amoo et al., 2014). Studies on the application of cytokinins individually support its role in shoot proliferation from PLBs in species of orchid, e.g., *Dendrobium* (Ferreira et al., 2006), *D. nobile* and *C. aloifolium* (Nayak et al., 2002), *Rhynchostylis gigantea* (Van Le et al., 1999), *C. ensifolium* (Chang and Chang, 1998), and *Dendrobium nobile* and *C. aloifolium* (Nayak et al., 1997b). TDZ has been found to stimulate shoot formation in orchids (Mahendran and Narmatha Bai, 2009; Zhao et al., 2007; Martin and Madassery, 2006; Ket et al., 2004; Huetteman and Preece, 1993). Kalimuthu et al. (2007) found BAP alone resulted in the formation of higher number of PLBs in *Oncidium* species compared to its combined application with NAA. Probably, the endogenous concentration of NAA is enough for induction of PLBs in this species. As cell messengers and signals, plant hormones have a synergistic or antagonistic effect on each other. The response to exogenous PGRs varies between cultivars and explant types. Also, the difference between the content of endogenous PGRs is the most important factor responsible for plant organogenesis. Hormonal pathways are interconnected by a complex network of interactions and feedback circuits that determines the final outcome of the individual hormone actions.

Conclusions

The growing popularity of orchids around the world has encouraged the propagators and breeders of these valuable plants to develop the orchid flower industry more than ever. Providing effective protocols using appropriate techniques, explants, and plant growth regulators is one way to achieve this goal. Concentration of 1.00 mg l^{-1} 2,4-D was found to be the best for PLB induction.

Acknowledgements

This work was supported by the Islamic Azad University, Rasht Branch. The authors would like to thank Dr. Naser Negahdar, Engineer Razieh Taghizadeh Andevari and Dr. Shima Seydi for their valuable technical assistance.

References

Amoo, S. O., A. O. Aremu, M. Moyo, L. Szüčová, K. Doležal and J. Van Staden, 2014. Physiological effects of a novel aromatic cytokinin analogue in micropropagated *Aloe arborescens* and *Harpagophytum procumbens*. *Plant Cell, Tissue and Organ Culture*, 116: 17–26.

Arditti, J. and R. Ernst, 1993. Micropropagation of orchids. *John Wiley and Sons, New York*.

Asa, M. and **B. Kaviani,** 2020. *In vitro* propagation of orchid *Phalaenopsis amabilis* (L.) Blume var. Jawa. *Iranian Journal of Plant Physiology*, 11 (1): 3457–3123.

Baker, A., B. Kaviani, Gh. Nematzadeh and N. Negahdar, 2014. Micropropagation of *Orchis catasetum* – A rare and endangered orchid. *Acta Scientiarum Polonorom-Hortorum Cultus*, 13 (2): 197–205.

Bhattacharyya, P., S. Kumaria and P. Tandon, 2016. High frequency regeneration protocol for *Dendrobium nobile*: A model tissue culture approach for propagation of medicinally important orchid species. *South African Journal of Botany*, 104: 232–243.

Bujarbarua, A. and C. M. Sharma, 1997. Micropropagation of *Cymbidium aloifolium* Sw from seeds. *Journal of Advances in Plant Science*, 1: 27–31.

Chang, C. and W. C. Chang. 1998. Plant regeneration from callus culture of *Cymbidium ensuifolium* var. Misericors. *Plant Cell Reports*, 17: 251–255.

Chugh, S., S. Guha and I. Usha Rao, 2009. Micropropagation of orchids: A review on the potential of different explants. *Scientia Horticulturae*, 122: 507–520.

Engelmann, F. 2011. Use of biotechnologies for the conservation of plant biodiversity. *In Vitro Cellular and Developmental Biology – Plant*, 47: 5–16.

Ferreira, W. D. M., G. B. Kerbauy and P. D. Costa. 2006. Micropropagation and genetic stability of a *Dendrobium* hybrid (Orchidaceae). *In Vitro Cellular and Developmental Biology – Plant*, 42: 568–571.

Huetteman, C. A. and J. E. Preece, 1993. Thidiazuron: A potent cytokinin for woody plant tissue culture. *Plant Cell, Tissue and Organ Culture*, 33: 105–119.

Kalimuthu, K., R. Senthilkumar and S. Vijayakumar, 2007. *In vitro* micropropagation of orchid, *Oncidium* sp. (Dancing Dolls). *African Journal of Biotechnology*, 6: 1171–1174.

Kaur, S. and C. M. Sharma. 1997. Application of *Rhizontonia* for establishment of plantlets of *Cymbidium aloifolium* Sw under natural conditions. *Journal of Advances in Plant Science*, 1: 17–20.

Kaviani, B., N. Negahdar, A. Baker and N. Mosafer, 2017. *In vitro* micropropagation of an endangered orchid species (*Orchis catasetum*) through protocorms: The effect of plant growth regulators and iron Nano-chelate. *Plant Research Journal*, 30 (1): 215–225 (In Persian with English Abstract).

Ket, N. V., E. J. Hahn, S. Y. Park, D. Chakrabarty and K. Y. Paek, 2004. Micropropagation of an endangered orchid *Anectochilus formosanus*. *Biologia Plantarum*, 48: 339–344.

Kuo, H. L., Chen, J. T. and W. C. Chang, 2005. Efficient plant regeneration through direct somatic embryogenesis from leaf explants of *Phalaenopsis* 'Little Steve'. *In Vitro Cellular and Developmental Biology – Plant*, 41 (4): 453–456.

Lee, Y. I. and E. C. T. Yeung, 2018. Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols, *Springer Protocols Handbooks*. **Luo, J. P., V. Wawrosch and B. Kopp,** 2009. Enhanced micropropagation of *Dendrobium huoshanense* C.Z. Tang et S.J. Cheng through protocorm-like bodies: The effects of cytokinins, carbohydrate sources and cold pretreatment. *Scientia Horticulturae*, 123: 258–262.

Luo, J. P., X. Q. Zha and S. T. Jiang, 2003a. Suspension culture of protocorm-like bodies from the endangered medicinal plant *Dendrobium huoshanenese*. *China Journal of Chinese Material Medicine*, 28: 611–614.

Mahendran, G. 2014. An efficient *in vitro* propagation, antioxidant and antimicrobial activities of *Aphyllorchis montana* (Reichenb.f.). *Journal of Ornamental Plants*, 4 (4): 1–16.

Mahendran, G. and V. Narmatha Bai, 2009. Mass propagation of *Satyrium nepalense* D.Don.—A medicinal orchid via seed culture. *Scientia Horticulturae*, 119: 203–207.

Martin, K. P. and J. P. Madassery, 2006. Rapid *in vitro* propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants and protocorm-like bodies. *Scientia Horticulturae*, 108: 95–99.

Mohammadi, M., B. Kaviani and Sh. Sedaghathoor, 2019. Micropropagation of two near threatened orchid. Part 2: *Phalaenopsis amabilis* Blume var. Grandiflora. *Advances in Horticultural Sciences*, 33 (4): 485–493.

Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Plant*, 15: 473–479.

Nayak, N. R., S. P. Rath and S. Patnaik, 1997b. *In vitro* propagation of three epiphytic orchids, *Cymbidium aloifolium* (L.) Sw., *Dendrobium aphyllum* (Roxb.) Fisch. and *Dendrobium moschatum* (Buch.-Ham.) Sw. through thidiazuron-induced high frequency shoot proliferation. *Scientia Horticulturae*, 71: 243–250.

Nayak, N. R., S. Sahoo, S. Patnaik and S. P. Rath, 2002. Establishment of thin cross section (TCS) culture method for rapid micropropagation of *Cymbidium aloifolium* (L.) Sw. and *Dendrobium nobile* Lindl. (Orchidaceae). *Scientia Horticulturae*, 94: 107–116.

Panwar, D., K. Ram and H. N. Shekhawat, 2012. *In vitro* propagation of *Eulophia nuda* Lindl., an endangered orchid. *Scientia Horticulturae*, 139: 46–52.

Park, S. Y., H. N. Murthy and K. Y. Paek, 2002. Rapid propagation of *Phalaenopsis* from floral stalk derived leaves. *In Vitro Cellular and Developmental Biology – Plant*, 38: 168–172.

Pati, R., S. P. Rath, M. Sharma, A. Sood and P. S. Ahuja, 2006. *In vitro* propagation of rose – A review. *Biotechnology Advances*, 24 (1): 94–114.

Roy, A. R., R. S. Patel, V. V. Patel, S. Sajeev and B. C. Deka, 2011. Asymbiotic seed germination, mass propagation and seedling development of *Vanda coerulea* Griff ex. Lindl. (Blue Vanda): An *in vitro* protocol for an endangered orchid. *Scientia Horticulturae*, 128: 325–331.

Seeni, S. and P. G. Latha, 1992. Foliar regeneration of the endangered red Vanda *Renanthera imschootiana* Rolfe (Orchidaceae). *Plant Cell, Tissue and Organ Culture*, 29: 167–172.

Sheelavanthmath, S. S. and N. N. Murthy, 2001. *In vitro* propagation of a terrestrial orchid *Habenaria marginata* Coleb. *Journal of Orchid Society, India*, 15: 85–88.

Sinha, P., M. Firoz Alam and M. Lokman Hakim, 2010. Micropropagation of *Vanda teres* (Roxb.) Lindle. pp. 76-86. In: Jain, M. S. and S. J. Ochatt (eds.). Protocols for *In Vitro* Propagation of Ornamental Plants. *Humana Press, Springer Protocols, UK*, pp. 400.

Sinha, P., M. Hakim and M. F. Alam, 2007. Efficient micropropagation of *Phalaenopsis amabilis* (L) BL. cv. 'Cool Breeze' using inflorescence axis thin sections as explants. *Propagation of Ornamental Plants*, 7: 9–15.

Talukdar, A. 2001. Multiple shoots induction in *Dendrobium aphyllum* Roxb. *Journal of Orchid Society, India*, 15: 35–38.

Van Lee, B., N. T. Hang Phuong, L. T. Anh Hong and K. Tran Thanh Van, 1999. High frequency shoot regeneration from *Rhynchostylis gigantea* (Orchidaceae) using thin cell layers. *Plant Growth Regulation*, 28: 179–185.

Winkelmann, T., G. Thomas and W. Priel, 2006. Commercial *in vitro* plant production in Germany in 1985–2004. *Plant Cell, Tissue and Organ Culture*, 85: 319–327.

Yam, T. W. and J. Arditti, 2018. Orchid micropropagation: An overview of approaches and methodologies. In: Lee, Y. I. and E. C. T. Yeung (eds.). Orchid Propagation: From Laboratories to Greenhouses—Methods and Protocols, *Springer Protocols Handbooks*.

Zakizadeh, S., B. Kaviani and D. Hashemabadi, 2019. Micropropagation of two near threatened orchid. Part 1: *Catasetum pileatum* cv. Alba. *Advances in Horticultural Science*, 33 (4): 475–483.

Zeng, S., K. Wua, J. A. Teixeira da Silva, J. Zhanga, Z. Chena, N. Xiaa and J. Duan, 2012. Asymbiotic seed germination, seedling development and reintroduction of *Paphiopedilum wardii* Sumerh., an endangered terrestrial orchid. *Scientia Horticulturae*, 138: 198–209.

.