***IN VITRO* CONSERVATION OF THE 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 contnuously on one quarter strength MS 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, randomly selected plantlets from different treatments 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, one-fourth strength MS medium with high sucrose (60 g l-1) showed a retarding effect on growth of *B. auricomum* plants.

**Key words:** *Bulbophyllum auricomum,* endangered species, *in vitro* conservation, nutrient medium, orchids

**Introduction**

Conservation of plant genetic resources is an essential component to sustain biodiversity for the agriculturally and horticulturally important species. It is estimated that genetic diversity of many plant species is being lost globally at a rate that is faster than at any previous time in history (IUCN 2004). This situation is even worse for orchid species partly because most of them have habitat preference, pollinator dependence for the completion of life cycle (IUCN/SSC Orchid Specialist Group 1996) and symbiotic association with a class of fungi for seed germination under natural condition (Arditti 2008, Yam and Arditti 2009). The vast majority of orchids, especially those native to tropical regions, are currently endangered because of extensive disturbance of their natural habitat and indiscriminate harvesting of the naturally growing plants (Koopowitz et al. 1993, Than et al. 2009). Thus, *in situ* conservation became rather impossible due to the disappearance of large wild areas. *Ex situ* conservation under natural condition is also limited due to labour costs for maintenance, trained personnel requirements, political and social issues, the risk of genetic wipe out as a result of natural disasters, etc. (Engelmann 1991). Hence, *in vitro* conservation of plant germplasm is becoming a complementary approach to conserve endemic and endangered plant species (Moges et al. 2003, Shibli et al. 2006). It is probably the only feasible way to maintain as a gene bank and has been applied widely for many plant species (Wannakrairoj 1998, Sarkar et al. 2001, Minoo et al. 2006, Mweetwa et al. 2007, Rangsayatorn et al. 2009).

*In vitro* germplasm conservation can be performed using different techniques depending on the storage duration, i.e. long-term conservation (cryopreservation) and short- to medium-term conservation (to reduce the growth) (Engelmann 1991, 1998). Aseptic conservation of germplasm by controlling plant growth is a desirable method for developing country. It needs less space requirements and reduction of the expenses in labour costs for maintenance. Conservation can be executed by manipulating temperature (Withers 1982), osmotic stress (Schnapp and Preece 1986), mineral concentration (Withers 1982) or gas availability (Bridgen and Staby 1981). Synthetic seed production in several orchids via alginate encapsulation has also been attempted as an alternate method for short- to mid-term storage (Saiprasad and Polisetty 2003, Mohanraj et al. 2009, Gantait et al. 2012).

*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 an economically orchid. This 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 seed germination and subsequent growth under natural condition. Due to its commercial importance and biological limitation to survive in natural environment, the species is now on the verge of extinction (Than et al. 2009, 2011, 2012). Consequently, *in vitro* conservation is of great interest for maintenance and storage of such endangered species.

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 addition of osmoticum in culture medium has been proved to be efficient in reducing growth and increasing the storage life of many *in vitro* grown explants of different plant species (Wilson et al. 2000). On the other hand, 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 *Lycopersicon esculentum* and *Dianthus caryophylus* microplants has been demonstrated by Schnapp and Preece (1986). They reported that shoot and root growth of microplants was significantly reduced when MS nutrient concentrations were decreased to one-fourth, half- or three-fourth strength in the culture medium. 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**

*B. 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) from a single donor plant were used in this study (Than et al. 2011). Seeds were aseptically cultured on semisolid MS medium (Murashige and Skoog 1962) (Fig. 1b). After two to three weeks of culture initiation, embryos swelled and turned into yellowish green spherule-like protocorms (Fig. 1c). Shoots emerged from protocorms (Fig. 1d) and shoot induction started within six to seven weeks of *in vitro* culture. Protocorm-derived shoots were maintained on MS (1962) medium by subculturing every two months.

**Effect of sucrose concentrations and light regime on shoot growth retardation**

*B. auricomum* shoots derived from *in vitro* raised seedlings (~11.0 mm long) with 19.73±2.43 mg fresh weight were used as source explants. To assess the slow growth of *B. auricomum* in culture, different concentrations of sucrose (0, 20, 30, 40 and 60 g l-1) in MS (1962) medium were tested. Medium pH was adjusted to 5.7 and gelled with 0.75% (w/v) agar. Each flask contained 5 shoots and 15 flasks per treatment. Cultures were maintained at 24±1º C under two different light regimes (16 h photoperiod or continuous illumination) with a photon flux density of 80 µmolm-2 s-1 for 18 weeks. A better minimal growth condition was selected and employed for the subsequent experiments.

**Effect of strength of MS basal salts**

*B. auricomum* shoots (~4.0 mm long) were used as explants for *in vitro* conservation. To standardize the suitable condition for maintaining the shoots under minimal growth condition, MS (1962) basal salts were used in full, half and one-fourth strength. The carbon source, sucrose, was compared at two different concentrations (30 and 60 g l-1). The 150 ml culture vessels containing 50 ml of medium were closed with aluminum foils. Each flask contained 4 shoots and there were 20 flasks per treatment. Cultures were maintained at 24±1º C under 16 h photoperiod (artificial fluorescent light of 80 µmol m-2s-1). Survival rate were recorded after 3, 6 and 12 months of conservation. Survival rate was determined as the percentage of cultures showing growth of 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 and growth rate was recorded after 8 weeks of culture.

**Hardening and transplantation**

Healthy *B. auricomum* plantlets with well-developed roots were taken out of the culture vessels and washed thoroughly under tap water to remove traces of adhering agar. These plantlets were transferred to the pots containing equal amount of charcoal and coconut husk. Pots were covered with polyethylene bags to maintain approximately 80-90 % humidity. Covers were withdrawn after 3-4 days and pots were maintained in a growth chamber at 25±1º C for 4 weeks. Seedling survival was recorded after 6 weeks of acclimatization.

**Statistical analyses**

Tabulated results were analysed using one-way ANOVA (Sokal and Rohlf 1987) and standard errors (SE) of replicate data were calculated. Statistical difference between mean values was computed with algorithms of Duncan’s Multiple Range Test using the Statistica Software v 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 different sucrose concentrations and light regime on shoot growth retardation**

Shoots began to proliferate after four weeks of culture under different light regimes. Growth of culture regenerants after 18 weeks of culture was dependent on sucrose concentration in the culture medium. The minimal growth 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 number of leaf and root formation per explant 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 in 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 not occur, and no significant differences were noted in these concentrations. The minimum GI of shoot was observed when grown on MS medium supplemented with 60 g l-1 sucrose or without sucrose supplementation under 16 h photoperiod.

**Effect of MS nutrient medium strength and plant recovery**

The effect of concentrations of sucrose and basal salts of MS nutrient medium on minimal growth of *B. auricomum* regenerants was studied. There was no significant difference in survival percentage of shoots (80-100 %) which were maintained on different culture medium after 3, 6 and 12 months of conservation (Table 2). The rate of shoot growth was higher on full strength MS medium supplemented with 30 g l-1 sucrose concentration when compared to half or one-fourth 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 (150 ml flask containing 50 ml medium) 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 one-fourth 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 one-fourth 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 root induction (~ 7 roots/plant) was obtained when the shoots were grown on the medium containing full strength MS 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 root induction was noted on the medium containing one-fourth strength MS nutrient with 60 g l-1 sucrose (1 root/plant) after 3, 6 and 12 months of culture. The shoots grown on this culture medium supported normal growth and development of plantlets. In general, one-fourth strength MS medium with high sucrose (60 g l-1) had a retardant effect on growth of *B. auricomum* plants.

Randomly selected plantlets from different treatments were transferred to normal MS medium with 30 g l-1 sucrose after 3, 6 or 12 months in order to determine the growth and viability of cultures maintained in the same culture vessel over long period without subculture. It was noted that all plantlets from different treatments were able to survive on MS basal medium without PGRs.

**Hardening and transplantation of *B. auricomum* plants**

*B. auricomum* plants were maintained on MS basal medium devoid of PGRs (Fig. 4c) for 3 months. Plantlets (4-5 cm) with well-developed roots were transplanted to the pots containing equal amount of charcoal and coconut husk for acclimatization. Charcoal was used as substrate together with coconut husk to avoid direct contact with the substrate, which retains excess moisture. Wood charcoal provides good drainage and adequate aeration to the roots, which is of primordial importance in the culture of orchids. These acclimatized plants survived and grew well but could not tolerate the high outdoor temperature during the summer season in Kolkata, India. The plantlets transplanted to the green house at Orchid Garden, Yangon, Myanmar with 80% success in establishment rate (Fig. 4d). The new shoot formation was initiated after 6 weeks of acclimatization.

**Discussion**

The main objective of *in vitro* conservation of plant genetic resources is to increase the intervals between the subcultures under sterile condition maintaining their clonal fidelity (Moges et al. 2003). Several types of plant materials such as bulblets (Kastner et al. 2001), nodal segments (Martin et al. 2007), protocorms (Rangsayatorn et al. 2009), PLBs (Kishi and Takagi 1997, Wannakrairoj 1998), plantlets (Miedema 1982), seeds (Mweetwa et al. 2007), seedlings (Ming et al. 2000, Zhong et al. 2000), shoots (Agrawal et al. 1992, Minoo et al. 2006) and shoot tips (El-Gizawy and Ford-Lloyd 1987) were used as explants for *in vitro* conservation. In the present study, protocorm-derived shoots were used as a source of explants for *in vitro* conservation.

Sucrose is widely used as carbon source in most of the tissue culture media (Hazarika 2003, Arditti 2008). It functions as energy source and osmotic agent. In the present study, sucrose is used as osmotic agent in the culture medium. The present findings indicated that the growth of shoot and root generally increased with increasing sucrose concentration until optimum and then decreased at very 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 accord with the findings of Van Waes and Debergh (1986) in Western European orchids, Rasmussen (1995) in terrestrial orchids, and Wotavová-Novotná et al. (2007) in *Dactylorhiza* species. Shibli et al. (1999) reported that the growth of bitter almond microshoots was significantly reduced in high sucrose concentration. The similar findings have been reported in other plant species such as *Solanum tuberosum* (Henshaw et al. 1980, Sarkar and Naik 1998), *Cymbidium* sp. (Homes et al. 1982), *Dendrobium crysenthum* and *D. ochreatum* (Tandon and Sharma 1986), *Campsis chinensis* (Paek and Kwang 1993) and *Anoectochilus formosanus* (Ket et al. 2004).

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. To the contrary, *Coffea arabica* shoots were unable to survive on the sucrose-free medium when maintained under 12 h photoperiod with a photon flux density of 50 µmolm-2 s-1 (Bertrand-Desbrunais et al. 1992). Galzy and Compan (1988) suggested that the reduction of carbohydrate content in the nutrient medium also induced photosynthetic activity of explants in some cases. Thus, further studies need to investigate the conservation of *B. auricomum* species in sucrose-free medium for prolong period.

Agrawal et al. (1992) have reported that *Vanilla walkeriae* shootswere conserved for 7 months when maintained on half-strength MS medium. In the present study, *B. auricomum* shoots were successfully maintained for 12 months even on the medium containing one-fourth strength MS nutrient. As observed in the present study, seedlings of *Dendrobium candidum* (Ming et al. 2000) and *D. officinale* (Zhong et al. 2000) were conserved *in vitro* for 12 months without subculture when maintained on one-fourth or half-strength MS medium. In contrast, sweet potato plantlets grown on half-strength MS medium resulted in low survival (36 % - 48 %) after 4 months of conservation (Aguilar and Lopez 1993).

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 nutrient 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 one-fourth strength MS nutrient with 60 g l-1 sucrose concentration. Thus, the reduction in concentrations of mineral nutrient and high sucrose concentration in the culture medium was sufficient to retard the *in vitro* growth in *B. auricomum.* In contrast, the rate of shoot growth was higher in full strength MS medium with 30 g l-1 sucrose. It was observed that these cultures grew faster and filled the culture vessel after 6 months of culture. 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). In the present investigation, shoots maintained on growth retardation medium (one-fourth strength MS medium with 60 g l-1 sucrose) survived after prolonged periods of storage and also fostered favourable plantlet to re-grow.

It may be concluded that one-fourth strength MS medium with 60 g l-1 sucrose showed a retarding effect on growth of *B. auricomum* plants. A simple and effective method for *in vitro* conservation of this endangered species has been developed in the present study.

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**Tables**

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 leaf/explant \* | Mean no. of root/explant \* | Mean no. of shoot/explant\* | Growth Index \* |
| 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 |

\* The mean of 15 replicates (n=15) ± standard error (SE). Values followed by different letters within a column are significantly different according to Duncan’s multiple range tests at *P* < 0.01.

Cultures were maintained for 18 weeks.

Table 2.Survival of *B. auricomum* shoots maintained on different culture media after 3, 6 and 12 months of conservation.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Basal medium | Sucrose concentrations (g l-1) | % of survival | | |
| 3 months | 6 months | 12 months |
| MS | 30 | 100±0 a | 100±0 a | 90±0.68 a |
| 1/2 strength MS | 30 | 100±0 a | 100±0 a | 100±0 a |
| 1/4 strength MS | 30 | 100±0 a | 95±0.58 a | 95±0.37 a |
| MS | 60 | 90±0.51 a | 90±0.81 a | 75±0.63 a |
| 1/2 strength MS | 60 | 90±0.98 a | 85±0.51 a | 85±1.03 a |
| 1/4 strength MS | 60 | 90±0.68 a | 80±0.86 a | 80±0.80 a |

\* Mean of 20 replicates and values followed by same letters within a column are not significantly different according to Duncan’s multiple range tests at P < 0.01.

Cultures were maintained under 16 h photoperiod.

**Legend of Figures**

Fig. 1 *In vitro* establishment of *B. auricomum* propagules*.* (a) field grown donor plant, Bar 26 mm; (b) isolated seeds *in vitro*, Bar 0.1 mm; (c) regenerated protocorms from germinated seeds, Bar 0.4 mm; (d) emergence of shoots form protocorms, Bar 0.4 mm.

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

Fig. 3Effects of different salt strength of MS medium and sucrose concentrations on growth parameters of *B. auricomum*; (a) shoot length, (b) number of shoots per explant and (c) number of roots per explant after 3, 6 and 12 months of *in vitro* culture without subculture.

Fig. 4 (a-b) Comparison of growth response of *B. 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; and (b) one-fourth strength MS nutrient medium with 60 g l-1 sucrose, Bar 10 mm. (c-d) Hardening and transplantation of *B. auricomum*. (c) pseudobulb formation from the base of shoots, Bar 5.0 mm; (d) well-developed plantlets grown under natural condition, Bar 9.0 mm.