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IN VITRO PROPAGATION OF *PAPHIOPEDILUM SPICERIANUM* (REICHB. F.) PFITZ. – A RARE AND ENDANGERED ORCHID SPECIES FROM NORTHEAST INDIA

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Abstract

Presently, an attempt was made to propagate *Paphiopedilum spicerianum* (Reichb. f.) Pfitz. *in* vitro using seed, leaf and shoot tip as explants. Murashige and Skoog (MS) medium with and without growth additives was used as nutritional recipe. The seeds procured from green capsules failed to respond, whereas, young leaf and shoot tip explants showed regeneration response via callusing. The formation and development of the callus was found to be extremely slow and poor. Though regeneration occurred on the leaf explants in MS medium supplemented with the combination of BAP (0.5 mgl^{-1}) + IAA (0.1 mgl^{-1}) + IBA (0.1 mgl^{-1}); KN (3.5 mgl^{-1}) + IBA (0.7 mgl^{-1}); BAP (2.5 mgl^{-1}) + 2, 4-D (0.5 mgl^{-1}), the size and weight of the callus varied with the growth stimulus (Fig. 2 A-L). In case of shoot tip explants, the regeneration occurred via callus formation in medium supplemented with only TDZ at different concentrations (*i.e.*, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 mgl^{-1}). The initiation and development of the callus was favored with increase in the concentration of TDZ; the best results were obtained in medium with TDZ at 0.20 mgl^{-1}. Leaf formation was reported in IAA (0.2 mgl^{-1}) supplemented medium and subsequently complete plantlets were successfully produced in this endangered orchid species.

Introduction

ORCHIDS CONSTITUTING an interesting group of flowering plants with beautiful flowers are economically important. They are grown almost all over the world mainly for cut-flower and pot-plant production. According to the IUCN Action plan (1999), orchids are identified as amongst the world's most diverse and widely distributed plants (cf. Sibin *et al.*, 2014). All the orchid species are protected under Wild Life (Protection) Act, 1972, and treated as Protected species under CITES (Convention on International Trade in Endangered Species of Wild Flora and Fauna) (Hegde, 2012).

Paphiopedilum commonly known as Slipper orchid with over 70 species is native to South and SouthEast Asia (Udomdee *et al.,* 2012). *Paphiopedilum spicerianum* (Rchb. f.) Pfitz., an endangered terrestrial species which flowers during November to January (Fig. 1), is in great demand because of unique beauty of its flowers. It is a terrestrial herb with small stem, leathery leaves, and very attractive colourful flowers developing individually; each flower is with a snow white upper sepal with a pink central stripe and a similarly coloured staminodium. It is an endangered plant species of Indian sub Himalayan region (Nayar and Sastry, 1987) and it is protected under the Appendix I of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES; CITES, 2013) (cf. ENVIS Centre on Floral Diversity, 2015). Kataki (1984) and Kataki *et al.* (1984) have mentioned that this orchid species is rare and endemic due to over collections as well as disturbance of its natural habitats. Due to the destruction of the natural habitat and anthropogenic pressure, its populations are very less in the natural habitats. Therefore, there is an urgent need for some conservation measures. *In vitro* propagation is one of the important advanced conservation initiatives which help to increase the population of a particular plant species within a particular period of time. This technique has been successfully used in many species by earlier researchers (Kaur and Pathak, 2014; Pathak and Vij, 2001, 2007; Pathak *et al.*, 1992, 2001, 2005, 2011, 2012; Vij and Pathak, 1988, 1990; Vij *et al.,* 1994, 1995). This *in vitro* technique for rapid and mass propagation offers possibilities for 'recovery' of the endangered species, thus reducing the risk of extinction (Nadeem *et al.*, 2000).

The presently selected *Paphiopedilum spicerianum* (Rchb. f.) Pfitz. is a fast disappearing species found in the Cachar District; it was earlier rediscovered by the research group under Prof. B. K. Dutta from Nimata Pahar (Borail Wild Life Sanctuary) during the year 2005 (Bhattacharjee *et al.*, 2005). However, after repeated surveys during the present work (2010-2013), no specimen of this plant could be collected from the recorded wild habitat. Subsequently, we procured two

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Fig.1. *Paphiopedilum spicerianum* (Rchb.f.) Pfitz.: plant in bloom.

specimens of the said plant from the Botanical Survey of India (Eastern Regional Centre), Shillong. Presently, an attempt was made to propagate the species using seeds, leaves and shoot tips as explants, so that it may be disseminated to its wild habitat for conservation purpose.

Materials and methods

Experimental Site

In the present work, experiments were carried out in the Sreedhar Apex Biotech, Bagbahar, Cachar, Assam.

Sample Collection

Two specimens of the orchid species were collected from the Botanical Survey of India (Eastern Regional Centre), Shillong and they were cultivated in pots under the green house conditions of the Department of Ecology and Environmental Science, Assam University, Silchar, Assam.

Explant Source

Shoot tips, leaves and seeds from green capsules (pods) were used as explants in the experiments and these were collected from the plant specimens growing in the green house of the Department of Ecology and Environmental Science, Assam University, Silchar.

Sterilization

The explants were thoroughly washed under slow running tap water for 15 min., washed in tween 80 (1 drop in 200 ml sterile distilled water), and subsequently rinsed 3-4 times with sterile distilled water (SDW) inside the Bio safety cabinet. Subsequently different explants were treated with 70% alcohol for varied time duration (leaves and shoot tips for 30 sec and the green capsules for 1 min), and rinsed in SDW.

Culture Medium

Murashige and Skoog (1962, MS) medium [readymade dehydrated medium (HIMEDIA)] was used for the *in vitro* propagation of *Paphiopedilum spicerianum* (Rchb. f.) Pfitz. The medium was supplemented with different concentration of IAA (Indole-3-acetic acid; 0.1-1 mgl^{-1}), IBA (Indole-3-butyric acid; 0.1-1 mgl^{-1}), BAP (6-benzyl-amino-purine; 0.5-5 mgl^{-1}), 2.4-D (2,4-dichloro-phenoxy-acetic acid; 0.1-1 mgl^{-1}), Kinetin (KN; 0.5-5 mgl^{-1}), and TDZ (Thiadiazuron; 0.1-0.4 mgl^{-1}) individually or in different combinations.

The pH of the medium was adjusted between 5.6 and 5.8. Agar was dissolved by boiling the mixture and about 20 and 50 ml medium was dispensed into each culture tube and flask respectively. After preparation, the culture medium was autoclaved at 121^{0}C for 20 min at 15 lb/sq. inch pressure. Then the medium was allowed to cool and kept under Bio safety cabinet for 42 hrs. If no contamination was observed, then the replicated media were used for inoculation.

Inoculation and Culture

The sterilized explants were prepared for culturing in varied ways: i) The leaf explants were prepared by aseptically removing the entire mid rib of the leaves. The resulting strips of the leaf were cut into small pieces (10 mm^2), and these explants were placed on the sterile tissue paper to dry, followed by their inoculation on MS medium and its different combinations; ii) the sterilized capsules were cut open longitudinally with a sharp sterilized surgical blade and subsequently the powdery mass of yellowish seeds was inoculated on the surface of the culture media with the help of a long spatula.

All these operations were done aseptically in a Bio-safety cabinet. All the culture vessels were kept at 25 $\pm$2 ^{0}C under 16 hour light and 8 hours dark period by white fluorescent tubes with an intensity of 1000 Lx. After every 15 days interval, explants were transferred into fresh medium for better growth.

Growth Parameters

The growth parameters taken for observation were days required for callus formation and weight of the callus.

Results and Discussion

The response of different explants used in the present experiment for *in vitro* propagation of *Paphiopedilum spicerianum* (Reichb. f.) Pfitz. varied with the nutritional recipe used. The seeds are

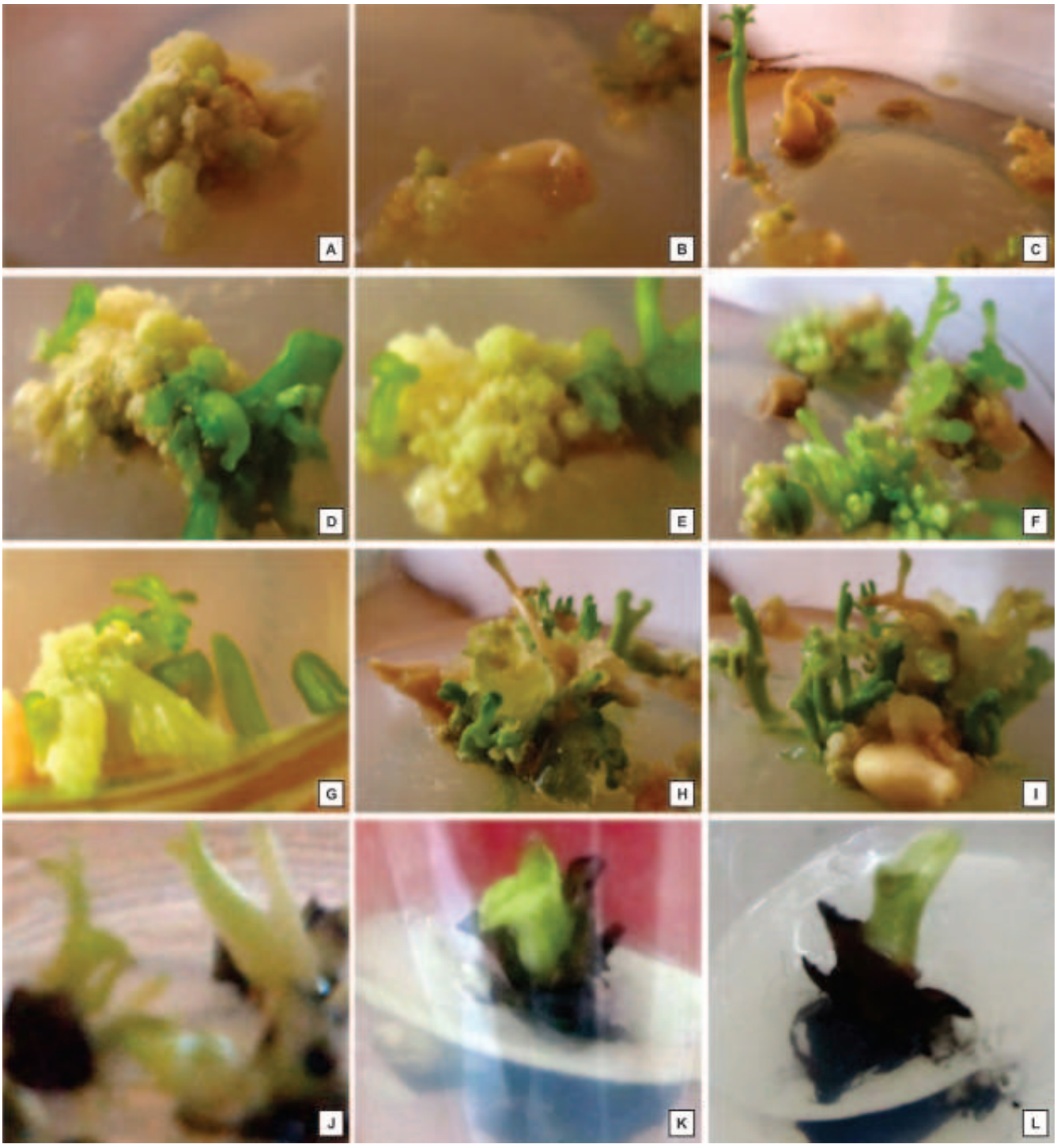

Fig.2.: *Paphiopedilum spicerianum* (Reichb. f.) Pfitz. *in vitro* propagation in MS Medium with Thiadiazuran (mgl^{-1}): A, B, C, Callus formation; D, E, F, Initiation of multiplication; G, H, I, Multiple shoot formation; J, Leaf initiation; K and L, development of leaf.

commonly used for *in vitro* propagation of *Paphiopedilum* orchids for the large scale production as the multiplication rate from the shoot tip derived explants is very low. Presently, the seeds obtained from undehisced capsules invariably failed to respond. According to Arditti and Ernst (1993), *Paphiopedilum* orchids have stringent requirements for seed germination. But little is known about their specific requirements.

Although young leaf and shoot tip explants had shown regeneration response via callusing, the formation and development of the callus was found to be extremely

slow and poor. It was reported earlier that the slow growth and low multiplication rate have been the important limiting factors of the *in vitro* culture of slipper orchids (Thongpukdee *et al.*, 2013). However, presently the shoot tip explants gave better results as compared to young leaf explants. Though regeneration occurred on the leaf explants in MS medium supplemented with the combination of BAP (0.5 mgl^{-1}) + IAA (0.1 mgl^{-1}) + IBA (0.1 mgl^{-1}); KN (3.5 mgl^{-1}) + IBA (0.7 mgl^{-1}); BAP (2.5 mgl^{-1}) + 2, 4-D (0.5 mgl^{-1}), the size and weight of the callus varied with the growth stimulus (Fig. 2 A-L).

In case of shoot tip explants, the regeneration occurred via callus formation in medium supplemented with only TDZ at different concentrations (*i.e.*, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35 mgl^{-1}). The initiation and development of the callus was favored with increase in the concentration of TDZ; the best results were obtained in medium with TDZ at 0.20 mgl^{-1}. The success of micropropagation of *Paphiopedilum* orchids through direct shoot regeneration depends on the optimization of culture media to a large extent (Udomdee, 2012), MS and MS modified medium is generally used in the *in vitro* propagation of *Paphiopedilum* orchid species (Chen *et al*. 2002; Chen *et al*, 2004; Hong *et al.,* 2008). Although some previous literature suggested that TDZ inhibits the shoot proliferation and rooting (Huang *et al.*, 2001), but Chen *et al.* (2002), reported that 0.45 μM TDZ and 4.52 μM 2,4-D supplemented in MS modified half strength medium enhanced the percentage of explants produced in the newly regenerated shoots from the stem nodal explants. Stewart and Button (1975) used shoot apex of *Paphiopedilum* to induce callus, while Chen *et al.* (2002, 2004) also reported that induction of multiple shoots could be achieved from the stem and leaf explants of *P. philippinense* (hybrids PH59 and PH60) cultured on MS medium supplemented with 4.52 µM 2,4-dichlorophenoxy acetic acid (2,4-D) and 4.54 ìM Thiadiazuran (TDZ). Lin *et al.* (2000) reported that the TDZ induced calli from the seed derived protocorm of *Paphiopedilum* hybrid orchid. TDZ seems to play a crucial role in the dedifferentiation of the orchid explants. Chang and Chang (1998) showed that presence of TDZ in the MS basal medium was essential to obtain the long term totipotent callus culture.

The time duration for the callus formation was found to vary with the explants and concentration of the plant growth regulator(s) used in the medium. The callus formation was observed within 34 days of inoculation from the shoot tip, but it took 48 days from the young leaf explants. The multiple micro shoots were transferred to MS medium with the addition of TDZ (0.2 mgl^{-1}), coconut water (40 mll^{-1}) and different plant growth regulator(s) (*i.e.*, IBA, IAA, BAP and KN) at different concentrations. The cultures were kept in culture room at 18 ^{0}C. The leaf formation was observed only in IAA (0.2 mgl^{-1}) supplemented medium. Leaf-like structure were also observed in medium containing BAP (0.3 mgl^{-1}), the micro shoots, however, could not survive and died after 22 days of their transfer in the culture medium.

Though *Paphiopedilum* orchids are propagated through the division of axillary buds from the mother plants. It is time consuming, extremely unproductive and unreliable for commercialization or conservation purposes (Liao *et al.*, 2011; Ng and Saleh, 2011). Nhut *et al*. (2007) reported that TDZ was the most effective than BA for the shoot induction in *Paphiopedilum delentii*. Shoot proliferation from leaf tissue is very common in ferns and dicotyledons, but it is very less in monocotyledons (Xiong and Wu, 2003). An appropriate medium, quality and quantity of plant growth regulators are important factors during seed culture and regeneration of leaf and stem nodal explants (Pathak *et al.,* 2001, 2012; Vij and Pathak, 1990; Vij *et al.,* 1994), in orchids and *Paphiopedilum* Delrosi, in particular (Thongpukdee *et al.*, 2013).

Conclusion

Presently, shoot tip and leaf explants were successfully used for regeneration in *Paphiopedilum spicerianum* (Rchb.f.) Pfitz. and the present study strongly supports that this *in vitro* propagation technique may help in mass propagation and conservation of this endangered orchid species.

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