Propagation of Ornamental Plants
Vol. 00, № 0, 2000: 00-00

IN VITRO CONSERVATION OF ENDANGERED ORCHID *BULBOPHYLLUM AURICOMUM* LINDL., THE ROYAL ORCHID OF MYANMAR

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Abstract

In vitro conservation method of *Bulbophyllum auricomum* Lindl. has been developed in the present study. Minimum shoot length (7.68 ± 0.71 mm) and least root formation (at least 1 root/plantlet) were achieved when protocorm-derived shoots were cultured continuously on quarter strength MS (Murashige and Skoog 1962) medium with 60 g l^{-1} sucrose for 12 months. In contrast, when the explants were maintained on full strength MS nutrient medium supplemented with 30 g l^{-1} sucrose, the cultures grew faster, filled the culture vessel after 6 months of culture if not subcultured. To determine the growth and viability of prolong cultures, 20 randomly selected plantlets cultured on full, half, and quarter strength MS medium with 30 or 60 g l^{-1} sucrose concentration were transferred to normal MS medium with 30 g l^{-1} sucrose. It was noted that all plantlets from different treatments were able to resume growth on MS basal medium after 3, 6 or 12 months of continuous culture on the same medium in the same culture vessel. There was no significant difference in survival percentage of shoots (80-100%) after culture for 3, 6, and 12 months on different strength of MS nutrient medium. While, quarter strength MS medium with high sucrose concentration (60 g l^{-1}) showed a retarding effect on growth of *B. auricomum* plants.

Key words: endangered species, growth retardation, nutrient medium, sucrose concentration

INTRODUCTION

Bulbophyllum auricomum, a sympodial epiphytic orchid, having a geographical distribution ranging from Myanmar, Thailand, Sumatra to Java, blooms once a year (November-January) and is a commercial important plant. It is 8-10 cm tall, ovoid-oblong pseudobulb carrying 1-2 leaves at the top and generally propagated through the division of pseudobulbs. However, the rate of multiplication is very slow as only one or two plants are produced per bulb per year (Myanmar Encyclopedia 1972). Moreover, as in other orchids, the minute seeds are non-endospermic and require the association with appropriate fungi for germination and subsequent growth under natural condition. Due to its biological limitation to survive in natural environment, the species is now on the verge of extinction (Than et al. 2011). Consequently, *in vitro* conservation is of great interest for maintenance and storage of such endangered species.

Aseptic conservation of germplasm by controlling plant growth is a desirable method. The growth rate of *in vitro* cultures could be retarded by using osmotic agents such as mannitol, sucrose, and sorbitol (Moges et al. 2003). Osmotic agents with high concentrations in the culture medium reduce mineral uptake by cells, thereby retarding plant growth (Thompson et al. 1986). The reduction of nutrient concentration in the culture medium has been found to be beneficial for storage of cultures (Kartha et al. 1981). The influence of low concentrations of nutrient elements on growth limitation in *Vanilla walkeriae* shoots has been demonstrated by Agrawal et al. (1992). The objective of the present study was to develop a simple and effective *in vitro* conservation method of *B. auricomum* which provides minimum growth with long storage life.

MATERIALS AND METHODS

Plant material

Bulbophyllum auricomum plants were collected from their natural habitat of Yakhine Yoma mountain ranges in Myanmar and maintained in pots (Fig. 1A). Seeds of immature capsules (~ 3 months old) were

Received: September 17, 2013

Accepted: November 19, 2013

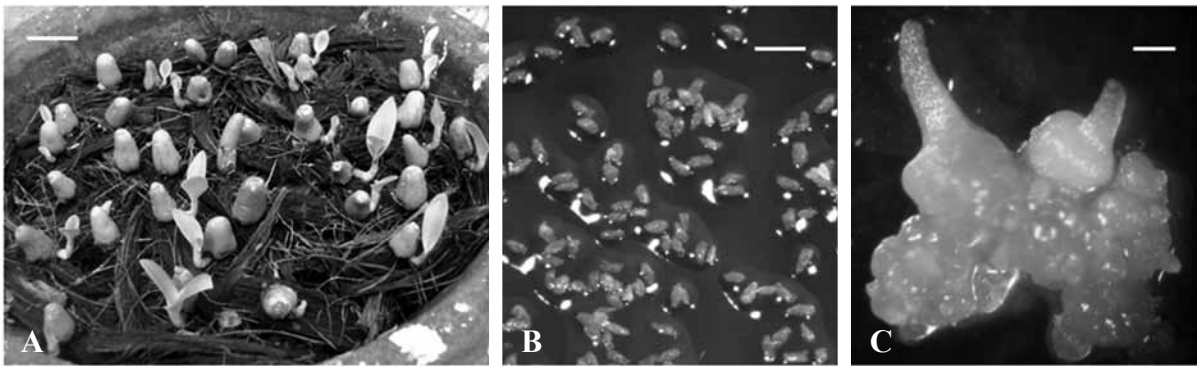

Fig. 1. *In vitro* establishment of *Bulbophyllum auricomum* propagules. A) Field grown donor plant, Bar = 26 mm, B) Isolated seeds *in vitro*, Bar = 0.1 mm, C) Emergence of shoots from protocorms, Bar = 0.4 mm.

aseptically cultured on half-strength MS (Murashige and Skoog 1962) medium (Fig. 1B). Protocorm-derived seedlings (Fig. 1C) were subcultured 3 times on the same medium in two month intervals (Than et al. 2011).

Effect of sucrose concentrations and light regime on shoot growth retardation

Bulbophyllum auricomum protocorm-derived shoots (~11.0 mm long) were used as source explants. The different concentrations of sucrose (0, 20, 30, 40, and 60 g l^{-1}) in MS medium were studied. Medium pH was adjusted to 5.7 and gelled with 0.75% (w/v) agar. The 150 ml culture vessels containing 50 ml of medium were closed with aluminum foils. Each treatment was repeated five times and each replication contained fifteen explants. Cultures were maintained at 24 ± 1°C 16 h photoperiod or continuous illumination with a photosynthetic photon flux density of 80 µmol m^{-2} s^{-1} for 18 weeks.

Effect of strength of MS basal nutrients

Bulbophyllum auricomum protocorm-derived shoots (~4.0 mm long) were used as explants for *in vitro* conservation. The effect of full, half, and quarter strenght MS basal medium was used to determine the suitable condition for mantaining the shoots under minimal growth condition. Based on previous results, sucrose was compared at 30 and 60 g l^{-1}. Each treatment was repeated four times and each replication contained twenty explants. To assess the recovery of storage plantlets, 20 randomly selected plantlets from different treatments were transferred to MS medium with 30 g l^{-1} sucrose concentration after 3, 6, and 12 months of conservation. Survival rate (% of cultures showing growth of explants) was recorded after 8 weeks of culture.

Statistical analyses

The results were analysed using one-way ANOVA (Sokal and Rohlf 1987) and standard errors (SE) were calculated. Statistical difference between mean values was computed with algorithms of Duncan's Multiple Range Test using the Statistica Software 5.0 (StatSoft 1995). Growth Index (GI) was calculated as the final fresh weight of the explants divided by the initial fresh weight.

RESULTS

Effect of sucrose concentrations and light regime on shoot growth retardation

Protocorm-derived shoots began to proliferate after four weeks of culture under different light regimes. Growth of explants after 18 weeks of culture was dependent on sucrose concentration in the culture medium. The minimal length of shoot (10.53 ± 0.58 mm) and root (1.73 ± 0.33 mm) was observed in the presence of 60 g l^{-1} sucrose concentration under 16 h photoperiod (Fig. 2 A,B). There were no significant differences in the mean number of leaves and roots among different sucrose concentrations under different light regimes (Table 1). Continuous illumination enhanced shoot multiplication and maximum number of shoots per explant (20 shoots) was obtained on MS medium containing 30 g l^{-1} sucrose. Under 16 h photoperiod, 0, 30, 40, and 60 g l^{-1} sucrose concentrations, multiple shoots formation did occur, but no significant differences were noted in these concentrations. The minimum GI of shoot was observed on MS medium supplemented with 60 g l^{-1} sucrose or without sucrose supplementation under 16 h photoperiod (Table 1).

Effect of MS nutrient medium strength and plant recovery

There was no significant difference in survival of shoots (80-100%) which were maintained on different culture medium after 3, 6, and 12 months of cultivation (data not shown). The rate of shoot growth was higher on full strength MS medium supplemented with

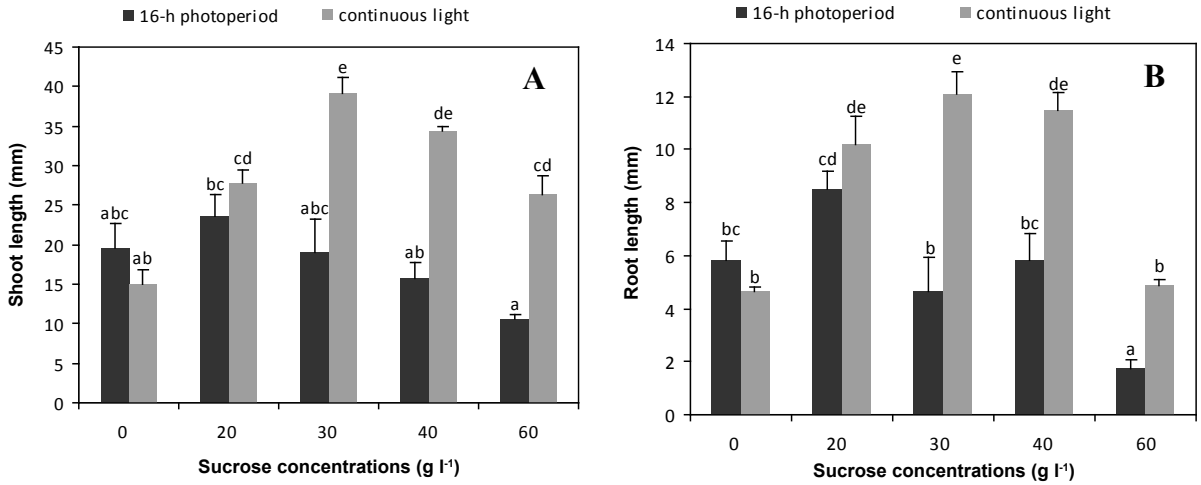

Fig. 2. Effect of different sucrose concentrations on length of (A) shoot and (B) root of *Bulbophyllum auricomum* under different light regimes after 18 weeks of *in vitro* culture.

30 g l^{-1} sucrose concentration when compared to half or quarter strength MS medium. The maximum shoot length (23.60 ± 2.74 mm) and shoot proliferation (~10 shoots) was observed on full strength MS nutrient medium supplemented with 30 g l^{-1} sucrose concentration after 12 months of culture (Fig. 3A,B; Fig. 4A). These cultures grew faster and filled the culture vessel after 6 months of culture if not subcultured. However, explants cultured on MS medium with 60 g l^{-1} sucrose resulted in retarded shoot growth. Minimum shoot length (7.68 ± 0.71 mm) was noted when explants were cultured on quarter strength of MS nutrient medium with 60 g l^{-1} sucrose, after 12 months of culture without any subculture (Fig. 4B). Minimum shoot proliferation was obtained on quarter strength MS medium with 30 g l^{-1} or 60 g l^{-1} sucrose concentration, when the explants were cultured continuously for 12 months in the same culture vessels.

The highest mean number of roots (~7 roots) was obtained when the shoots were grown on the medium containing full-strength MS medium with 60 g l^{-1} sucrose after 6 months of culture (Fig. 3C). It was observed that prolonged period of culture inhibited new root induction and some roots turned brown when shoots were grown on the same medium for 12 months. The minimum mean number of roots (1 root) was recordered on the medium containing quarter strength MS nutrients with 60 g l^{-1} sucrose after 3, 6, and 12 months of culture. The shoots grown on this culture medium supported normal growth and development of plantlets. In general, quarter strength MS medium with high su-

Table 1. Effect of different sucrose concentrations on *in vitro* shoot proliferation and root induction of *B. auricomum* under different light regimes.

Sucrose concentrations (g l^{-1})	Mean no. of leaves	Mean no. of roots	Mean no. of shoots	Growth index (GI)
16 h photoperiod				
0	1.27 ± 0.17 a	4.53 ± 0.17 a	2.67 ± 0.24 a	5.0 ± 0.70 a
20	1.33 ± 0.24 a	6.27 ± 1.09 a	4.67 ± 0.35 ab	9.0 ± 2.03 b
30	1.20 ± 0.11 a	3.47 ± 0.69 a	3.20 ± 0.20 a	9.0 ± 1.36 b
40	1.20 ± 0.20 a	3.00 ± 0.72 a	3.20 ± 0.46 a	8.0 ± 2.83 b
60	0.93 ± 0.33 a	0.93 ± 0.07 a	2.33 ± 0.69 a	6.0 ± 2.68 a
Continuous light				
0	1.53 ± 0.59 a	3.80 ± 2.01 a	6.20 ± 0.87 b	9.0 ± 1.45 b
20	1.07 ± 0.26 a	4.07 ± 0.35 a	3.27 ± 0.17 a	9.0 ± 0.40 b
30	1.67 ± 0.17 a	5.27 ± 0.25 a	20.27 ± 1.35 c	28.0 ± 3.67 c
40	1.40 ± 0.05 a	4.93 ± 1.79 a	4.80 ± 0.87 ab	14.0 ± 0.27 b
60	1.07 ± 0.48 a	4.47 ± 1.96 a	4.47 ± 0.63 ab	14.0 ± 0.53 b

Mean ± SE within a column followed by the same letter are not significantly different according to Duncan's multiple range tests at $p < 0.01$.

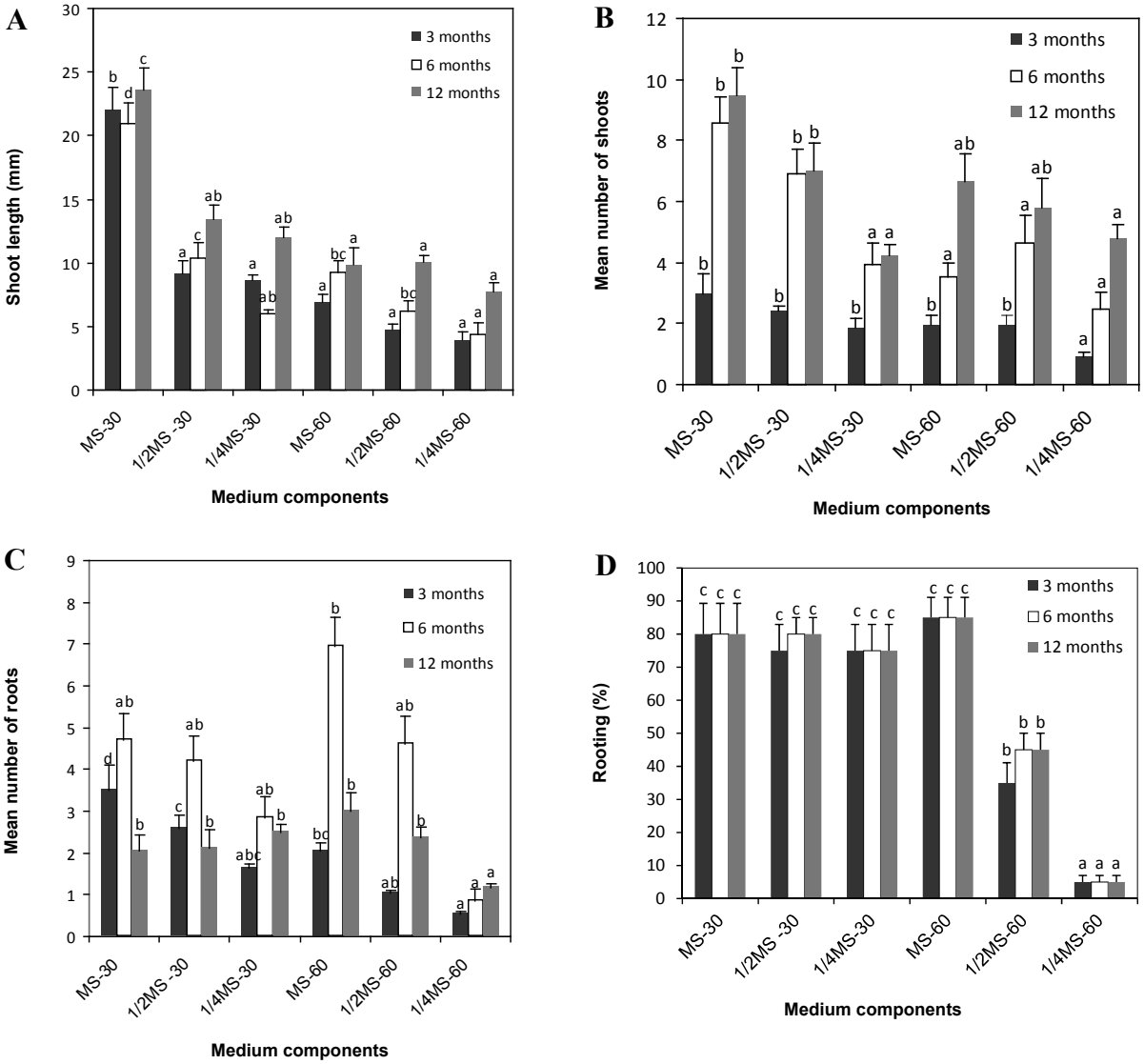

Fig. 3. Effects of different nutrients strength of MS medium and sucrose concentrations on growth parameters of *Bulbophyllum auricomum.* A) Mean shoot length, B) Mean number of shoots, C) Mean number of roots, D) Rooting of the shoots after 3,6, and 12 months of *in vitro* culture without subculture.

crose concentration (60 g l^{-1}) had a retardant effect on growth of *B. auricomum* plants.

It was noted that all plantlets from different treatments were able to survive on MS basal medium without PGRs (Fig. 4C).

DISCUSSION

Sucrose is widely used as carbon source in most of the tissue culture media (Arditti 2008). It functions as energy source and osmotic agent. The present findings indicated that the growth of shoot and root generally increased with increasing sucrose concentration until optimum and then decreased at high concentration. This may be due to the negative water potential relating with sucrose concentration in the medium (Ket et al. 2004). Such stress condition might be inhibited the growth of *B. auricomum* shoots cultured *in vitro*. This result is in concords with the findings of Homes et al. (1982) in *Cymbidium* species, Tandon and Sharma (1986) in *Dendrobium crysenthum* and *D. ochreatum,* Van Waes and Debergh (1986) in Western European orchids, Rasmussen (1995) in terrestrial orchids, Ket et al. (2004) in *Anoectochilus formosanus*, and Wotavová-Novotná et al. (2007) in *Dactylorhiza* species.

In the present study, it was observed that *B. auricomum* shoots were able to survive on sucrose deprived medium when cultured under different light regimes for 18 weeks. Kartha et al. (1981) have been reported that coffee shoot tips were successfully retained on the medium without sucrose when maintained under 7500 lux. Galzy and Compan (1988) suggested that the reduction of carbohydrate content in the nutrient me-

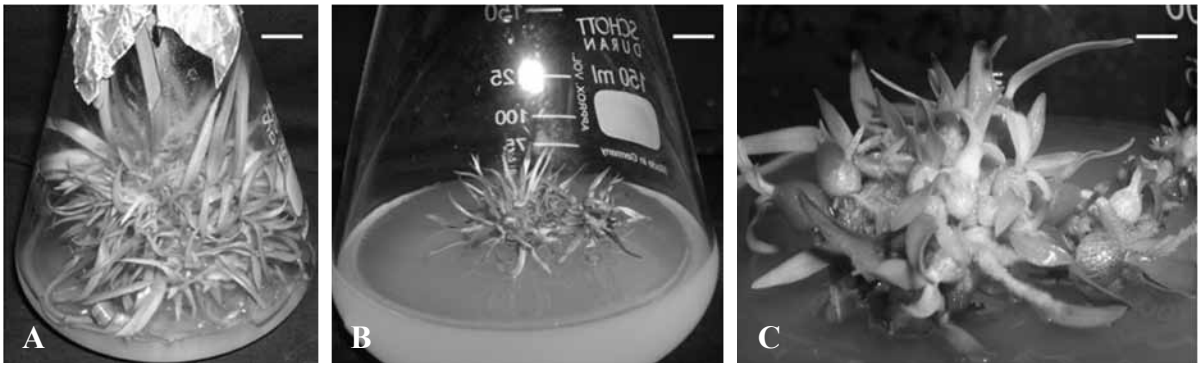

Fig. 4. Comparison of growth response of *Bulbophyllum auricomum* shoots maintained after 12 months of culture without any subculture. Shoots grown on A) Full strength MS nutrient medium supplemented with 30 g l^{-1} sucrose concentration, Bar = 14 mm, B) Quarter strength MS nutrient medium with 60 g l^{-1} sucrose, Bar = 10 mm, C) Pseudobulb formation from the base of shoots, Bar = 5.0 mm.

dium also induced photosynthetic activity of explants in some cases.

The shoot of endemic orchid species, *Ipsea malabarica,* was maintained for 20 months on half strength MS medium, without the addition of sucrose or PGRs (Martin and Pradeep 2003). In *Vanilla* sp., shoots were maintained for one year on full or half strength MS nutrients with 15 g l^{-1} each of sucrose and mannitol (Minoo et al. 2006). In the present study, *B. auricomum* shoots could be successfully maintained for 12 months on the medium containing quarter strength MS nutrients with 60 g l^{-1} sucrose concentration. In contrast, the rate of shoot growth was higher in full strength MS medium with 30 g l^{-1} sucrose. This overgrowth resulted in exhaustion of nutrients and drying up of some leaves after 6 months of culture if not subcultured.

Availability of green shoots (for further micropropagation *vis-à-vis* regeneration) after protracted periods of slow-growth conservation is one of the most important features in any *in vitro* conservation programme (Roca et al. 1989). Too much reduction in microplant growth during conservation may result in limited number of viable plantlets for further subculturing or regeneration (Sarkar et al. 2001).

It may be concluded that quarter strength MS medium with 60 g l^{-1} sucrose showed a retarding effect on growth of *B. auricomum* plants.

Acknowledgements: The author is thankful to the Department of Biotechnology, Goverment of India and The World Academy of Sciences, (Italy) for the award of Postdoctoral fellowship and Prof. Sumita Jha, mentor, CAS, Department of Botany, University of Calcutta and Prof. Amita Pal, Bose Institute for their constant encouragement and supports.

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