International Journal of Science and Research (IJSR)
ISSN (Online): 2319-7064
Index Copernicus Value (2013): 6.14 | Impact Factor (2015): 6.391

Role of Basal Media, Carbon Sources and Growth Regulators in Micropropagation of Two Valuable Medicinal Orchids of Bangladesh

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Abstract: ***An efficient, protocol for in vitro germination seeds of and micropropagation of two the valuable indigenous medicine Orchids namely; Bulbophyllum lilacinum Ridl. and Cymbidium aloifoloum (L) Sw. were developed by enhanced shoot multiplication, nodal and leaf segments was designed. The medium type and various carbon sources, plant growth regulators markedly influenced in vitro propagation of B. lilacinum Ridl. and C. aloifoloum (L) Sw. The in vitro protocorms production system was investigated on three 0.8% (w/v) agar solidified basal media namely; Phytomax Sigma Chemical Co. (PM), Modified Vacin and Went (MVW), Murashige and Shook,1962 (MS) medium with the synergistic combination of Auxin (IAA 1.0-2.0 mgl^{-1}, IBA 2 mgl^{-1}, NAA 1.0-2.0mgl^{-1}, 2,4-D 1.0mgl^{-1}) , Cytokinins (BAP 0.5-2.0mgl^{-1}, Kn 0.5-2mgl^{-1}) and 2% -3% sucrose which induced maximum number of shoots as well as beneficial shoot length. Sub culturing of nodal segments and leaf segments on similar medium enabled continuous production of healthy shoots with similar frequency. Rooting was highest (2.96 cm) on half strength MS medium containing 1.5% sucrose. The in vitro growing seedling were then transferred to outside natural environment through successive phases of acclimatization.***

Keywords: Micropropagation, Protocorms, *Bulbophyllum lilacinum, Cymbidium aloifoloum,* Shoot multiplication and Growth regulators

1. Introduction

Orchids are economically important group of plants for their uses in floriculture, medicine and food industries. The diversity and population of orchid is decreasing due to human activities such as habitat destruction, degradation and fragmentation and over harvesting of selected orchids for commercial trade (Pant *et al.,* 1999). Highly exploited species immediately require *ex situ* conservation by tissue culture method.

The application of tissue culture in multiplication of orchids dates back to second decade of twentieth century. Knudson (1922) became successful in germination of *Cattleya*, *Laclia* and *Epidendrum* seeds on agar medium containing sugar and concluded that mycohorizal fungus is not obligatory for orchid seed germination. After this discovery many other scientists became successful in *in vitro* germination of orchid seeds. At present a good number of improved media are used for *in vitro* germination of orchid seeds.

Micropropagation of orchids can also be done with the use of the aseptically grown seedling. The history of *in vitro* micropropagation of orchids through tissue culture dates back to 1960`s when Morel (1960) developed the technique of shoot apex culture in *Cymbidium* sp subsequently Ito (1960), Sagawa (1962), and Isreal (1963) demonstrated that *in vitro* germinated seedlings could be used in micropropagation. Later on different kinds of explants viz. shoot tips (Agrawal *et al*., 1992; Nagashima, 1999; Seeni and Lath, 2000), axilary buds (Wang,1988), root tips (Sanchez,1988), Leaf and nodal segments (Barua and Bhadra , 1999) rhizome tips (Takahashi, 1998; Sheelavantmath *et al*., 2000) were used for quick propagation of many commercially important orchid species .

The plant growth regulators has an important role on growth and differentiation of cultured tissues and explants under *in vitro* conditions accordingly different kinds of PGRs of different nature are used in tissue culture media. The process of differentiation has been proved to be dependent of the ratio of auxins and cytokinins used in the culture media. In case of orchids tissue culture a good number of experiments have been carried out for understanding the role of different PGRs of auxin, and cytokinin group on the regeneration of different orchids from cultured tissues and organs.

Although the research in the area of orchid tissue culture has progressed tremendously in many countries, in Bangladesh only a very limited number of research study in this area have so for been carried out. The main constraints in the area of orchid micropropagation in Bangladesh area:

In view of the importance of rapid propagation of different indigenous orchids of Bangladesh, this research programme was undertaken with a view to develop a reproducible micropropagation technique for rapid multiplication of two medicinally important orchid species namely;, *Bulbophyllum lilacinum,* and *Cymbidium aloifolium* . The technique of *in vitro* seed germination, elongation, rooting and establishment in outside environment will be developed.

2. Materials and Methods

Plant Material

Selected two different indigenous orchids species namely; *Bulbophyllum lilacinum* Ridl. and *Cymbidium aloifolium* (L.) Sw, were selected for the study. The fruits of these species were collected from naturally grown plants of different region of Bangladesh, especially from Chittagong and Cox's Bazar, Collected fruits of were washed with

running tap water to remove dust particles and then washed with sterile distilled water 3-4 times. The capsule was surface sterilized by immersing it in the solution of 0.2% (w/v) $HgCl_2$ for 10 min, 70% ethanol for 1 min and then washed for 2-3 times with double sterile distilled water.

In case of all species, the technique of *in vitro* seed germination and micropropagation were developed. Three different media namely, MS (Murashige and Skoog, 1962 with 3% (w/v) sucrose, PM (Phytamaxe) with 2% (w/v) sucrose and MVW (Modified after Vacin and Went 1949) with 2% (w/v) sucrose were used for the aseptic culture of the seeds of *Bulbophyllum lilacinum* Ridl. and *Cymbidium aloifolium* (L.) Sw. For micropropagation MS and PM media were used as basal media with different combinations of different PGRs. The P^H of all media was adjusted to 5.8 before autoclaving. Agar (0.8% w/v) was added as a gelling agent. Agar was dissolved by boiling the mixture and about 20ml media was dispensed into each culture tube (150 x 25mm) and autoclaved at 120 ^{0}C for 15 min/15lb. The cultures were maintained at 25±2 ^{0}C and 200-300 lux under 16 hrs photoperiod.

Subculture of seedlings
When orchid seeds germinated and protocorms came out, these were taken out aseptically from the culture vessels and the masses of protocorms were sub cultured at a lower density on the same culture media for further growth. The sub-cultured materials were maintained in the same culture room under the same light and temperature conditions as used for germination. Further sub culturing was done at every 20-25 d interval when the masses of seedlings were further thinned.

Subculture of tiny seedlings for rapid elongation
Although in germination media the seedlings continued its growth but the growth rate was very slow. In order to induce rapid elongation, seedlings with 1-2 leaves were transferred to the elongation media. Here also regular subculturing was done at a lower density.

Subculture of seedlings for induction of better root system
In elongation media the seedlings produced weak root system. In order to induce strong and stout root system, the seedlings of 2-3 cm size were aseptically taken out of the culture vessels, separated from each other and sub-cultured on rooting media. Culture vessels were maintained in the same conditions.

Transplantation and establishment of *in vitro* grown seedlings outside of the culture room
In rooting medium the plantlets grew further and developed good root system, these were taken out of the cultural vessels and shifted out side the culture room following successive phases 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 in the third day. Finally the seedlings were taken out of the culture vessels and washed with running tap water for removal of agar attached to the roots. The seedlings were then transferred to small pots containing moisten coconut coir, coir dust and coal where those were grown for 20-30d. Finally the seedlings were transferred to wooden orchid pots filled up with moistened coconut coir, coal and small brick pieces. Here the seedlings eventually grew well.

Culture of explants of *in vitro* grown seedlings for micropropagation
Different explants *viz.* nodal and leaf segments of *in vitro* grown seedlings were used for micropropagation. For the purpose 0.8% (w/v) agar solidified MS, PM and MVW media supplemented with 2-3% (w/v) sucrose and different concentrations and combinations of PGRs were used.

3. Results and Discussion

In vitro plant tissue culture is the most commercially successful aspect of plant biotechnology which has introduced an exciting new phase into plant propagation (Prakash *et al.*,1996) and orchid is the first horticultural plant multiplied by tissue culture methods on a commercial scale. Many researchers have reviewed the strategic approaches that may be applied in the development of reliable orchid regeneration system (Goh 1990; Sagawa 1990; Arditti and Ernst 1993; Prakash *et al.*, 1996). In Bangladesh Ismat (1987); Hoque *et al.*, (1994) and Das Gupta and Bhadra (1998) reported *in vitro* seed culture and propagation of *Cymbidium.* Das Gupta *et al.*, (1998) reported on micropropagation of *Dendrobium crepidatum* but growth performance of survived plants and their flowering condition were not mentioned. Very recently a number of NGOs and private horticulture farms are trying to establish orchid industry in Bangladesh.

The present research study was under taken to develop protocols for rapid *in vitro* germination of important medicinal orchids species namely *Bulbophyllum lilacinum* Ridl. and *Cymbidium aloifolium* (L.) Sw. In the present investigation three different kinds of basal media, namely; MS, PM and MVW were used for *in vitro* germination of all selected orchid species. The results on germination indicate that PM was better than other two media for all these selected orchid species (Table 1). The germination of orchids seeds are medium dependent and interact with genotype. The emerged protocorms turned in to mini seedling and continued their growth in the germination media very slowly. For enhancement of growth rate, germinated seedlings were transferred to elongation media. Different types of PGRs (BAP, NAA, IBA, IAA, Pic. Kn and 2,4-D) and various combinations and concentration were used for the purpose (Table 2 &4). Different hormone combination were found to be better for elongation of different species of *Bulbophyllum lilacinum* on MS + 1.0 mgl^{-1} IAA + 2.0 mgl^{-1} BAP + 3% (w/v) sucrose and *Cymbidium aloifolium* (L.) Sw. on MS + 2.0 mgl^{-1} NAA + 3.0 mgl^{-1} BAP + 1.0 mgl^{-1} Kn + 3% (w/v) sucrose was better for elongation. It is relevant to mention here enhancement of the elongation of germinated seedling has been done with the supplementation of plant growth regulators in the medium in many orchid species (Barua and Bhadra,1999; Kusumoto, 1980; Kokubu *et al.*, 1980; Geetha and shetty, 2000).

Although the seedlings developed roots in germination and elongation media, those were very weak. In order to induce strong and stout root system the seedling of two species namely; *Bulbophyllum lilacinum* Ridl. and *Cymbidium aloifolium* (L.) Sw. were transferred to two different rooting media while one Half MS + 1.5% (w/v) sucrose was most effective for development of strong root system. Another media MS + 0.5 mgl^{-1} IAA + 3% (w/v) sucrose was proved most efficient for induction of root system (Table 2). It is indicated that for induction of strong and stout root system either stress condition or auxin supplements promoted root growth. Similar finding was also noted in same orchid species by Gupta *et al.*, (1998) and Bhadra *et al.*, (2002).

Different explants such as nodal segment and leaf segment were used of *in vitro* culture of seedling on different media fortified with different combination and concentration of PGRs. The efficiency of the media was estimated in terms of number of multiple shoot buds produced from each explants. The overall results MS + 2.0 mgl^{-1} BAP + 1.5 mgl^{-1} IAA + 3% (w/v) sucrose, MS + 1.0 mgl^{-1} IAA + 1.0 mgl^{-1} Kn + 2.0 mgl^{-1} BAP + 3% (w/v) sucrose, MS + 2.0 mgl^{-1} BAP + 1.5 mgl^{-1} IAA + 3% (w/v) sucrose, MS +1.0 mgl^{-1} NAA + 2.00 mgl^{-1} BAP + 3% (w/v) sucrose. In case of *C. aloifolium* multiple shoot buds obtained from both nodal segment and leaf segment when grow on MS +1.0 mgl^{-1} BAP + 2.0 mgl^{-1} IAA + 3% (w/v) sucrose and MS + 2.5 mgl^{-1} NAA + 3% (w/v) sucrose(Table 4). Similar result from other plant have been reported previously by many scientist (Bhadra *et al.*,2002; Bhadra and Hossain 2003).

In order to induce good root system the multiple shoot buds at a height of 1 to 2 cm were individually cultured on two different 0.8%(w/v) agar solidified media namely; i) Half strength MS + 1.5% (w/v) sucrose ii) Full strength MS + 0.5 mgl^{-1} NAA + 3% (w/v) sucrose. Half strength MS + 1.5% (w/v) sucrose was more effective for rooting in case of all these species(Table 5). Similar results was noted by Bhadra *et al.*, (2002); Bhadra *et al.*, (2006). *In vitro* developed seedlings were finally transferred to outside and acclimatized in natural environment through sequential steps of acclimatization. The survival rate was 50% for *C. aloifolium*, 36% for *B. lilacinum* respectively. Similar finding was also noted in same orchid species by Geetha and shetty (2000), Seeni and Latha (2000) and Teixira da Silva *et al.*, (2006). This protocol can be used in commercial and research laboratories for both rapid propagation and conservation of this medicinal orchid species.

4. Conclusion

The results indicated that the two species 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.

Table 1: Percentage of *in vitro* germination of seeds of two orchid species on 0.8% (w/v) agar solidified MS, PM and MVW basal media

Name of the species	Name of medium	% of seed germination	Time required for germination (days)	Remarks
Bulbophyllum lilacinum	MS	-	-	Not germinated.
	PM	80	30-35	Green seedlings were produced.
	MVW	-	-	Not germinated.
Cymbidium aloifolium	MS	90	30-35	Green protocorms were produced
	PM	90	30-35	Green seedlings were produced
	MVW	-	-	Not germinated.

'-' Indicates no germination

Table 2: Results * on the elongation of the germinated seedlings of *Bulbophyllum lilacinum* Ridl., *Cymbidium aloifolium* (L.) Sw. and on 0.8% (w/v) agar solidified media supplemented with different kinds of PGRs.

Culture media	Seedlings length (cm)** after 60d of germination (mean length)		Seedlings length (cm) after 30d of culture on elongation medium(mean length)		Seedlings length(cm) increased within 30d of culture on elongation medium	
	B lilacinum	*C aloifolium*	*B lilacinum*	*C aloifolium*	*B lilacinum*	*C aloifolium*
MS + 2.0 mgl-^{1}IAA + 3% (w/v) sucrose	1.20	1.40	2.30	2.70	1.10	1.30
MS + 1.0 mgl^{-1} IAA + 2.0 mgl^{-1} BAP + 3% (w/v) sucrose	1.30	1.50	3.10	3.40	1.80	1.90
MS + 2.0 mgl^{-1}BAP + 3% (w/v) sucrose	1.20	1.50	2.50	2.90	1.30	1.40
MS + 3.0 mgl^{-1}BAP + 1.0 mgl^{-1} NAA + 1.5 mgl^{-1} Kn + 3% (w/v) sucrose	1.20	1.50	2.60	3.10	1.40	1.60
MS + 2.0 mgl^{-1} NAA + 3.0 mgl^{-1} BAP + 1.0 mgl^{-1} Kn + 3% (w/v) sucrose	1.20	1.40	1.80	3.50	2.00	2.10
MS + 2.0 mgl^{-1} NAA + 2.0 mgl^{-1} BAP +3% (w/v) sucrose	1.40	1.40	2.70	2.70	1.30	1.30
MS + 1.0 mgl^{-1} NAA + 0.5 mgl^{-1} BAP + 3% (w/v) sucrose	1.2	1.40	2.6	3.10	1.4	1.70
MS + 2.0 mgl^{-1} BAP + 0.1 mgl^{-1} Pic + 3% (w/v) sucrose	1.40	1.40	3.00	3.10	1.60	1.70
PM + 1.0 mgl^{-1} 24-D + 2.0 mgl^{-1} Kn + 3% (w/v) sucrose	1.5	1.50	2.6	3.40	1.10	1.90
PM + 2.0 mgl^{-1} IBA + 2% (w/v) sucrose	1.20	1.50	1.90	2.80	0.70	1.30
PM + 2.0 mgl^{-1} BAP + 2% (w/v) sucrose	1.20	1.50	2.20	2.70	1.00	1.20

***Based on observations recorded from 50 seedlings taking 5 at random from each of 10 culture vessels.**
**** Only the length of shoot system.**

Table 3: Results* on the induction of roots in *Bulbophyllum lilacinum* and *Cymbidium aloifolium* seedlings when grown on 0.8% (w/v) agar solidified rooting media.

	Rooting media	Seedling roots length (cm) and number before culture on rooting medium (mean value)		Seedling roots length (cm) and number after 30 d of culture on rooting medium (mean value)		Seedling roots length (cm) and number increased after 30 d of culture on rooting medium (mean value)	
		Length (cm)	No.of roots / seedling	Length (cm)	No.of roots / seedling	Length (cm)	No.of roots / seedling
Bulbophyllum lilacinum	**Half strength MS + 1.5% (w/v) sucrose**	**1.65**	**3.25**	**2.95**	**5.00**	**1.60**	**1.95**
	MS + 0.5 mgl^{-1} IAA+ 3% (w/v) sucrose	1.52	3.65	2.60	5.05	1.28	1.60
Cymbidium aloifolium	**Half strength MS + 1.5% (w/v) sucrose**	**1.65**	**2.65**	**2.97**	**5.58**	**1.52**	**2.43**
	MS + 0.5 mgl^{-1} IAA+ 3% (w/v) sucrose	1.52	2.43	2.62	4.56	1.30	2.33

***Based on observations recorded from 50 seedlings taking 5 at random from each of 10 culture vessels.**

Table 4: Results** on the development of multiple shoot buds in nodal and leaf segments of *Bulbophyllum lilacinum* Ridl. and *Cymbidium aloifolium* (L.) Sw. on 0.8% (w/v) agar solidified media supplemented with different PGRs.

Culture media	*Name of the explants**		*Days (d) required for initiation and sprouting of multiple shoot buds.*		*Number of shoot buds sprouted (mean/explants)*	
	B lilacinum	*C aloifolium*	*B lilacinum*	*C aloifolium*	*B lilacinum*	*C aloifolium*
MS + 2.0 mgl^{-1} 2,4-D + 0.5 mgl^{-1} Kn + 3% (w/v) sucrose	NS	NS	45-50	35-40	2.60	4.35
	LS	LS	-	-	-	-
MS + 2.0 mgl^{-1} BAP + 1.5 mgl^{-1} IAA + 3% (w/v) sucrose	NS	NS	40-45	35-40	3.50	4.75
	LS	LS	-	-	-	-
MS + 2.0 mgl^{-1} BAP + 2.0 mgl^{-1} NAA + 3% (w/v) sucrose	NS	NS	40-45	30-35	2.80	4.40
	LS	LS	-	-	-	-
MS + 2.0 mgl^{-1} NAA + 3% (w/v) sucrose	NS	NS	40-45	30-35	2.50	4.40
	LS	LS	-	-	-	-
MS + 1.0 mgl^{-1} NAA + 0.5 mgl^{-1} BAP + 3% (w/v) sucrose	NS	NS	40-45	35-40	2.70	4.35
	LS	LS	-	-	-	-
PM + 1.0 mgl^{-1} BAP + 0.5 mgl^{-1}IAA + 2% (w/v) sucrose	NS	NS	40-45	30-35	2.70	4.50
	LS	LS	-	-	-	-
PM + 2.0 mgl^{-1} BAP + 1.5 mgl^{-1}IAA + 2% (w/v) sucrose	NS	NS	40-45	40-45	2.70	4.35
	LS	LS	-	-	-	-

***NS= Nodal Segment, LS= Leaf Segment, '-' Indicates no response**
****Based on observations recorded from 20 cultured segments.**

Table 5: Results* on the development of roots of individual multiple shoot buds of *Bulbophyllum lilacinum and Cymbidium aloifolium* when grown on 0.8% (w/v) agar solidified rooting media

Name of the species	Rooting media	Roots length (cm) after 30d of culture on rooting medium (mean value)	Mean number of root/seedlings
Bulbophyllum lilacinum	MS + 0.5 mgl^{-1} IAA+ 3% (w/v) sucrose	2.2	3.5
	Half strength MS + 1.5% (w/v) sucrose	**2.4**	**3.8**
Cymbidium aloifolium	MS + 0.5 mgl^{-1} IAA + 3% (w/v) sucrose	2.36	2.86
	Half strength MS + 1.5% (w/v) sucrose	**2.96**	**3.8**

***Based on observations recorded from 25 seedlings taking 5 at random from each of 5 culture vessels.**

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