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Review Article

MICROPROPAGATION AND *IN VITRO* CONSERVATION OF THREATENED ORCHIDS: A BRIEF REVIEW

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ABSTRACT
Orchids are the most beautiful flowers in the God's creation acting as jewels among the floriculture crops exhibiting an incredible range of diversity in size, shape, colour and long shelf life. In the 21st century, the major concerns for orchid conservation revolved around unsustainable harvest for the orchid trade, habitat destruction, and more importantly, global climate change. Recent rapid changes in the Earth's climate have been linked to changes in physiology, disturbance in pollination and distributions of the orchids. It is, however, likely that many orchid populations will be affected adversely and that *in situ* conservation techniques by themselves will not be sufficient to prevent the extinction of many species. A range of complimentary *ex situ* strategies based on *in vitro* techniques are discussed. Large scale multiplication using tissue culture methods opened up a number of possibilities over the past few years. The application of these techniques such as production of quality plants in mass scale and propagation of exquisite and amazing hybrids have catapulted orchids among the top ten cut flowers in the international market. *In vitro* techniques and storage methods enabled the establishment of extensive collection using minimum space and make it safe for sustainable usage.

Keywords: *Climate Change, Conservation, In vitro, Orchids, Propagation*

INTRODUCTION
Orchids comprise of the largest family of flowering plants with 25,000 species belonging to over 800 genera (Bell, 1994). In almost all aspects, Orchidaceae stands apart from the rest of the plant families, maintaining an intriguing individuality, all its own. The word orchid is derived from a Greek word Orchis meaning testicle because of the appearance of subterranean tubers of the genus *Orchis*. Orchids are perennial herbs; and are either terrestrial (growing on soils), epiphytes (growing on plants but not parasitizing on them) or lithophytes (growing on rocks and sand grains), (Focho *et al.*, 2010). Most of the orchids are epiphytes or terrestrial, rarely saprophytic. Living epiphytically has some advantages. Apart from increased light and air, they get variety of pollinators which include insects, small birds or even bats. This unique partnership between orchid and their pollinator has evolved over ages creating amazing shapes and sizes of the floral parts. Terrestrial orchids are distributed in diverse habitats such as sandy hot deserts or in marshy swamps, in grassland, cool forests, or in areas close to the Arctic Circle. Lithophytic orchids e.g. *Paphiopedilum* and *Phragmipedium* can grow in rock crevices and draw moisture and nutrients from mossy coverings.

During the last few years population decline and failure in flowering has been noted among the orchid species of most of the regions where they grow naturally. Based on worldwide evidence, scientists have declared that the orchids are facing severe threats. The orchid requires an undisturbed habitat, but it faces a variety of threats. It is affected by aggressive non-native species and has been over collected because of its beauty. In addition, the insects that pollinate the plant, agricultural pests, are being eliminated by insecticides. Climate change poses a new threat, and as the local climate dries and warms, it is possible that the species may go extinct. Extrinsic factors are the reasons most frequently cited in the extinction of orchids. Competition from invasive species, increasing soil salinity, and climate change has reduced orchid numbers. Many species are thus, in danger of becoming extinct while some others have already become so. It is, however, likely that these orchid populations will be affected adversely and that *in situ* conservation techniques by themselves will not be sufficient to prevent the extinction of many species.

Currently, a range of complimentary *ex situ* strategies are available. Amongst these, orchid seed germination and propagation through *in vitro* techniques have been highlighted as invaluable tools for conserving the maximum amount of genetic diversity in the minimum space and have the potential to enable the conservation of valuable material for possible re-introduction and habitat restoration programs in future. Particular techniques are mentioned here for propagation and *ex situ* conservation of orchids, which need urgent for the species that are most likely on the verge of extinction.

In vitro Propagation of Orchids

The orchid seeds are minute, non-endospermic, and with a reduced embryo enclosed within more or less transparent coat (Arditti, 1992). So, the rate of germination in nature is very low. Apart from the difficulties of seed germination, in natural conditions, the life cycle of orchids is very long. It takes about 5-10 years for a plant to bloom and produce fertile seeds. Vegetative propagation of orchids is also an extremely slow process. Therefore, mass scale production of orchids depends heavily on different *in vitro* methods. Such methods could be effectively used for conservation purposes in both direct and indirect ways. Many workers have made valuable contributions in the field of orchid micropropagation.

Asymbiotic Seed Germination: The seeds of the orchids, produced in large numbers in each capsule (Figure A, B), are highly fragile and possess virtually no stored food material or endosperm (Mitra, 1971). In nature they cannot utilize their own scanty lipid reserves, breaks down starch or photo-synthesize. Following water uptake, which causes swelling, orchid seeds may turn green, but fail to develop further in the absence of fungal infection (symbiotic germination). Consequently, they germinate very poorly in nature (less than 1%). However, successful *in vitro* germination methods of orchid seeds took place following the formulation of Knudson 'B' and 'C' medium (Knudson, 1922, 1946). The culture of immature ovules often referred to as embryo/green-pod/ green-fruit culture has opened new vistas in conservation and commercialization of orchid genetic resources and has been successfully employed in a large number of commercially important and endangered taxa representing both epiphytic and terrestrial habits (Zeng *et al.,* 2015; Mondal *et al.,* 2016; Utami *et al.,* 2017). The germination potential of embryos varies with their developmental stage. The immature embryos, in general, germinate readily, and much better than the mature ones. Very young orchid ovules fail to germinate and hence, do not form suitable explants, due to dormancy, pH, and other metabolic factors. The epiphytes germinate better than the terrestrials probably due to the nutritional complexities of the latter taxa. Stoutamire (1974) attributed the impaired germination of these taxa to their greater mycorrhizal requirements in nature; the fungus probably provides the growth substances to activate their enzyme system (Pathak *et al.,* 2001). Orchid seed germination and subsequent development into seedlings is usually accompanied by an intervening protocorm stage (Figure C). The word "Protocorm" was derived by Melchior Treub, who was the director of the Bogor Botanic Gardens in Indonesia (Arditti, 1992). In general, orchid seed germination includes swelling of embryos and their emergence through an apical or lateral slit in the seed coat as globular/elongated spherules which subsequently form chlorophyllous, hairy, and pear-shaped protocorms. The orchid protocorm has been variously considered as an undifferentiated callus or a differentiated shoot primordium structure (Kanase *et al.,* 1993) whereas it is also believed that it acts as a cotyledon for supplying nutrients during development of embryo and its subsequent growth into seedlings (Lee, 1987). The protocorm budding characterizes orchids and it leads to the development of multiple plantlets (Figure D).

Micropropagation of Orchids: As orchids are out breeders, their propagation using seeds leads to the production of heterozygous plants. Hence, protocols providing regeneration from various vegetative parts of mature plants i.e. micropropagation are essential. After 1960, revolution took place in the field of propagation of orchids when Georges Morel (1960) cultured *Cymbidium* shoot tips and obtained protocorm-like bodies (PLBs) to generate mosaic virus free *Cymbidium* plants from infected ones. Morel first used the term "Protocorm like Body (PLB)" in the work that was reported in the American Orchid Society Bulletin (Arditti, 2010). Subsequently, successful results were also obtained with a large number of orchid species including *Cattleya*, *Dendrobium*, *Lycaste*, *Miltonia*, *Ondontoglossum, Phaius* and *Vanda* (Arditti and Ernst, 1993).

Plant regeneration of orchids via various *in vitro* techniques may be achieved through callus formation (Figure E, F) or direct shoot bud formation or PLB mediation (Figure G). The various modes of orchid micropropagation are described as follows:

Shoot tip culture: Shoot tips have been effectively used for the induction of shoot buds and PLBs of many orchids (Winarto and Teixeira da Silva, 2015). The production of virus free *Cymbidium* by Morel (1960) was an important advance in itself and also led to the clonal propagation of orchids through shoot tip culture technique in particular. Later Morel extended this technique for the production of virus free *Cymbidium* plants to *Odontoglossum, Miltonia,* and *Phaius* (Morel, 1963). This technique has provided efficient regeneration through vegetative multiplication of these orchids. Utilization of shoot tip culture technique was resulted in rapid multiplication of *Vanda coerulea* and successful establishment of clonal plants in forest segments of the Western Ghats (Seeni and Latha, 2000). Shoot tips from both *in vitro* and mature plants responded by the formation of buds on medium containing 8.8µM BAP and 4.1µM NAA. Combined effect of NAA and BAP was also more effective for multiple shoot formation from shoot tips of *Vanda tessellata* than the single effect of NAA, IAA or Kinetin (Rahaman *et al.*, 2009). Mondal *et al.,* (2013) reported that the tissue cultured shoot primordium of *Doritis pulcherrima* could maintain a high ability for rapid propagation and regeneration of plantlets and high chromosome stability.

Leaf culture: Young leaves and leaf tips were successfully cultured *in vitro* to propagate orchids (Wimber, 1965; Arditti, 1977; Vij *et al.,* 1986, 1994b; Vij and Aggarwal, 2003). The first unambiguous and well documented report that leaves can produce protocorm like bodies (somatic embryos) was made in cultures derived from *Cymbidium* shoot tips (Wimber, 1965).

Mathews and Rao (1985) reported, in leaf explants of *Vanda* hybrid (*Vanda* TMA x *Vanda joaquim*) that the leaf base was the most amenable region for growth with over 80% of the isolated leaf base cultures showing proliferation.

The presence of leaf base was a deciding factor even for the distal end proliferation (Figure H). Similarly, in other vandaceous orchids the leaf base exhibited a greater proliferative potential than leaf tips in *Ascocenda* and *Vanda* (Fu, 1978, 1979), *Renanthera* (Seeni and Latha, 1992) and *Acampe* (Nayak *et al.,* 1997). Young leaves responded better than the older ones. Similar conclusion was made by Kaur and Bhutani (2009) where they assessed the regeneration potential of leaf segments of *Vanda testacea*. *Vanda coerulea* leaves of the mature plants did not form shoot buds or PLBs (Seeni and Latha, 2000) *in vitro*, though the shoot tips of mature plants could differentiate. Frequency of response in foliar cultures of *Vanda* Kasem's Delight 'Tom Boykin' (Vij *et al.,* 1994b) and *V. coerulea* (Vij and Agarwal, 2003) was also markedly influenced by the juvenility of the tissues in terms of size of the explants. In contrast the regeneration potential of leaf explants from mature plant of *V. spathulata* (L.) Spreng was complemented significantly by more number of shoots harvested per explant compared to *in vitro* explants on Mitra *et al.,* (1976) medium supplemented with 66.6µM BAP + 28.5 µM IAA. Successful micropropagation using leaf explants depends on many factors like nutrient composition of medium, growth hormones, source of the leaves (*in vivo/in vitro*), part of the leaf taken, explant orientation and most importantly the age of the leaf (Chugh *et al.,* 2009).

Axillary bud culture: In orchid micropropagation axillary bud culture also acted as an important tool for plant regeneration through various morphogenetic pathways. Wang (1990) obtained PLBs from axillary buds of *Cymbidium ensifolium*.

Multiple shoot formation was observed when axillary buds of five species of *Dendrobium* (*D. crumenatum, D. fimbriatum, D. moschatum, D. nobile, and D. pierardii*) were cultured *in vitro* (Sobhana and Rajeevan, 1993).

The regeneration potential of these explants has been positively evaluated in several orchids including *Arudina* (Mitra, 1971), *Bletilla* (Vij and Dhiman, 1997), *Cattleya*, *Miltonia*, *Cymbidium*, *Phaius* (Morel, 1964; Vajrabhaya, 1978), *Cymbidium* (Vij *et al.,* 1994a), *Dendrobium* (Vij and Sood, 1982), and *Mormodes* (Arditti and Ernst, 1993) etc. The regeneration potential seems to be influenced by the age of mother plant, position on the donor axis and growth stimulus in the nutrient pool as has also been suggested by Vajrabhaya (1978) (Prakash and Chaudhary, 2001).

Inflorescence stalk and floral buds culture: Out of various *in vitro* techniques that have been developed for propagation of orchid, culturing the dormant buds, present at the basal part of the inflorescence, is also an important one. The first species in which young flower buds or inflorescence cultured were *Ascofinetia, Neostylis, and Vascostylis* (Intuwong and Sagawa, 1973). Those of *Phalaenopsis, Phragmipedium*, and *Cymbidium* (Kim and Kako, 1984) were cultured subsequently. *In vitro* propagation procedures involve exposing buds to either high auxin levels (Zimmer and Peiper, 1977), or high cytokinin levels (Tanaka and Sakanishi, 1978) or anti-auxin levels (Reisinger *et al.,* 1976). Age of the explant is another factor which affects regeneration response of orchid inflorescence explants and explants from younger sources have given better results in *Dendrobium Miss Hawaii*, *Phalaenopsis capitola*, *Oncidium Gower Ramsey*, *Ascofinetia* and *Ponerorchis graminifola Rchb.f.* (Nuraini and Sahib, 1992; Intuwong and Sagawa, 1973; Mitsukuri *et al.,* 2009). In contrast, Goh and Wong (1990) showed that developing inflorescence of *Aranda deborah*, at the later phase of exponential growth, can be used as explants for clonal propagation in monopodial orchids.

Root and Rhizome segment culture: The possibility of using aerial roots of orchids was earlier suggested by Beechey (1970) even though capacity of orchid roots to form shoots is low (Kerbauy, 1984). Later roots of *Catasetum* (Kerbauy, 1984), *Rhyncostylis* (Sood and Vij, 1986), and *Cyrtopodium* (Sanchez, 1988) have been cultured successfully and used to produce plantlets. Chaturvedi and Sharma (1986) reported a very high rate of proliferation in excised root tips of *Vanda* hybrid and *Rhyncostylis* in medium containing 1 mg/L BA, 1 mg/L IAA, and 200 mg/L of casein hydrolysate. Vij (1993) reported that the degree of responsiveness and the number of propagules produced was species specific during regeneration of roots. In general, the frequency of embryo formation of root derived callus was higher than stem and leaf derived callus (Jen and Chang, 2000). However, in *Vanda* sp. the frequency of embryo formation of stem derived callus was higher than that of root and leaf derived callus (Lang and Hang, 2006).

Rhizome tips have also been cultured, with the earliest success being reported by H. Torikata (Udea and Torikata, 1972). A highly efficient method for the induction of whole plantlets of *Cymbidium kanran* from rhizomes has been reported (Shimasaki and Uemoto, 1990). Protocorm-like shoots were induced on medium supplemented with high concentrations of BAP whereas proliferation of *C. kanran* rhizomes was enhanced when higher levels of NAA were employed in the medium. Shimasaki and Uemoto (1990) suggested that the application of exogenous plant growth regulators is not indispensable for the normal plantlet production in this terrestrial *Cymbidium* species. The fact that cytokinins are effective in induction of shoots from rhizome segments is further supported by reports on *Cymbidium forrestii* (Paek and Yeung, 1991) and *Geodorum densiflorum* (Lam.) Schltr. (Sheelavantmath *et al.,* 2000; Roy and Banerjee, 2002). However, in BAP-containing medium the rhizome growth and branching was reduced and some rhizome tips gradually turned up and developed into shoots (Paek and Yeung, 1991). Auxins, especially NAA, are known to stimulate rhizome formation and branching in cultures of many orchids like *C. kanran* Makino (Shimasaki and Uemoto, 1990), *G. densiflorum* (Roy and Banerjee, 2002), *C. forrestii* (Paek and Yeung, 1991) and these rhizome branches can readily give rise to shoots in the presence of cytokinins. Paek and Kozai (1998) have reported BAP to be the best cytokinin for inducing shoot formation and for switching rhizome tissues into PLBs in most of the commercially important rhizomatous orchids like *Cymbidium*.

Callus culture: Success in callus cultures in which the callus can be maintained for a long period through subsequent subculture has been limited to a few orchids (Chang and Chang, 1998; Ishii *et al.,* 1998; Roy and Banerjee, 2003). This is primarily due to difficulty in induction, limited growth and severe necrosis of callus (Roy *et al.,* 2007). In contrast, Lang and Hang (2006) reported that the embryogenic calli derived from the segment of roots, stem and leaves of *Vanda* orchid are able to form PLB or somatic embryo in PGR free MS medium. An efficient protocol for callus production is of much significance in some orchids like *Dendrobium chrysotoxum* as an alternative source of secondary metabolites like bisbenzyl erianin, which has tremendous therapeutic potential as an antioxidant, antiangiogenic and antitumour agent (Roy *et al.,* 2007).

Suspension culture: F. C. Steward and M. O. Mapes produced the first orchid (*Cymbidium*) from cell suspensions (Steward and Caplin, 1952; Steward and Mapes, 1971). According to Steward, the cell lumps and embryos in the suspension and eventually the plants produced from them originated from single cells isolated by the shaking. It has been discovered that in the meristems, cultured in a liquid medium and kept in rotary motion, the number of PLBs are greatly increased and maximum plants can be produced from a single explants. Wang (1990) obtained PLBs from shoot meristem and axillary buds of *Cymbidium*, following this technique. When PLBs were cultured on half strength MS medium without any growth regulator under continuous illumination, a 'habituated mericlone' can be established which could be multiplied and sub cultured without differentiation of buds or roots. On half strength liquid MS medium with 10% coconut milk, PLBs multiplied and differentiated into plantlets.

Thin cell layer culture: The thin cell layers consist of explants of small size excised from different plant organs, either longitudinally or transversely from different plant parts like leaves, stems, floral primordia, or PLBs. Longitudinal sections consist of only one type of tissue, such as monolayer of epidermal cells, whereas, transverse sections include a small number of cells from different tissue types: epidermal, cortical, cambium, perivascular and medullary tissue, parenchyma cells (Tran Thanh Van, 1980). According to Rout *et al.,* (2006), the efficiency of thin cell layer culture is very high compared to the conventional technique of tissue culture. Lakshmanan *et al.,* (1995) reported rapid regeneration of a monopodial orchid hybrid *Aranda deborah* a hybrid of *Arachnis hookeriana* and *Vanda lamellata*, using thin sections measuring 0.6-0.77 mm in thickness obtained from the shoot tips of *in vitro* grown plantlets as explants on Vacin and Went medium (1949) enriched with 20% (v/v) coconut water. Transverse thin cell layers excised from the stem internodes and nodes from *in vitro* grown plantlets of *Dendrobium* have also been used to obtain shoot buds and PLBs (Teixeira da Silva *et al.,* 2015).

Protoplasts culture: The latest development in orchid propagation has been the successful isolation of protoplasts from different explants such as stem, root, leaf disc, petal and protocorm. Production and fusion of orchid protoplasts was first reported by the Malaysian orchid scientist Chris K. H. Teo and the German botanist K. Neumann (Teo and Neumann, 1978 a, b). Seeni and Abraham (1986) screened more than 24 orchid species for protoplast culture and successfully achieved it from juvenile leaf bases of *Cymbidium aloifolium*. Subsequent isolation of the orchid protoplast and cells were carried out in The United States (Price and Earle, 1984), Singapore (Loh and Rao, 1985; Hew and Yip, 1986; Koh *et al.,* 1988) and India (Seeni and Abraham, 1986; Gopalakrishnan and Seeni, 1987).

Ex-vitro establishment of the regenerants: When plantlets and seedlings were sufficiently large to handle conveniently, they were transferred for acclimatization (Figure I, J). Larger plantlets are easier to handle and have better chance of survival. After taking out from flasks, the seedlings and well rooted plants were washed thoroughly to remove traces of nutrient agar, and then transferred to clay pots containing a potting mixture. Seedlings generally grow better in a group rather singly due to community effect. The potting media was generally a mixture of finely chopped dried coconut husk, small pieces of dead tree bark and broken bricks or charcoals in various ratios. Peat moss was found to be best for improving stem diameter and height for increasing root number. Immediately after transplant, the plantlets should be sprayed with very low dosages of broad spectrum fungicide to avoid the fungal attack. It is recommended to keep transplants in a well-ventilated location under subdued light and water regularly for further growth and development.

In vitro Conservation *of Orchids*

At present the orchids figure prominently in the Red Data Book prepared by International Union of Conservation of Nature and Natural Resources (IUCN). In fact, the entire family is now included in Appendix-II of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), where the international trade is strictly controlled and monitored (Chugh *et al.,* 2009). It is, however, likely that many populations will be affected adversely by the adverse climatic change and that *in situ* conservation techniques by themselves will not be sufficient to prevent the extinction of many species. A range of complimentary *ex situ* conservation strategies based on *in vitro* techniques are discussed below. The different *in vitro* conservation methodologies, such as *in vitro* micropropagation

and medium and long term storage of germplasm are applicable for sustainable production of quality planting material of important, rare, endangered, threatened as well as exotic hybrid orchids.

Asymbiotic seed germination techniques, which have been applied for the conservation of endangered and threatened taxa, proved to be useful in the reintroduction of many orchids (Pedroza - Manrique *et al.,* 2005; Stewart and Kane, 2006; Deb and Imchen, 2006). Since the population size of endangered orchid *Vanda coerulea* is steadily declining, asymbiotic seed germination technique was used to increase the population size as because orchid seed germination is a slow process (Devi *et al.,* 1998; Roy *et al.,* 2011). For terrestrial orchid seed in particular, considering the appropriate symbiotic fungi alongside the orchid seed, techniques have been successfully discovered for storing both orchid seed and its fungal partner in alginate beads at sub-zero temperatures (Wood *et al.,* 2000; Sommerville *et al.,* 2009). *In vitro* (both symbiotically and asymbiotically) raised seedlings of these orchid species were acclimatized and re-introduced in natural habitat. In Latin America possible model is a project aimed at conserving and re-introducing *Cattleya quadricolor* (Seaton and Orejuela, 2009).

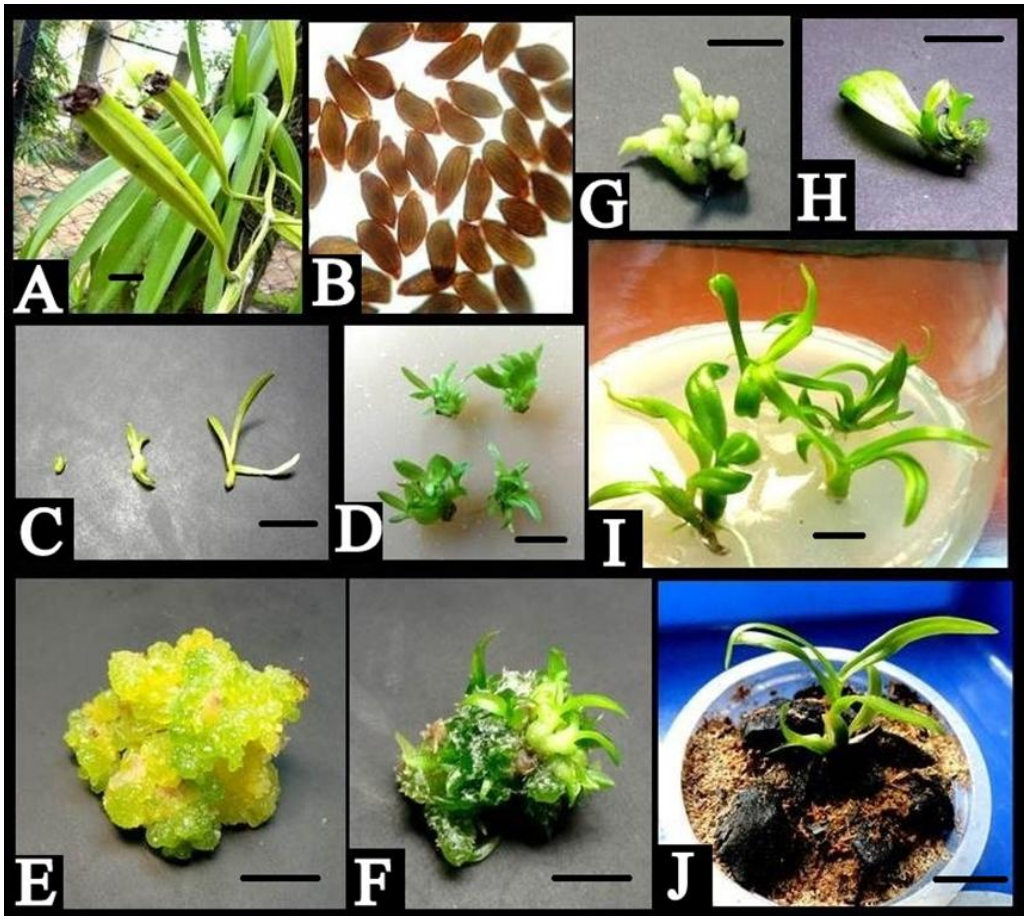

Figure: *In vitro* Propagation of Orchids: A Orchid Fruits; B Orchid Seeds; C Developmental Stages of Seedlings; D Developed Seedlings; E Embryogenic Callus; F Plant Regeneration from Callus; G Shoot Tip Derived PLBs; H Plantlets Regeneration from Leaf Explant; I Regenerated Plants under *in vitro* Condition; J *Ex vitro* Establishment of the Regenerated Plants (Bar = 1 cm)

Generally, *in vitro* storage of cultured propagules could be achieved through two different methods. At first, a plant material was subjected to direct storage, where established cultures of protocorms and somatic embryos were transferred to growth chambers with various low temperature and light intensity. Low-temperature storage of orchid callus also proved to be of considerable importance for the

preservation of genetic diversity and specific clones (Sivasubramaniam *et al.,* 1987). Similarly, Na and Knodo (1995) reported the tissue culture shoot primordium method that holds promise for long term conservation of the gene resources of the Yunnan threatened orchid *Vanda pumila* and can be furthermore, applied to stable, clonal mass propagation of ornamentally important strains after artificial selection.

In the second method, the plant materials were encapsulated by sodium alginate, using $Ca(NO_3)_2$ as gelling agent to develop synthetic seeds. These synthetic seeds were subsequently stored at various temperatures, with or without growth retardants (e.g. ABA and p-coumaric acid etc.). The alginate coat protects micropropagule and thus has the practical application for the germplasm conservation of an elite plant species and exchange of axenic plant materials between laboratories (Fabre and Dereuddre, 1990; Hasan and Takagi, 1995; Hung and Trueman, 2011; Ahmad *et al.,* 2012). Alginate-coated, non-embryogenic micropropagules were relatively inexpensive to produce, easy to handle, transport and plant. Furthermore, they can be used for cryopreservation via encapsulation dehydration and encapsulation vitrification techniques (Pennycooke and Towil, 2001; Wang *et al.*, 2002). During storage, encapsulated propagules require no transfer to fresh medium, thus, reduce the cost of maintaining germplasm *in vitro* (West *et al.,* 2006).

Conclusion

Recent rapid changes in the Earth's climate have been linked to changes in physiology, disturbance in pollination and distributions of the wild orchids. Projected future climate change will undoubtedly result in even more dramatic declination of these jewels of plant kingdom i.e. the beautiful orchids. To cope up with this climate change, there is a growing awareness of the need to take prompt action and an increasing number of different propagation and *ex situ* conservation strategies for the orchid population around the world. Living collections are currently being utilized as a conservation tool, and there is a need to do more to include members of the wider orchid community.

The past decades have witnessed significant advancement of *in vitro* techniques and currently new methods are available to conserve the threatened orchid germplasm. The recently developed techniques offer new options and facilitate propagation via various types of *in vitro* cultures. *In vitro* techniques and storage methodologies enable the establishment of extensive collection using minimum space. The development of successful storage methods enables the establishment of extensive basal collections, with representative genetic diversity. Establishing and maintenance of medium and long term *in vitro* plant stock of orchid germplasm is extremely beneficial since this results in significant reduction in collection pressure on the wild orchid populations. These collections further allow continuous supply of valuable material for wild population recovery, molecular investigations, ecological studies, or commercial uses in the floriculture industry.

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