IN VITRO CONSERVATION OF ENDANGERED ORCHID *Bulbophyllum auricomum* Lindl., THE ROYAL ORCHID OF MYANMAR

Myo Ma Ma Than[1]*

Centre of Advanced Study, Department of Botany, University of Calcutta,

35, Ballygunge Circular Road, Kolkata-700 019, India

[1]Senior Researcher, Network Activities Group, Yangon, Myanmar

*E-mail: myomamathan3@gmail.com

Telephone no.: 91-33-2461-5445

Fax: 91-33-2461-4849

Abstract

In vitro conservation method of *Bulbophyllum auricomum* Lindl. has been standardized/ developed in the present study. Minimum shoot proliferation (7.68±0.71 mm in length) and at least 1 root/plantlet developed after 12 months when shoots germinated from protocorms were cultured incessantly on one-fourth strength MS nutrient medium with 60 gm l^{-1} sucrose. In contrast, when the explants were maintained on full strength MS nutrient medium supplemented with 30 gm l^{-1} sucrose, the cultures grew faster and filled the culture vessel (150 ml flask with 50 ml medium) and difficult to maintain without subculture after 6 months. 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 %) which were maintained on different strength of MS nutrient medium after 3, 6 and 12 months of conservation. While, one-fourth strength MS medium with high sucrose (60 gm l^{-1}) showed a retardant 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 are 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 these have

Kommentar [P1]: I think "developed"

Formatiert: Hervorheben

Formatiert: Hervorheben

Kommentar [P2]: Growth?

Kommentar [P3]: After acclimatization?

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 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 a profit-making 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 (Myanma 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 in 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 objective of the present study was to develop method for *in vitro* conservation of endangered orchid *Bulbophyllum auricomum*, the royal orchid of Myanmar.

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 spherules like protocorms (Fig. 1c). Shoots emerged from germinated protocorms (Fig. 1d) and shoot proliferation started

within six to seven weeks of *in vitro* culture. Axenic shoots of *B. auricomum* were maintained on MS (1962) nutrient medium by subculturing every two months.

Effect of sucrose concentrations and light regime on shoot 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 explant. To assess the slow growth of *B. auricomum* in culture, MS (1962) medium with 0, 20, 30, 40 and 60 gm l^{-1} sucrose concentrations and light conditions were tested. Medium pH was adjusted to 5.7 and gelled with 0.75% w/v agar. Each flask contains 5 shoots and 15 replicates 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 next.

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 gm l^{-1}). The 150 ml culture vessels containing 50 ml of medium were closed with aluminum foils to minimize the evaporation rate. Each flask contained 4 shoots and there were 20 replicates per treatment. Cultures were maintained at 24±1º C under 16 h photoperiod (artificial fluorescent light 80 µmol $m^{-2}s^{-1}$). Survival rate and viability 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

Kommentar [P4]: different

Kommentar [P5]: contained

Kommentar [P6]: flasks or shoots

Formatiert: Hervorheben

Formatiert: Hervorheben

Kommentar [P7]: type?

MS medium with 30 gm 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 retardation

Shoots began to proliferate after four weeks of culture under different light regimes. Growth of culture regenerants is dependent on sucrose concentration in the culture medium. The minimal growth rate of shoot (10.53±0.58 mm) and root (1.73±0.33 mm) length was observed in the presence of 60 gm 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 among different sucrose

Kommentar [P8]: was

concentrations under different light regimes (Table 1). Continuous illumination enhanced shoot multiplication and maximum number of shoots (20 shoots) was obtained in MS medium containing 30 gm l^{-1} sucrose. Under 16 h photoperiod, 0, 30, 40 and 60 gm l^{-1} sucrose concentrations inhibited multiple shoots formation and there was no significant difference noted in these concentrations. The minimum GI of shoot was retained when grown on MS medium supplemented with 60 gm 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 gm 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 gm 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 sucrose 60 gm l^{-1} 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 gm 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 gm l^{-1} or 60 gm l^{-1} sucrose concentration, when the explants were cultured continuously for 12 months in the same culture vessels.

Kommentar [P9]: which ones?

Kommentar [P10]: What do you mean with recovery?

Kommentar [P11]: Not expected under this headline

Kommentar [P12]: It seems you like to have strong shoot growth. Did you explain what is the rate of shoot growth?

The optimum root induction (~ 7 roots/plant) was obtained when the shoots were grown on the medium containing full strength MS with 60 gm 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 gm 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 gm l^{-1}) had a retardant effect on growth of *B. auricomum* plants.

Randomly selected plantlets from different treatments were transferred to normal MS medium after 3, 6 or 12 months in order to determine the growth and viability of cultures maintained in 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 pot 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

Kommentar [P13]: From where do you know this is optimum?

Kommentar [P14]: Had they the same vigor?

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. Among them, shoot explants were most suitable for *in vitro* conservation due to high survival rate and high genetic stability (Reed et al. 1998). In the present study, axenic *B. auricomum* shoots were used as source of explants for *in vitro* conservation.

The growth rate of *in vitro* cultures could be retarded by using osmotic agents such as mannitol, sucrose, sorbitol, malic hydrazide, succinic acid and ancymidole (Moges et al. 2003). In the present study, sucrose is used as osmotic agent in the culture medium. Osmotic agents in the culture medium reduce mineral uptake by cells due to difference in osmotic pressures, thereby retarding plant growth (Thompson et al. 1986). Such stress condition inhibits shoot induction in cultured explants (Brown et al. 1979). 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).

Kommentar [P15]: What is the standard interval. I guess for such slow growing cultures more than 4 weeks.

Kommentar [P16]: You used seed and therefore genetically heterogenic material and not clones.

Kommentar [P17]: Belongs to the introduction

Kommentar [P18]: introduction

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. Sucrose can also be used to retard the plant growth *in vitro* (Moges et al. 2003). In the present study, shoot growth was retarded in the presence of 60 gm l^{-1} sucrose in the culture medium, which is consistent with the observations 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).

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 microplant height and root growth was significantly reduced when MS nutrient concentrations were decreased to one-fourth, half- or three-fourth strength in the culture medium. Agrawal et al. (1992) also reported that *Vanilla walkeriae* shoots were conserved for 7 months when maintained on half-strength MS medium. In the present study, *B. auricomum* shoots were successfully maintained 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-

Kommentar [P19]: introduction

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 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 gm 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 gm 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.*

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 be resulted 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 gm l^{-1} sucrose) survived after prolonged periods of storage and also fostered favourable plantlet to re-grow.

It may be concluded that a method for *in vitro* conservation of *B. auricomum* plants have been developed in the present study.

Acknowledgements

MMMT is thankful to the DBT-TWAS (Department of Biotechnology, Govt. 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.

References

Agrawal D.C., Morwal G.C., Mascarenhas A.F. (1992). *In vitro* propagation and slow growth storage of shoot cultures of *Vanilla walkeriae* Wight, an endangered orchid. Lindleyana, 7(2): 95–99.

Aguilar M, Lopez M (1993) *In vitro* conservation of sweetpotato clone N-1437 with minimal growth rate. In: Reunion annual de la Sociedad del Programa Cooperativo Centro Americano de Cultivos y Animales (PCCMCA), Guatemala, 276-278 pp.

Arditti J. (2008). Micropropagation of orchids. Second Edition, John Wiley and Sons, New York, 1-88 pp.

Bridgen M.P., Staby G.L. (1981). Low pressure and low oxygen storage of *Nicotiana tabacum* and *Chrysanthemum Morifolium* tissue cultures. Plant Science Letters, 22: 177-186.

Brown D.C.W., Leung D.W.M., Thorpe T.A. (1979). Osmotic requirements for shoot formation in tobacco callus. Physiologia Plantarum, 46: 36-41.

El-Gizawy A.M., Ford-LIoyd B. (1987). An *in vitro* method for the conservation and storage of garlic (*Allium sativum*) germplasm. Plant Cell, Tissue and Organ Culture, 9: 147-150.

Engelmann F. (1991). *In vitro* conservation of tropical plant germplasm - a review. Euphytica, 57: 227-243.

Engelmann F. (1998). *In vitro* conservation of horticultural genetic resources review of the state of the art. ISHS: World Conference on Horticultural Research, Rome, Italy, 17-20 June.

Gantait S., Bustam S., Sinniah U.R. (2012). Alginate-encapsulation, short-term storage and plant regeneration from protocorm-like bodies of *Aranda* Wan Chark Kuan 'Blue' x *Vanda coerulea* Grifft. ex. Lindl. (Orchidaceae). Plant Growth Regulation, 68(2): 303-311.

Hazarika B.N. (2003). Acclimatization of tissue-cultured plants. Current Science, 85(12): 25.

Henshaw G.G., O'Hara J.F., Westcott R.J. (1980) In: Ingram D.S., Helgelson J.P. (Eds) Tissue Culture Method for Plant Pathologists. Blackwell Scientific Publications, UK, 71-76 pp.

Homes J., Dubus F., Bourdon J.L. (1982). Cold storage of plant tissue cultures. In: Fujiwara A. (Eds). Proceeding of 5th International Congress in Plant Tissue and Cell Culture, Tokyo, 801-802 pp.

IUCN (2004). IUCN Red List of Threatened Species. www.iucnredlist.org.

IUCN/SSC Orchid Specialist Group (1996). Orchids-Status Survey and Conservation Action Plan. Gland and Cambridge: IUCN, 3-4 pp.

Kartha K.K., Mroginski L.A., Pahl K., Leung N.L. (1981). Germplasm preservation of coffee (*Coffea arabica* L.) by *in vitro* culture of shoot apical meristems. Plant Science Letters, 22: 301-307.

Kastner U., Klahr A., Keller E.R.J., Kahane R. (2001). Formation of onion bulblets *in vitro* and viability in medium-term storage. Plant Cell Reports, 20: 137-142.

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

Kishi F., Takagi K. (1997). Efficient method for the preservation and regeneration of orchid protocorm-like bodies. Scientia Horticulturae, 68: 149-156.

291 Koopowitz H., Thornhill A., Anderson M. (1993). Species distribution profiles of the neotropical
292 orchids *Masdevallia* and *Dracula* (Pleurothallidinae, Orchidaceae); implications for
293 conservation. Biodiversity and Conservation, 2: 681–690.
294 Martin K.P., Pradeep A.K. (2003). Simple strategy for the *in vitro* conservation of *Ipsea*
295 *malabarica* an endemic and endangered orchid of the Western Ghats of Kerala, India. Plant Cell,
296 Tissue and Organ Culture, 74: 197–200.
297 Martin M.T., Pedranzani H.E., Sierra De Grado R. (2007). Behavior and preservation of an *in*
298 *vitro* collection of European aspen in Spain. Biocell, 31(1): 41-49.
299 Miedema P. (1982). A tissue culture technique for vegetative propagation and low temperature
300 preservation of *Beta vulgaris*. Euphytica, 31 : 635-643.
301 Ming X., Ye Q., Guo L., Zhong S.Y. (2000). *Dendrobium candidum* germplasm resources from
302 the low-temperature preservation. Chinese Journal of Applied and Environmental Biology, 6(4):
303 326-330.
304 Minoo D., Nirmal Babu K., Peter K.V. (2006). Conservation of *Vanilla* species, *in vitro*. Scientia
305 Horticulturae, 110: 175–180.
306 Moges A.D., Karam N.S., Shibli R.A. (2003). Slow growth *in vitro* preservation of African
307 violet (*Saintpaulia ionantha* Wendl.) shoot tips. Advances in Horticultural Science, 17: 1-8.
308 Mohanraj R., Ananthan R., Bai V.N. (2009). Production and storage of synthetic seeds in
309 *Coelogyne breviscapa* Lindl. Asian Journal of Biotechnology, 1: 124–128.
310 Murashige T., Skoog F. (1962). Revised medium for rapid growth and bioassays with tobacco
311 tissue cultures. Physiologia Plantarum, 15: 473-497.
312 Mweetwa A.M., Welbaum G.E., Tay D. (2007). Orchid seed storage for germplasm preservation.
313 Acta Horticulturae, 760: 2.

Formatiert: Deutsch (Deutschland)

314 Myanma Encyclopedia (1972). Tha Zin Pan. In: Myanma Encyclopedia, Yangon, Myanmar, 12:
315 465-466.
316 Paek K.Y., Kwang H.J. (1993). *In vitro* growth control of horticultural plants by manipulating
317 water potential of media with sucrose, manitol and agar. In: Quynh N.T., Uyen N.V. (Eds):
318 Adapted Propagation Techniques for Commercial Crops of the Tropics. IFS, Hochi Minh City,
319 126-136 pp.
320 Rangsayatorn N., Jina K., Pampasit S. (2009). Slow growth *in vitro* conservation of *Dendrobium*
321 *draconis* Rchb.f.: Effect of black Kwao Krua (*Mucuna collettii*, Lace). Acta Horticulturae, 829:
322 355-358.
323 Rasmussen H.N. (1995). Terrestrial orchids, from seed to mycotrophic plant. Cambridge
324 University Press, Cambridge, 184-196 pp.
325 Reed B.M., Paynter C.L., Denoma J., Chang Y. (1998). Techniques for medium- and long-term
326 storage of pear (*Pyrus* L.) genetic resources. Plant Genetic Resources Newsletter, 115: 1-5.
327 Roca W.M., Chavez R., Marin M.L., Arias D.I., Mafia G., Reyes R. (1989). *In vitro* methods of
328 germ-plasm conservation. Genome, 31: 813-817.
329 Saiprasad G.V.S., Polisetty R. (2003). Propagation of three orchid genera using encapsulated
330 protocorm-like bodies. In Vitro Cellular and Developmental Biology-Plant, 39: 42–48.
331 Sarkar D., Chakrabart S.K., Naik P.S. (2001). Slow-growth conservation of potato microplants:
332 efficacy of ancymidol for long-term storage *in vitro*. Euphytica, 117: 133-142.
333 Sarkar D., Naik P.S. (1998). Factors affecting minimal growth conservation of potato
334 micoroplants *in vitro*. Euphytica, 102: 275-280.
335 Schnapp R.S., Preece J.E. (1986). *In vitro* growth reduction of tomato and carnation microplants.
336 Plant Cell, Tissue and Organ Culture, 6: 3-8.

337 Shibli R.A., Shatnawi M.A., Ajlouni M.M., Jaradat A., Adham Y. (1999). Slow growth *in vitro*
338 conservation of bitter almond (*Amygdalus communis* L.). Advances in Horticultural Science, 13:
339 133-134.
340 Shibli R.A., Shatnawi M.A., Subaih W.S., Ajlouni M.M. (2006). *In vitro* conservation and
341 cryopreservation of plant genetic resources: A review. World Journal of Agricultural Sciences,
342 2(4): 372-382.
343 Sokal R., Rohlf F.J. (1987). Introduction to Biostatistics, Second Edition, Freeman W.H. and
344 Company, San Francisco, 132-183.
345 StatSoft INC. (1995). Statistica for Windows (Computer program). Statsoft INC., Tulsa,
346 Oklahoma, USA.
347 Tandon P., Sharma J. (1986). Regeneration of *Dendrobium* from cold preserved protocorms. In:
348 Somers D.A., Gengenbach B.G., Biesboer D.D., Hackett W.P., Green C.E. (Eds). Proceeding of
349 6th International Congress in Plant Tissue and Cell Culture, Minneapolis, USA, 425 pp.
350 Than M.M.M., Pal A., Jha S. (2009). *In vitro* flowering and propagation of *Bulbophyllum*
351 *auricomum* Lindl., the royal flower of Myanmar. Acta Horticulturae, 829: 105-111.
352 Than M.M.M., Pal A., Jha S. (2011). Chromosome number and modal karyotype in a
353 polysomatic endangered orchid, *Bulbophyllum auricomum* Lindl., the royal flower of Myanmar.
354 Plant Systematics and Evolution, 294: 167–175.
355 Than M.M.M., Pal A., Jha S. (2012) Plant regeneration from callus cultures in endangered orchid
356 *Bulbophyllum auricomum* lindl. Propagation of Ornamental Plants, 12(2): 102-108.
357 Thompson M.R., Douglas T.J., Obata-Sasamoto H., Thorpe T.A. (1986). Mannitol metabolism in
358 cultured plant cell. Physiologia Plantarum, 67: 365-369.

Van Waes J.M., Debergh P.C. (1986). *In vitro* germination of some Western European orchids. Physiologia Plantarum, 67: 253-261.

Wannakrairoj S. (1998). *In vitro* preservation of *Vanda coerulea* Giff. Protocorm-like-bodies. Kasetsart Journal: Natural Sicience, 32: 329-332.

Wilson S.B., Rajapakse N.C., Young R.E. (2000). Media composition and light affect storability and post storage recovery of micropropagated hosta plantlets. Journal of Horticultural Sciences, 35: 1159-1162.

Withers L.A. (1982). Institutes Working on the Tissue Culture for Genetic Conservation, A Revised List. IBPGR Secretariat, Rome, Italy, 104 pp.

Wotavová-Novotná K., Vejsadová H., Kindlmann P. (2007). Effects of sugars and growth regulators on *in vitro* growth of *Dactylorhiza* species. Biologia Plantarum, 51(1): 198-200.

Yam T.W., Arditti J. (2009). History of orchid propagation: a mirror of the history of biotechnology. Plant Biotechnology Reports, 3: 1–56.

Zhong S.Y., Pan R., Wang X., Ye Q., Guo L. (2000). *In vitro* conservation of *Dendrobium officinale* at low temperature. Chinese Journal of Applied and Environmental Biology, 6(4): 326-330.

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 (gm l^{-1})	Mean no. of leaf/plant *	Mean no. of root/plant *	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 $P < 0.01$.

Formatiert: Hervorheben

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

Basal medium	Sucrose concentrations (gm l^{-1})	% of survival		
		3 months	6 months	12 months
MS	30	100 [a]	100 [a]	90 [a]
1/2 strength MS	30	100 [a]	100 [a]	100 [a]
1/4 strength MS	30	100 [a]	95 [a]	95 [a]
MS	60	90 [a]	90 [a]	75 [a]
1/2 strength MS	60	90 [a]	85 [a]	85 [a]
1/4 strength MS	60	90 [a]	80 [a]	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 $P < 0.01$.

Kommentar [P20]: these are qualitative data and therefore Duncans test is not possible.

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

Kommentar [P21]: You cannot connect the results by lines, they are independent data. Please use columns.

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 gm l^{-1} sucrose concentration, Bar 14 mm; and (b) one-fourth strength MS nutrient medium with 60 gm 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.