International Journal of Agriculture, Environment and Biotechnology
Citation: IJAEB: 9(4): 659-666 August 2016
DOI Number: 10.5958/2230-732X.2016.00086.3

PLANT PHYSIOLOGY

Regeneration of plantlets from *in vitro* Root and Leaf culture of Vandaceous orchid, *Ascocentrum ampullaceum* (Roxb.) Schlter

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Paper No. 489 Received: 22-1-2016 Accepted: 14-8-2016

Abstract

The paper describes *in vitro* culture protocol of commercially important Vandaceous orchid from North East India, *Ascocentrum ampullaceum* using leaves and roots as explants. Leaves and roots of *in vitro* raised 5-6 months old *Ascocentrum ampullaceum* were inoculated in Murashige and Skoog (MS) medium supplemented with additive growth hormone (15%) Coconut milk and growth regulators such as NAA, IAA, BAP and Kn (0.5, 1.0, 2.0, 2.5 mgl^{-1}), both singly and in combination. Leaf culture produced highest protocorm like bodies (Plbs) (40%) in the medium supplemented with 0.5 mgl^{-1} BAP, followed by 30% Plbs supplemented with 15% CM and BAP 2.0 mgl^{-1}, whereas the highest (60%) multiple shoot bud formation was noticed in MS medium supplemented with 15% CM and 0.5 mgl^{-1} BAP, followed by 30% in ½MS medium supplemented with 0.5 mgl^{-1} Kn; Callus formation (30%) was observed in ½MS medium supplemented with 2.0 mgl^{-1} NAA. Roots when cultured in ½ MS, only elongation was observed, but in full strength MS medium, it produced both Plbs and shoot buds. The highest Plbs and shoot buds (40%) was observed in the media supplemented in combination of CM (15%) + BAP (0.5 mgl^{-1}) followed by 25% in CM (15%) + BAP (1.0 mgl^{-1}).

Highlights

- The paper describes *in vitro* culture protocol of commercially important Vandaceous orchid from North East India, *Ascocentrum ampullaceum* using leaves and roots as explants.
- Root culture suggested that quality and quantity of growth regulators play an important role for further development of protocorm like bodies (plbs) and shoot bud.
- Results from leaf culture showed best development of Plbs and shoot bud in MS medium supplemented with 15% CM, BAP and NAA.

Keywords: *Ascocentrum ampullaceum*, *In vitro* culture, Murashige and Skoog medium, Orchid, Regeneration

Ascocentrum ampullaceum (Roxb.) Schlter is an epiphytic orchid belonging to sub-tribe Sarcanthinae under the tribe Epidendreae of the family Orchidaceae. This species is considered extremely rare ornamental plant found in the hills of Manipur and Arunachal Pradesh, India. The species is known for its lovely bloom with erect axillary inflorescence with number of brightly pink coloured flowers and attractive dwarf vegetative form. In case of Vandaceous orchids like *Ascocentrum*, production of seedlings and flowers is still a problem, mainly because of their monopodial habit and long gestation period required for flowering. The shoot meristem culture has now emerged as an important technique for mass multiplication of desired genotypes. The technique, however, requires the sacrifice of the

entire new growth or the only growing point and has a limited utility in monopodium taxa where it endangers the survival of the mother plant. Efforts have, therefore, been directed to identity an alternate but equally effective explant for the purpose. Churchill *et al*. (1972) applied root culture technique for the first time in orchid micropropagation in an attempt to induce *in vitro* formation of protocorm like bodies (plbs) of *Epidendrum*. Later on various authors used other meristematic tissues from leaf, root tips, flower bud, inflorescence, seeds etc. (Chaturvedi and Sharma 1986; Ahmed *et al.* 1998; Rao *et al.* 1998; Hegde and Sakia 2001; Sinha and Hegde 2001; Deb *et al.* 2006; Shadang *et al.* 2007a,b, 2009). Almost every morphological structure in higher plants, owing to its cellular totipotency characteristics, can be induced to dedifferentiate into an unorganized callus, produce protocorms and can be maintained indefinitely on a nutrient medium of appropriate constitution. The potential of leaf tissue to produce protocorms like bodies (Plbs) *in vitro* was first demonstrated by Wimber (1965) in *Cymbidium* culture. This opened up new possibilities in orchid regeneration. Arditti (1977) indicated at the possibility of raising a large number of identical clones from single leaf through direct or callus-mediated organogenesis. The present investigation was undertaken so as to develop high frequency plantlet formation of *Ascocentrum ampullaceum*, an economically important orchid from North East India, using *in vitro* culture of root and leaf as explants.

Materials and Methods

In order to find out the possibilities of micropropagation using root tips and leaves, *in vitro* grown seedlings were used. The explants were rinsed in the sterile double distilled water for 2-3 min and placed over sterile filter paper in order to absorb remaining water. Cut the explants into 0.5-1.0 cm with sterilized surgical blade and peeled the leaf with the help of forceps. Finally, the leaf explants were inoculated in basal Murashige and Skoog (MS) and ½MS media supplemented with 2% sucrose and pH maintained at 5.6. (control). Root segment, entire and peeled off leaves were inoculated into MS and ½MS medium supplemented with α-Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), Benzyl amino purine (BAP) and Kinetin (Kn) in various concentrations (0.5, 1.0, 2.0, 2.5 mgl^{-1}), either singly or in combination, along with additive growth hormone (15% Cocunut Milk, CM). The cultures were incubated at 25°C ± 1°C under 14 h photoperiod (50 µmol $m^{-2}s^{-1}$ light intensity). The experiments were repeated thrice with several replicates per treatment, and observations recorded. Statistical analysis was done for one way and two way ANOVA following SYSTAT 10 package.

Results and Discussion

In the nutrient media tested, root tips elongation was observed but none showed any other morphological changes (Plate I; Fig: a). The highest Plbs (40%) and shoot buds (30%) was observed in the media supplemented in combination of CM (15%) and BAP (0.5 mgl^{-1}) followed by 25% in CM (15%) and BAP (1.0 mgl^{-1}). (Table 1; Plate I; Fig. b, c and d). Development of shoot buds from entire leaf and peeled leaf was observed in ½MS after 30 days, but after 50 days the shoots became dark brown and died. The inoculated peeled leaf remained unchanged till 40 - 50 day, then became yellowish and died (Table 2).

Culturing of entire leaf in ½MS medium supplemented with NAA, IAA, BAP and Kn in various concentrations (0.5, 1.0, 2.0 mgl^{-1}), both singly and in combination, responded differently. The highest (40%) response for Plbs and shoot bud formation was observed in presence of BAP (0.5 mgl^{-1}), callus (20%) in NAA (1.0 mgl^{-1}), shoots buds (30%) in Kn (0.5 $mgl^{-1.}$), and no response at all in BAP (1.0, 2.0 mgl^{-1}) and kn (2.0 mgl^{-1}). Plbs, callus and shoot bud (30%) was observed in the NAA (2.0 mgl^{-1}) supplemented media. (Table 3; Plate II; Fig. a & b). Different responses were observed in the ½ MS medium when supplemented in combination of NAA and BAP. Plbs (25%) were obtained in the medium supplemented with NAA and BAP (0.5 mgl^{-1} each). The highest (25%) shoot was induced in combination of NAA (1.0 mgl^{-1}) and BAP (2.0 mgl^{-1}) (Table 4; Plate II; Fig. c & d).

When coconut milk (15%) was added to the media with entire leaf cultured, shoot bud response was best observed in presence of 0.5 mgl^{-1}BAP. The combined effect of BAP and NAA on shoot bud was less marked. However, both Plbs and shoot buds were formed in the medium supplemented with 15% CM, 1.0 mgl^{-1} BAP, 0.5 mgl^{-1} NAA (20%). NAA

Table 1: Root culture of *Ascocentrum ampullaceum* in MS media supplemented with combination of NAA, BAP and CM

Nutrient Medium	Growth regulators (mgl^{-1})			Degree of response		
MS	15% CM	BAP	NAA	Callus	Plbs	Shoot bud
		0.5		-	40	30
		1.0		-	25	-
		2.0		-	5	5
			0.5	-	-	-
			1.0	-	-	-
			2.0	-	-	-
		0.5	0.5	-	-	-
		1.0	0.5	-	10	-
		2.0	0.5	-	-	-
		0.5	1.0	-	-	-
		1.0	1.0	-	-	-
		2.0	1.0	-	20	-
		0.5	2.0	-	10	-
		1.0	2.0	-	-	-
		2.0	2.0	-	-	-

Plbs responses: n = 150, df = 14, F= 85.668, $p < 0.001$ [p = 0.000]

Shoot bud responses: n = 150, df = 14, F = 73.929, $p < 0.00$ [p = 0.000]

Based on response of callus, Plbs and shoot bud formation among all the replicates, % response is mentioned. (-) No response; (+) Response < 49 %.

BAP: Benzyl amino purine; CM: Coconut Milk; mgl^{-1}: Milligram per liter; MS: Murashige and Skoog; NAA: α-Naphthalene acetic acid.

Table 2: Entire and peel off Leaf culture responses of *Ascocentrum ampullaceum* in MS and half strength MS basal media

Nutrient Media	Explants	Observation for Callus/Plbs/ Shoot bud formation (Days after)	% Response	Degree of response
MS	Entire leaf	15	0	Remained unchanged
		30	0	Remained unchanged
		40	0	Remained unchanged
		50	0	Remained unchanged
		60	0	Remained unchanged
		70	0	Explants become yellowish
		80	0	All died
	Peeled Leaf	15	0	Remained unchanged
		30	0	Remained unchanged
		40	0	Remained unchanged
		50	0	Explants become yellowish
		60	0	All died
½ MS	Entire leaf	15	0	Remained unchanged
		30	5	Shoot bud from base
		40	0	Remained unchanged
		50	0	The shoot become dark brown
		60	0	Remained unchanged

	70	0	Remained unchanged
	80	0	All died
Peeled leaf	15	0	Remained unchanged
	30	0	Remained unchanged
	40	0	Remained unchanged
	50	0	Explants become yellowish
	60	0	All died

Table 3: Entire Leaf culture response of *Ascocentrum ampullaceum* in half strength MS medium supplemented with various concentrations of auxin and cytokinin

Nutrient Medium	Growth regulators (mgl^{-1})				Degree of response		
½ MS	BAP	NAA	IAA	Kn	Callus	Plbs	Shoot buds
	0.5				-	40	30
	1.0				10	10	-
	2.0				-	-	-
		0.5			10	-	10
		1.0			20	-	-
		2.0			30	20	20
			0.5		-	-	10
			1.0		5	-	-
			2.0		-	-	5
				0.5	-	-	30
				1.0	-	-	-
				2.0	-	-	-

Callus development: n = 120, df = 11, F = 61.003, $p < 0.001$ [p = 0.000]

Plbs development: n = 120, df = 11, F = 103.807, $p < 0.001$ [p = 0.000]

Shoot bud responses: n = 120, df = 11, F = 71.636, $p < 0.001$ [p = 0.000]

Based on response of callus, Plbs and shoot bud formation among all the replicates, % response is mentioned. (-) No response; (+) Response < 49 %.

Table 4: Leaf culture of *Ascocentrum ampullaceum in* half strength MS medium supplemented with NAA and BAP

Nutrient Medium	Growth regulators (mgl^{-1})		Degree of responses		
½ MS	NAA	BAP	Callus	Plbs	Shoot buds
	0.5	0.5	-	15	25
	1.0	0.5	-	-	20
	2.0	0.5	-	-	20
	0.5	1.0	-	-	20
	1.0	1.0	-	-	-
	2.0	1.0	-	-	-
	0.5	2.0	-	-	25
	1.0	2.0	-	-	-
	2.0	2.0			

Plbs responses: n = 90, df = 8, F 101.250, $p < 0.001$ [p = 0.000]

Shoot bud responses: n = 90, df = 8, F = 35.779, $p < 0.001$ [p = 0.000]

Based on response of callus, Plbs and shoot bud formation among all the replicates, % response is mentioned. (-) No response; (+) Response < 49 %.

Plate 1

Fig a: ½ MS supplemented with various strength of NAA and BAP. Elongation observed

Fig b: MS media supplemented with CM (15%) + BAP (0.5 mgl^{-1}). Plbs and shoot buds responded.

Fig c: MS media supplemented with CM (15%) + BAP (0.5 mgl^{-1}). shoot buds responded

Fig d: MS media supplemented with CM (15%) + BAP (1.0 mgl^{-1}). Highest Plbs responded.

BAP: Benzyl amino purine; IAA: Indole-3-acetic acid; Kn: Kinetin; mgl^{-1}: Milligram per liter; MS: Murashige & Skoog; NAA: α-Naphthalene acetic acid.

Plate 2

Fig.a: ½ MS + NAA (1.0 mgl-1). Highest callus responded.

Fig. b: ½ MS + Kn (0.5 mgl-1). Shoot buds responded.

Fig. c: ½ MS + NAA + BAP (0.5mgl-1 each). Plbs responded.

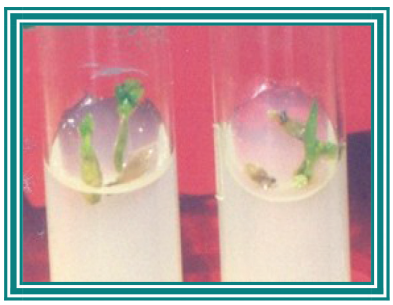

Fig. d: ½ MS + NAA (1.0 mg/l-1) +BAP (0.5mgl-1). Plbs responded

Fig.e: MS + 15% CM + BAP (0.5 mgl-1). Highest shoot buds responded.

Fig. f: MS + 15% CM + BAP (1.0 mgl-1) +NAA (0.5mgl-1). Plbs and shoot responded.

Table 5: Entire Leaf culture of *Ascocentrum ampullaceum* in MS medium supplemented with combination of NAA, BAP and CM

Nutrient Medium	Growth regulators (mgl^{-1})			Degree of response		
MS	15% CM	BAP	NAA	Callus	Plbs	Shoot bud
		0.5	-	-	-	60
		1.0	-	-	-	20
		2.0	-	-	-	10
		-	0.5	-	-	-
		-	1.0	-	10	-
		-	2.0	-	30	-
		0.5	0.5	-	-	-
		1.0	0.5	-	20	20
		2.0	0.5	-	-	20
		0.5	1.0	-	-	-
		1.0	1.0	-	-	-
		2.0	1.0	-	-	20
		0.5	2.0	-	10	-
		1.0	2.0	-	-	-
		2.0	2.0	-	-	-

Plbs responses: n = 150, df = 14, F= 52.625, $p < 0.001$ [p = 0.000]

Shoot bud responses: n = 150, df = 14, F = 68.491, $p < 0.001$ [p = 0.000]

Based on response of callus, Plbs and shoot bud formation among all the replicates, % response is mentioned. (-) No response; (+) Response < 49%; (++) Response > 50%

($2.0\ mgl^{-1}$) could show some effect by producing shoot bud (30%). (Table 5; Plate II; Fig. e & f).

Elongation of root tips in ½MS medium supplemented with BAP and NAA was observed. There was no formation of callus, shoot bud and plbs. Similar result was noticed in root tip culture of *Aerides rosea* when inoculated in ½MS medium, which failed to induce morphogenetic changes except for elongation (Sinha 2000). But when root tips were cultured in full strength MS medium, it produced both Plbs and shoot buds; the root tip culture produced better response in terms of Plbs (40%) and shoot bud (30%) formation. Paek *et al.* (1990), Yam and Weatherhead (1991) also found that development of callus and shoot bud from root tip of *Bletilla atriata*, *Cleisostoma fordii* and *Pholidota*

chinensis was favoured in MS medium. In the present study, Plbs and shoot buds were produced more when BAP and NAA were supplemented in the medium singly along with 15% CM, rather than in combination.

In leaf culture, highest Plbs (40%) was observed in ½MS medium supplemented with 0.5 mgl^{-1} BAP, followed by 30% Plbs in MS medium supplemented with 15% CM and BAP 2.0 mgl^{-1}, whereas highest (60%) multiple shoot bud formation was noticed in MS medium supplemented with 15% CM and 0.5 mgl^{-1} BAP, followed by 30% in ½MS medium supplemented with 0.5 mgl^{-1} Kn; Callus formation (30%) was observed in ½MS medium supplemented with 2.0 mgl^{-1} NAA. However, Mathew and Rao (1985) reported that auxin or cytokinin alone had no role in stimulating proliferation of leaf culture, and the explants produced Plbs and callus only in the medium supplemented with both auxin and cytokinin. However, in the present investigation, enrichment of ½ MS medium with NAA and BAP in combination did not increase the response. Leaves from *in vitro* source of *Aerides maculosum* failed to respond regardless of the chemical stimulus in the nutrient pool (Kulkarni and Surwase 1998). The highest response of *Aerides rosea* and *Renanthera imschootiana* leaf culture produced Plbs and shoot when supplemented with BAP in MS medium. (Sinha 2000).

Thus, present experiment with root tip culture suggested that quality and quantity of growth regulators play an important role for further development of Plbs and shoot bud, whereas results from leaf culture showed best development of Plbs and shoot bud in MS medium supplemented with 15% CM, BAP and NAA.

Acknowledgements

Grateful thanks are due to the Department of Biotechnology, Govt. of India, New Delhi for the financial assistance to carry out this work. Thanks are also due to the Director, State Forest Research Institute (SFRI), Itanagar for facilities and encouragement.

References

Ahmed, N., Rao, A.N. and Hegde, S.N. 1998. Clonal propagation of Renades Arunodaya through aseptic culture of inflorescence. *Arunachal Forest News* **16**(1/2): 25-31.

Arditi, J. 1977. Clonal propagation of orchids by means of leaf cultures *in vitro*. *The Orchids Rev* **85:** 102-103.

Chaturvedi, H.C. and Sharma, A.K. 1986. Mericloning of orchids through culture of tip of leave and roots. In: Biology Conservation and Culture of Orchids (ed. S.P. Vij). Affiliated East-West Press, New Delhi, pp 469-478.

Deb, C.R. and Temjensangba 2006. *In Vitro* mass multiplication of a rare and threatened monopodial orchid, *Arachnis labrosa* (Lindl. Ex paxt.) Reichb. using alternative explant sources. In: Proc 8th Natl. Sem. on Orchid Conservation, Improvement and Commercialization, organized by the Orchid Soc. of India, Dept. of Botany, Punjab University, Chandigarh, India, 18-20 March, 2006, p. 43.

Hegde, S.N. 2001. Orchids from lab to field in Arunachal Pradesh. *Arunachal Forest News* **9**(1)**:** 27-31.

Hegde, S.N. and Saikia, D.K. 2001. Cultivation of Dendrobiums in Arunachal Pradesh. In: Orchids Conservation, culture, farming and trade (ed. S.N. Hegde). Himalaya Publisher, Itanagar, India.

Kulkarni, A.R. and Surwase, B.S. 1998. *In vitro* propagation of *Aerides maculascum* Lindl. *J Orchid Soc India* **12:** 47-51.

Mathew, V.H. and Rao, P.S. 1985. *In vitro* culture of *Vanda* Hybrid (Vanda TMA x Vanda Miss Joaquim). II. Studies on seedling explants. *Proc Indian Natl Sci Acad* **51:** 496-501.

Paek, K.Y., Shim, G.B. and Kim, J.J. 1990. Exploitation of temperate *Cymbidiums* and establishment of micropropagation system-II. Effect of natural products and BAP exposure period on organogenesis from rhizome of temperate *Cymbidium*. *J Kor Soc Hort Sci* **311:** 74-80.

Rao, A.N., Ahmed, N. and Hegde, S.N. 1998. Culture notes from ORDC, Tipi, *in vitro* culture of some rare and endangered orchids of Arunachal Pradesh. Souvenir, International Festival of Orchids, Itanagar, p. 16-27.

Shadang, R., Padmanabh Dwivedi, Hegde, S.N. and Ahmed, N. 2007a. Effects of different culture media on seed germination and subsequent *in vitro* development of protocorms of *Hygrochilus parishii* (Veith & Rchb.f.) Pfitz (Orchidaceae). *Indian J Biotech* **6:** 256-261.

Shadang, R., Padmanabh Dwivedi and Hegde, S.N. 2007b. Shoot formation through *in vitro* culture of axillary buds in *Ascocentrum ampullaceum* (Roxb.) Schltr. *J Orchid Soc. India* **21:** 71-73.

Shadang, R., Padmanabh Dwivedi and Hegde, S.N. 2009. Regeneration competence of *Hygrochilus parishii* inflorescence segments and axillary buds: a study *in vitro*. *J Orchid Soc India* **23:** 75-78.

Sinha, S.K. 2000. *In vitro studies on the effect of Colchicine treatment and Gamma irradiation in Renades 'Arunodaya' Hybrid and its parent species Aerides rosea Loddiges Ex Paxt.* (A. Williamsii Warn.*) and Renanthera imschootiana* Rolf. Ph. D. Thesis. Gauhati University, Guwahati, India.

Sinha, S.K. and Hegde, S.N. 2001. Mericloning *Renades* 'Arunodaya' through root-tip culture. In: Conservation, Culture Farming and Trade (ed. S.N. Hegde). Himalaya Publisher, Itanagar, India, pp 107-111.

Wimber, D.E. 1965. Additional observations on clonal propagation of *Cymbidium* through culture of shoot-meristem. *Cym Soc News* **20:** 7-10.

Yam, T.W. and M.A. Weatherhead. 1991. Root-tip culture of several native orchid of Hong Kong. *Lindleyana* **6**(3): 151-153.