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Research Article

CONSERVATION OF *PHOLIDOTA PALLIDA* LINDL MEDICINAL ORCHID – IN VITRO

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ABSTRACT

An efficient and reproducible protocol developed for micropropagation of *Pholidota pallida Lindl*. an endangered terrestrial orchid; using 4mm section of axillary bud segment has been developed. Axillary bud section were cultured on Murashige and Skoog (MS) medium supplemented with different concentration of harmones (BAP), 5,1.0,2.0,mg/L, [α-naphthalene acetic acid (NAA), indole-3-acetic acid (IAA), 0.5, 1.0, 2.0 and 5.0 µM] and Casein hydrolysetes (CH: 5, 10 and 15%).,2,4-D,.2,.5,1.0, 2.0.The explants developed protocorm like bodies (PLBs) within 6–8 weeks on the growth medium. MS+2 mg/l 2,4-D supplemented medium was found best for the induction of PLBs. Upon subculture on basal MS medium, the PLBs differentiated plantlets within 6–8 weeks with in vitro tuber and roots. This simple protocol will be useful for large-scale propagation of *Pholidota pallida Lindl*

Key words

Endangered, Orchid, PLB, *Pholidota pallida Lindl*

INTRODUCTION

Among the flowering plants, orchids represent one of the largest groups, with highly colorful, attractive flowers exhibiting a long shelf-life with varied shapes and sizes. They are of great value to the floriculture industry as cut flowers and potted plants. Orchid seeds are microscopic and nonendospermous with undifferentiated embryos. They are produced in large numbers and their germination in nature depends upon a suitable association with mycorrhizal fungus, thus making it difficult to observe their further development after they are released from the seed capsule soil (Temjensangba and Deb, 2006). Currently, orchids are the second most popular potted floriculture

crop and the production and sales of native orchids are on rise. The small market for some native terrestrial orchids is due to the difficulties in propagation methods and the long time period required to obtain flowering (USDA, 2004).

The orchid *Pholidota pallida Lindl* (D.Don) So is indigenous to the Himalayas and is exclusively found in the Ladakh region of Jammu and Kashmir, India. It has been identified as a critically endangered species (CAMP status) and is listed under Appendix II of Convention of International Trade in Endangered Species (Uniyal et al., 2002). *Pholidota pallida Lindl* is also a high-value medicinal orchid used in the Indian system of medicine, particularly in Ayurveda, Siddha, and Unani medicine (Pant and Rinchen, 2012). It is widely used to cure various diseases including dysentery, diarrhea, chronic fever, cough, stomachache, wounds, cuts, burns, fractures, and general weakness. The tubers of *Pholidota pallida Lindl* contain glucoside, a bitter substance, and starch, mucilage, albumen, a trace of volatile oil, and ash (Dutta and Karn, 2007). Chemically, dactylorhins A to E, dactyloses A and B, lipids, and so on are found as major constituents. The tubers of this species yield a high quality salep, which is used as a tonic to increase immunity (immunomodulator) and as a vajikaran drug (aphrodisiac); it increases sexual performance as well as serving as a nervine tonic (Baral and Kurmi, 2006). The annual consumption of *salep* obtained from the species in India is about 7.38 t (valued at about 5,000,000 rupees or US$83,333) and has great demand in national and international markets (Badola and Pal, 2002).

The orchid species are rapidly declining because of their low rate of propagation, poor seed germination, and ongoing collection from nature. Careless collection of these species has led to serious genetic and ecological erosion; many have already been listed as endangered species (Machaka-Houri et al., 2012). The rate of vegetative propagation in *Pholidota pallida Lindl* is very slow and seed germination in nature is very poor, i.e. 0.2%–0.3% (Vij, 2002). In vitro germination of seeds can be an important mode for orchid multiplication and conservation programs, since the dust seeds are tiny and contain few food reserves. Both in situ and ex situ approaches are important for the protection of rare and endangered orchid species. Tissue culture is one of the most important measures in ex situ conservation of terrestrial orchids (Jakobsone et al., 2007). Knowledge of the physiological and morphological aspects of the germination and development of particular orchid species is of critical importance for the establishment of tissue culture. The current study reports for the first time on the optimization of increased in vitro immature seed germination, seedling development, and plantlet formation, as well as multiplication of *Pholidota pallida Lindl* plants

grown under in vitro and in vivo conditions, for rescuing their declining population by reintroducing them to their native habitats

MATERIALS AND METHODS

SEED SOURCE

Seeds of *Pholidota pallida Lindl* were obtained from immature capsules collected from naturally pollinated plants growing in a farm at Tirith village (altitude 3183 ± 8.2 m, 34°32′09.977″N, 77°38′52.386″E), in the Nubra valley of the Ladakh region of India. The seeds were air dried for 2 weeks at room temperature and stored in small bottles at 4 °C until use.

SEED VIABILITY TEST

Immature seeds were soaked in filtered triphenyl tetrazolium chloride (TTC) solution (1 g in 100 mL of phosphate buffer, pH 6.5–7.0) for 48 h in darkness at 20 ± 2 °C and rinsed 5 times in sterile distilled water. Seeds were agitated between cover slides to remove testa and viewed using a Nikon microscope. The embryos that stained pink to red were considered viable, while seeds with partially colored, white, or brown embryos were assumed to be nonviable (Van Waes and Deberg, 1986).

FDA staining

The immature seeds were soaked in *fluorescein diacetate* (FDA) solution composed of equal volumes of distilled water and FDA stain (0.5 g in 100 mL of absolute acetone) for 15 min and viewed with an Olympus BH-2 (UV light) fluorescence microscope. Seeds with embryos completely stained (fluorescent) were considered viable (Rasmussen, 1995). The same test was repeated for viability of seeds collected from stored seed samples at 4 °C at 1-week intervals for 1 month.

NUTRIENT MEDIA

Ten different basal media were tested for immature seed germination. Five of the media were commercial preparations with modifications from HiMedia Laboratories Pvt. Limited, Mumbai, India: terrestrial orchid medium (BM-1); Vacin and Went modified (VW) medium (Vacin and Went, 1949); Malmgreen modified terrestrial orchid (MM) medium (Malmgren, 1996); Mitra (PT139) orchid medium (Mitra et al., 1976), and Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). Three of the media were modified by Titan Biotech Limited, Rajasthan, India: Knudson C (KC) orchid medium (Knudson, 1946); Heller (TP039) medium (Heller, 1953), and Lindemann (LD) orchid medium (Lindemann et al., 1970). Two of the media were procured from Phyto Technology

Laboratories, LCC (Shawnee Mission, KS, USA): Fast (F522) terrestrial orchid medium (Fast, 1976) and orchid seed sowing medium (P723). The pH of the media was adjusted to 5.7 with 0.1 N HCl and 0.1 N NaOH, after the addition of sucrose and agar; 15 mL of medium was dispensed in each test tube (25 × 150 mm), and tubes were plugged and autoclaved for 20 min at 121 °C and 1.05 kg cm–2 pressure

SEED SURFACE STERILIZATION AND INOCULATION

Immature seeds were surface-sterilized for 2 min in 5 mL of ethanol (100%) and for 15–20 min in 5 mL of 0.1% NaOCl, followed by rinsing in 90 mL of sterile distilled deionized water for 15–20 min. Solutions were removed from the surface sterilization vials using disposable 1000-µL sterile pipette tips that were replaced after each use. Seeds were then suspended in sterile deionized distilled water and a sterile inoculating loop was used for inoculating culture tubes. The inoculating loop was immersed once into the seed suspension and seeds were placed in the culture tubes. One replicate consisted of 1 test tube containing 100–150 seeds.

CULTURE CONDITIONS

Cultures were incubated in a growth chamber maintained at 25 ± 1 °C under a 16/8-h photoperiod with illumination of 3000 lx intensity of white light.

IN VITRO SEED GERMINATION AND DEVELOPMENT OF PROTOCORMS

The seeds were germinated and differentiated into protocorm-like bodies (PLBs) on the same germination medium. The cultures were monitored regularly and the data were scored at weekly intervals. After 2 and 3 weeks of germination, the protocorm development was assessed using a dissection stereoscope. Germination and protocorm development were scored on a scale of 0–5 (Zettler and McInnis, 1994): stage 0, no germination; stage 1, rupture of test a due to swelling of embryo (i.e. germination); stage 2, protocorm formation and emergence of rhizoids; stage 3, emergence of leaf primordium (shoot); stage 4, appearance of first leaf; stage 5, elongation of leaf and root differentiation. The percentage germination was calculated by dividing the number of seeds in each individual germination and development stage by the total number of viable seeds in the sample. The remaining seeds were dried in petri dishes with anhydrous CaCl2 for 2 weeks at 5 °C. Dried seeds were transferred into *Eppendorf microtubes* and stored in hermetically closed containers at –20 °C. All experiments were repeated 3 times. The experimental design was completely randomized.

PLANTLET REGENERATION AND MASS MULTIPLICATION

The PLBs developed from cultured immature embryos were maintained on optimum basal germination medium for further differentiation to form leaf primordia. The PLBs with leaf primordia were separated from the germination medium and cultured on regeneration media. For regeneration of plantlets and their multiplication, BM-2 and MS media supplemented with combinations of growth regulators [0–3 mg/L indole butyric acid (IBA) and 0–3 mg/L kinetin (Kin)] were tested. The resulting microshoots were separated from the regeneration medium for further mass multiplication. During plantlet regeneration, data were recorded for shoot number, shoot length, root number, root length, and number of days required for shoot/root formation. The well-developed plantlets were maintained on the same regeneration medium before transferring for hardening.

ACCLIMATIZATION AND IN VIVO MULTIPLICATION OF PLANTLETS

Fully grown plantlets were taken from the regeneration medium and traces of agar were removed with a soft brush. Plantlets of 5–10 cm in shoot length, with 5–7 roots, were successfully transplanted to controlled greenhouse conditions into 8 combinations of potting mixtures: C-1 (sand + soil, 1:1), C-2 (sand + soil + farmyard manure, 1:1:1), C-3 (sand + soil + cocopeat, 1:1:1), C-4 (sand + soil + vermiculite, 1:1:1), C-5 (sand + soil + perlite, 1:1:1), C-6 (cocopeat + vermiculite, 1:1), C-7 (vermiculite + perlite, 1:1), and C-8 (cocopeat + vermiculite + perlite, 1:1:1). Initially, for 10–15 days the plantlets were covered with glass jars to provide sufficient humidity and avoid desiccation until the plantlets showed new growth. During the hardening process, glass jars were taken off every day for 1–2 h so as to acclimatize the plantlets to the external environment, and data were recorded for percent survival, number of shoots and roots, and length of shoots and roots.

DATA ANALYSIS

Univariate statistical procedures were used to analyze the data. Descriptive analysis of the data was performed using SPSS 19. Analysis of variance with comparative Duncan's multiple range tests at 5% was used to determine the significance of differences between treatments

RESULTS

SEED VIABILITY

The interpretation of seed viability using TTC was very difficult. Seeds of *Pholidota pallida Lindl* are shown in Figure 1A. Viewing the immature embryos was only possible after removing the testa, a tedious and delicate process, since terrestrial orchid seeds are very minute (200 to 1700 µm). Staining colors that distinguished viability were very difficult to determine and the method was

abandoned. By contrast, FDA was an excellent stain for observing viable seeds. Fluorescence was easily recognized and stained seeds were easily counted without removing the testa, which was transparent. With the FDA method the highest percentage (24%) of viable embryos was observed in the first week of sampling, followed by a significant reduction (13%) in the fourth week of sampling, whereas the TTC method showed high viability (22%) in the first week and 11% in the fourth week of sampling.

ASYMBIOTIC SEED GERMINATION

Seeds began swelling within 2 weeks after inoculation and germinated to form protocorms within the third week of inoculation, as is shown in stage 1 and 2A–2C. Of the 10 media tested, seed germination was highest (37.12%) in LD medium, followed by 35.48%, 34.48%, and 31.68% in TP039, BM-1, and 0.5 MS media, respectively. Maximum protocorm formation (23.40%) occurred in LD medium, followed by 21.40%, 20.4%, and 18.6% on TP039, BM-1, and 0.5 MS media, respectively. Seed germination was minimal in MM, KC, VW, F522, and orchid seed media, even after 4 weeks of inoculation. Protocorm formation occurred on the rest of the media after 7 weeks of incubation. The germinating seeds converting into PLBs were maintained on the same germination medium for further differentiation into leaf primordia. Formation of first leaf from PLBs was developed in all media except PT139, as is shown in stage 3 and Figures 2D and 2E.

REGENERATION AND MASS MULTIPLICATION

The protocorms with first leaf primordia were cultured on BM-2 and 7 different MS media supplemented with different concentrations and combinations of IBA and Kin for shoot formation. The protocorm with first leaf primordia developed into multiple shoots within 15 to 20 days of incubation in shoot regeneration media and further developed into plantlets with shoots and roots within 28 to 42 days of incubation. Growth and development of plantlets with maximum number of shoots/protocorm (18.12 ± 0.3), shoot height (17.80 cm ± 2.16), number of roots (8.25 ± 0.69), and maximum root length (8.02 cm ± 1.45) occurred on MS medium supplemented with IBA (3 mg/L) + Kin (1 mg/L) within 28 to 30 days of incubation. MS medium containing Kin at 3 mg/L showed relatively fewer numbers of shoots (16.00 ± 1.26) and other respective parameters of growth and development until 34 to 40 days of culturing (Figures 3A–3C).

ACCLIMATIZATION AND IN VIVO MULTIPLICATION OF PLANTLETS

The plantlets grown in vitro with 2–3 shoots were transferred to different potting mixtures for their acclimatization to field conditions and for further growth and multiplication. The highest

plantlet survival (100%) was in the C-8 potting mixture consisting of cocopeat + vermiculite + perlite (1:1:1), with a significantly higher number of shoots (75), maximum number of plantlets (25), highest shoot length (18.8 cm), maximum number of roots (23), and highest root length (44.7 cm) after 1 month of transplantation in the greenhouse. Satisfactory percentage survival of plantlets was observed on the potting mixtures, whereas plantlets' multiplication parameters were lowest in C-1 potting mixture with a survival percentage of 52.5%, 29 shoots, 9.66 plantlets, 13.2 cm shoot length, 15 roots, and 33.2 cm root length.

DISCUSSION

Habitat destruction, fragmentation, overexploitation, and the effects of global climate change are the main threats to the survival of orchids (Bubb et al., 2004). *Pholidota pallida Lindl* is indigenous to the temperate regions of Sikkim, Uttaranchal, and parts of Jammu and Kashmir. Very little information is published on the growth and seedling development of *Pholidota pallida Lindl* in its natural habitat and as of today no information is available on in vitro seed germination and mass multiplication of plantlets. Ramsay and Stewart (1998) suggested that the period of time when immature seeds germinate efficiently in axenic conditions is short, approximately 7–10 days, and should be experimentally estimated for each species. Previous studies revealed that no single nutrient medium is universally suitable for asymbiotic seed germination for most of the orchid taxa; PT139 medium was found suitable for Cymbidium macrorhizon Lindl. (Vij et al., 1998) and Goodyera biflora Lindl. (Pathak et al., 1992), KC medium for Cymbidium elegans (Lindl.) and Cioelogyne punctulata Lindl. (Sharma and Tandon, 1990), VW medium for Vanda coerulea Lindl. (Devi et al., 1998), Nitsch medium for Cymbidium iridioides D.Don (Jamir et al., 2002), and KC, VW, and MS media for Aerides rosea ex Lindl. (Sinha et al., 1998). In vitro germination of terrestrial orchid species seeds is often a long and slow process due to considerably longer required periods for germination and specific mycorrhiza as reported by De Pauw and Remphery (1993), and as in *Dactylorhiza majalis* (Rchb.) seeds that started to germinate after 6 weeks of culture on F522 medium (Znaniecka and Łojkowska, 2004). *Dactylorhiza ruthei* (M.Schulze) and *Dactylorhiza prastermissa* (Druce) seeds started to germinate after 4 months of culture on Norstog medium (Vaasa and Rosenberg, 2004). Germination of Dactylorhiza ruthei (M.Schulze) and *Dactylorhiza praetermissa* (Druce) seeds depended on the concentration of MnSO4 in the media (Vaasa and Rosenberg, 2004). Seeds of *Dactylorhiza maculata* (Lindl.) started to germinate after 3.5 months of incubation in soil (Kinderen, 1995). Vassa and Rosenberg (2004) found that a high percentage of seeds of Dactylorhiza baltica (Klinge), *Dactylorhiza praetermissa* (Druce), and *Dactylorhiza ruthei*

(M.Schulze) germinated within 3 months of culture, and seed germination decreased afterwards. Bektaş et al. (2013) found the highest germination rate (44.2%) of *Orchis coriophora* Lindl. on Orchimax medium. Aggarwal and Zettler (2010) found 100% germination in *Pholidota pallida Lindl* within 10 days after sowing and plants developed seedlings after 3 months. Giri and Tamta (2012) used 4 media for the germination of Pholidota pallida Lindl and found a better response on MS medium supplemented with peptone (1.0 g/L), morpholino ethanesulfonic acid (1.0 g/L), and activated charcoal (0.1%). However, in our study, seeds of *Pholidota pallida Lindl* germinated within 1 week on LD and TP039 media, which has MnSO4 as an important component. The percentage of in vitro protocorm formation varied on different media, along with differences in duration, such as MS and KC media. Protocorm formation was 30%–40% in 49 days of culture in *Cleisostoma racemiferum* Lindl. (Temjensangba and Deb, 2006), while in Malaxis khasiana (Hook.f.) Kuntze, 20%–30% of protocorm formation occurred on MS medium with 500 mg/L casein hydrolysate + 1 µM BA in 107 days (Deb and Temjensangba, 2006). Similarly, in Ophrys species, 23% of protocorms were formed after 107 days of culture on Malmgreen medium (Kitsaki et al., 2004). We observed 22%–23% of protocorm formation within 17 days of culture on LD medium. There are reports of multiple shoot formation under in vitro conditions, such as in epiphytic orchid *Cleisostoma racemiferum* Lindl. within 30 days on MS medium supplemented with 10.0 µm naphthalene acetic acid (NAA) + 8.0 µm 6-benzylaminopurine (BAP) (Temjensangba and Deb, 2006), in Cymbidium eburneum Lindl. (MS + 15 µm NAA + 15 µm BAP) in 28 days (Gogoi et al., 2012), and in Coelogyne nervosa Lindl. (MS + 3 mg/L BAP + 1 mg/L NAA) in 90 days (Abraham et al., 2012). Multiple shoots were formed in MS + 6 µm IAA + 18 µm BAP + 18 µm Kin in 49–56 days in *Malaxis khasiana* (Hook.f.) Kuntze (Deb and Temjensangba, 2006). Significant differences in multiple shoot formation on MS medium supplemented with 3 mg/L IBA and 1 mg/L Kin in a shorter duration of 28 to 30 days of incubation occurred in our study. Differences in the survival of plantlets in potting mixtures have been reported, such as 65% survival of transplants of *Malaxis khasiana* (Hook.f.) Kuntze in charcoal pieces + coconut husk + sterilized forest litter (1:1:1) (Deb and Temjensangba, 2006), and 90% survival of transplants of *Pholidota pallida* Lindl. in charcoal chips + coconut husk + and broken tiles (2:2:1) (Mulgund et al., 2012). We observed 100% survival in the C-8 potting mixture; other potting mixtures (C-3 and C-6) also gave good shoot and root growth because of the presence of cocopeat and vermiculite, which provided enough moisture, aeration, and micronutrients for the profuse growth of plants. This observation is supported by

Hartmann et al. (2007), who found that vermiculite, when combined with perlite and cocopeat, promotes faster shoot/root growth and gives quick anchorage to young plants.

CONCLUSION

In the present study, a successful attempt has been made to culture immature seed embryos of *Pholidota pallida Lindl* for developing protocorms, shoot regeneration, and mass multiplication. The growth and multiplication of plants further continued after their transplantation to the greenhouse in potting mixtures, thereby resulting in the development of a micropropagation technology to meet the growing demand for *Pholidota pallida Lindl*. This technology will help not only in multiplying the plantlets but can also play a major role in its conservation and metabolite production through tissue/cell culture systems.

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