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CONSERVATION OF RARE AND THREATENED THERAPEUTICALLY IMPORTANT ORCHID *Pleione maculata(Lindl.)* Lindl. & Paxton THROUGH PSEUDOBULB CULTURE *IN VITRO*

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ABSTRACT

The regeneration response of pseudobulb explants procured 40 wks after their emergence in axenic seedlings, was tested in Mitra medium and its various combinations with growth adjuncts. Development of neoformations in the explants was markedly influenced by their position on the donor axis (apical half responded better) and the chemical stimulus in the nutrient mix. The regenerative competence of pseudobulb segments was best achieved in Mitra medium supplemented with KN. The apical segments responded via formation of multiple shoot buds and callus wherein 12 healthy plantlets can be obtained after 20wks of culture.

KEYWORDS: *Pleione maculata*, Endangered, Pseudobulb, Neoformations, Regeneration, Axenic, PLBs

Medicinal plants, since times immemorial, have been used as a source of medicine in virtually all culture forms. The use of herbal remedies mentioned in ancient texts such as Vedas and Bible are obtained from wild and naturally occurring medicinal plants. Medicines, in several developing countries, using local traditions and beliefs, are still widely used to conserve human health. Developed countries, in recent times, are turning to the use of traditional medicinal systems like acupuncture and ayurveda involving the use of herbal drugs and remedies which are in practice especially in China and India.

WHO (World Health Organization) reports (2002), over 80% of world population relies on traditional medicines, largely plant based, for their primary health care needs. Such popularity of healthcare plant-derived products has been traced to their increasing acceptance and use in the cosmetic industry as well as to increasing public costs in the daily maintenance of personal health and well being. Nearly twenty one thousand species of flowering plants are used in 21 countries with a global market of US $ 62billion with an average annual growth rate of export of medicinal plants between 1996-97 to 2000-01 was 8.9% and it is still increasing. China with exports of over 1,20,000 tonnes p.a., and India with some 32,000 tonnes p.a. dominate the trade. It is estimated that Europe, annually imports about 400,000 tonnes of medicinal plants with an average market value of US$1billion from Africa and Asia(Hoareau and DaSilva, 1999). The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka and Thailand. In China, about 40% of the total medicinal consumption is attributed to traditional tribal medicines. In the mid-90s, it is estimated that receipts of more than US$2.5 billion have resulted from the sales of herbal medicines. It has been reported that in Europe alone 10000 medicinal species are endangered (Vines, 2004). Globally, about 15,000 medicinal plant species are threatened, the reasons including loss of habitat, commercial over-harvesting, invasive species and pollution (Hamilton, 2008).

India, one among 12 Bio-diverse countries of the world, is abode of 45000 floral species, out of which 15000 are those of Medicinal Plants. Approximately 85% to 90% of these come from the wild. Department of Indian System of Medicine & Homeopathy (ISM&H), Ministry of Health & Family Welfare, Government of India, has identified 1500 medicinal plants of which 500 are commonly used in the preparation of herbal drugs. Nearly 150 species have been categorized as endangered. 90% drugs used in ISM&H are plant based.

However, the genetic diversity of traditional medicinal plants is continuously under erosion because of extensive collections, poor harvesting techniques, and progressive loss of growth habitats and unmonitored trade of medicinal plants. Stocks of several medicinal plants are fast diminishing and are in danger of extinction as a result of growing trade demands for cheaper health care products and new plant-based therapeutic markets in preference to more expensive target-specific drugs and biopharmaceuticals. It is by no doubt very necessary to push every possible effort into strategies for the conservation of these invaluable resources. It is imperative to develop the tradition of cultivating medicinal plants within the traditional cropping system to

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avoid extinction of most of the plants (Amujoyegbe *et al*, 2012)

Orchids have been described as the "Royal Family" of plants by those captivated by their exquisite flowers of myriad shapes, sizes, and colours. The Chinese were the first to cultivate and describe orchids for medicinal use. The Greeks referred to testicles as *Orchis*, and Theophrastus (370-287 BC) named the orchids from that word, as the underground tubers of many European terrestrial orchids resemble a pair of testicles. He made mention about the medicinal properties of orchids in his book "Enquiry into Plants". In India, the earliest mention of Orchids is found in Charaka Samhita (A.D.100), which describes the medicinal properties of *'Vanda'*. The therapeutic significance of orchids has been attributed to their huge reservoir of phytochemicals (alkaloids, flavonoids, glycosides, carbohydrates). The orchids are habitat specific and their characteristic interdependence often of very specific nature with their ecosystem makes them more vulnerable to destruction than any other group of plants. Besides contributing tremendously to the growth and development of international trade in floriculture, the orchids are also therapeutically significant.

Pleione maculata is a lithophytic/epiphytic herb with two leaves and barrel-shaped pseudobulbs. It flowers when leafless, during October and November. Flowers white; lip white with purple streaks at base and yellow patches at apex. It requires a well drained substratum and dwells in a variety of habitats, ranging from humus rich soils, rocks and tree trunks, within an altitudinal range of 1300-1700m in the North Eastern Himalayas (Arunachal Pradesh, Meghalaya, Manipur, Nagaland and Sikkim). The species is well known in the orchid trade for its beautiful white and fragrant flowers. It is medicinally also important as its pseudobulb/rhizome is used in liver complaints and stomachache (Teoh, 2016). It is one of the Indian orchid species which is in great demand in international market for breeding material due to their inherent attractiveness coupled with their ability to transmit these characters to hybrids (Bose and Bhattacharjee, 1980; Kumar and Sheela, 2007). It is considered as extremely rare or possibly extinct orchid mainly due to its excessive collections, progressive depletion of its natural habitat, global warming, soil erosion and encroachment. Plant tissue culture could be one of the most suitable alternative tools to minimize the pressure on natural population of medicinal orchids and their sustainable utilization. Orchids are highly heterozygous and their vegetative propagation through division of clumps of rhizomes, bulbs or by the rooting of off shoots also takes long time and difficult to obtain desired number of orchids. This difficulty in natural population drives the many orchids including medicinal orchids to be threatened and some are reached to extinction. It is therefore important to take initiative for their mass propagation and reestablish them in nature. *Ex situ* conservation measures can be complementary to *in situ* methods as they provide an "insurance policy" against extinction. These measures also have a valuable role to play in recovery programmes for endangered species. In this context, *ex situ* conservation is very important aspect of orchid conservation which can include both seed banks and *in vitro* culture plant tissue collections (Pant, 2013).

MATERIALS AND METHODS

In Vitro sourced 40wks old pseudobulbs were excised into apical and basal segments and their regenerative response was tested employing M medium (Fig.a) and its combinations with growth adjuncts. The medium was adjusted for its pH 5.6 prior to autoclaving at 121ºC at 1kg cm^{-2} for 20min. The cultures were maintained under a 12-hr photoperiod of 30µ$molm^{-2}s^{-1}$ light intensity and a temperature of 25±2ºC, and observed regularly. The problem of phenolic exudates was overcome by frequent subculturing. All the experimental manipulations were carried out under aseptic conditions and for each experiment at least 4 replicates were used and experiment is repeated thrice. The data were analyzed statistically using one-way analysis of variance (ANOVA), and the data means ±SE of at least three different experiments were represented and compared using Duncan's multiple range test with the level of significance set at 5%.

RESULTS AND DISCUSSION

The results are summarized under Table1; Figs.1-8 and some of the pertinent findings are as follows:

In the basal medium cent per cent explants responded; both segments responded via shoot bud formation in 3.00±0.00wks. Complete plantlets with 2-3 leaves and 1-2 roots were obtained in 7wks (Figs.4&8). When organic growth supplements YE was used, in the medium, apical/proximal segment responded via shoot bud formation while the distal one through multiple shoot

buds in 4.75±0.50wks (Fig.3), which grew rapidly to form complete plantlets in another 3 wks. Both P and CH exhibited similar response (Figs. 5-6) though it was delayed in P enriched medium (6.00±0.00wks) but response was cent per cent as compared to 75.00±0.00 per cent in CH containing medium. The apical segments responded via direct differentiation of shoot primordia while callus was initiated at cut ends of the basal segment. Callus was creamish, compact and non-organogenetic and could not be maintained for more than 8 wks. IAA, in the medium failed to induce regeneration response while in NAA supplemented combination, 50.0±0.00 per cent explants responded in 4.00+0.00wks. It induced regeneration via callus formation only in the apical segment (Fig.2).Amongst cytokinins used, BAP failed to evoke any regeneration response. Replacement of BAP with KN, on the other hand elicited cent per cent response from the explants. Multiple shoot buds and callus were formed within 3.50±0.00 wks on apical segment (Fig.7).Callus was green and organogenetic and complete plantlets were obtained in 8 wks.

Fig.1 Fig.2 Fig.3 Fig.4

Fig.5 Fig.6 Fig.7 Fig.8

Figs. 1-8. *Pleione maculata,* Regeneration through pseudobulb segment culture: 1, pseudobulb segments with apical and basal halves at the time of inoculation(M);2, callus mediated regeneration at the apical half (M+NAA);3, Shoot bud mediated response in both the halves(M+YE);4, plantlets with differentiating leaves and roots on both apical and basal segments(M);5, Apical segments showing developing plantlet via shoot bud formation while basal segment showing callus(M+CH);6, Plantlet formation at the apical segment(M+P);7, multiple shoots and callusing at the apical segment (M+KN);8, Multiple shoot bud mediated plantlet formation(M).

Pseudobulbs segments are known to have responded positively in *Arundina, Cattleya, Cymbidium, Miltonia and Phaius* (Morel,1964 and 1970), *Bletilla striata* (Vij and Dhiman,1997), *Bulbophyllum careyanum* (Vij *et al*,2000), *Cattleya* (Vajrabhaya,1978), *Cymbidium* (Chang and Chang,1998), *Dendrobium moschatum* (Vij and Sood,1982), *D. chrysanthum* (Vij and Pathak,1989), *Malaxis acuminata* (Vij and Kaur,1998) and *Thunia* (Arora,1990). Presently, the regeneration potential of *in vitro* sourced pseudobulbs was positively tested.

Table 1. *In vitro* regeneration response of pseudobulb segments of *Pleione maculata* on Mitra medium.

Additive(s)	Explant responded (%)	Time taken in wks for initiation of response	No. of meristematic loci invoked/explant	Regeneration pathway	No. of plantlets observed/explant after 20wks of culture	Remarks
-	100.00±0.00[a]	3.00±0.00[a]	2	SP-Pl	8	Shoot bud mediated regeneration
P	100.00±0.00[a]	6.00±0.00[c]	2	SP- Pl	8	Shoot bud mediated regeneration
YE	100.00±0.00[a]	4.75±0.50[b]	3	SP- Pl	12	Shoot bud mediated regeneration
CH	75.00±0.00[b]	4.00±0.00[b]	2	SP- Pl /Ca	6	Shoot bud mediated regeneration in apical and callus formation in basal segment
IAA	-	-	-	-	-	No regeneration response
NAA	50.0±0.00[c]	4.00±0.00[b]	1	Ca-Pl	2	Callus mediated regeneration in apical segment
BAP	-	-	-	-	-	No regeneration response
KN	100.00±0.00[a]	3.50±0.00[a]	3	SP- Pl	12	Multiple shoots and Callus mediated regeneration

*Pl, Plantlet; SP, Shoot primordia; Ca, Callus; YE, Yeast Extract; CH, Caesin Hydrolysate; P, Peptone; IAA, Indole Acetic Acid; NAA, Naphthalene Acetic Acid; BAP, Benzyl Amino Purine; KN, Kinetin

However this potential was markedly influenced by both the position of the donor axis [proximal (basal)/distal (apical)] and the growth stimulus in the nutrient pool in *Pleione maculata*. In *Pleione maculata,* either apical (M+NAA/KN)/or both apical and basal segments (M, M+P/YE/CH)regenerated; the apical half in general responded better as compared to basal half. The explants regenerated via both shoot bud and/or callus formation. The positions of the explant on the donor axis and growth stimulus in the nutrient pool were important factors affecting the regenerative pathway in *Pleione maculata.* Such a differential response was attributed to the genetic and/or source related physiological intricacies of the explants (Sharma,1996).While axillary buds in the present cultures could be activated in the basal medium, induction of additional meristematic loci was obligatory to PGR treatment as reported in *Thunia* (Arora,1990) and *Bletilla* (Vij and Dhiman,1997).

CONCLUSION

Presently, in *Pleione maculata* the regenerative competence of pseudobulb segments was best achieved in Mitra medium supplemented with KN. The apical segments responded via formation of multiple shoot buds and callus and wherein 12 healthy plantlets can be obtained after 20 wks of culture.

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