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In vitro seed germination and histology of protocorm development in *Spathoglottis plicata* blume

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Abstract
Most terrestrial orchid seeds are difficult to germinate *in vitro* and *ex vitro* condition due to its specific nutrient and environmental requirements. Present study aimed to investigate the germination of seeds, development and histology of protocorm. Tissue culture studies were carried out in MS medium with BA (0.1-2.00 mg/l), Kin (0.1-2.00 mg/l), TDZ (0.1-2.00 mg/l) or in basal MS medium. During sowing seeds in a culture medium, seed germination occurred after the imbibition, which resulted in swelling of the embryos, and then the seed coats were ruptured by the emerging of germinated embryos, so the embryo enlargement and the papillae appeared on the one end of protocorms after the second subculture. MS medium supplemented with BA (0.5 mg/l) resulted in 98% of seed germination within the third week of inoculation. Histological study revealed the presence of vascular tissue in the central region at early development and later developmental stages showed well-developed shoot and root pole. In the present study, we reported a simple, quick, structured, and dependable *in vitro* revival system for mass propagation of *S. plicata* through the culture of seeds. The protocol established in this species will facilitate *in vitro* propagation for commercial purposes, sustentation, and metamorphic studies.

Keywords: protocorm, histology, mature seeds

Introduction
The ground orchid *Spathoglottis plicata* is one of the most frequently cultivated orchids in Southeast Asia and could be found in gardens throughout the region. It is suitable as a potted orchid because it is very pleasant, rapidly - growing with plentiful flowers all through the year. Each peduncle usually carries 10 - 40 flowers and blooms for several months and flowers were open for 2 weeks. Most members of this genus are terrestrial and most grow at low to moderate altitudes, although a few taxa occur at extreme altitudes in grasslands and arid forests in humid areas [1]. Primary center of origin of this species was tropical Asia and was distributed throughout China, Malay Archipelago, New Guinea, Thailand, Borneo, Philippines, Australia and the islands of the Southwest Pacific Ocean [2].
Spathoglottis plicata is commonly used as a decorative variety because of its nice leaves and attractive flowers. It has the ability to adapt to the unfavorable environment and thus making it easy to grow in all regions. Prevalent proliferation is mainly through the uncoupling of the pseudobulb. For the cultivation and generation of seedlings of endangered and subtle orchids, many scientists have suggested *in vitro* seed sprouting as an acceptable multiplication strategy [3]. However, most terrestrial orchid seeds need specific nutrient and environmental requirements otherwise it is dormant in both *in vitro* and *ex vitro* conditions. In nature, orchids pre occupy mycorrhizal fungi as a source of energy to evoke seed germination process [4]. The embryo within the seed expands and appeared as the protocorm, which represented a non - chlorophyllous and heterotrophic phase of the orchid life cycle [5]. Occurrence of mycorrhizal colonization and fungi consumption have an outstanding position in orchid nourishment. For embryological studies, orchid embryos were selected because of the heterogeneity in the developmental pathway and organization of the megagametophyte, different patterns of polyembryony. The present study also describes seed germination, maturation, and histology of protocorm development.

Materials and methods
Mature capsules were selected and dipped in 1% (v/v) Labolene® (Qualigens, India) for 10 min and kept under running tap water for 60 minutes. The capsules were surface disinfected in 0.1 % (w/v) mercuric chloride for 5 min followed by three rinses in autoclaved double-distilled water, 4-5 min for each rinse. Under aseptic conditions, by using a clean knife or scalpel, the capsule was dehisced and the seeds were scraped out and transferred into the culture medium. MS^6 liquid medium supplemented with BA (0.1-2.00mg/l) KIN (0.1-2.00 mg/l) TDZ (0.1-2.00 mg/l) or in basal MS medium with $30gl^{-1}$ sucrose, and pH of the media was adjusted to 5.8 before autoclaving at 120°C for 18 min. The cultures were maintained at a temperature of 25±2°C with a photoperiod of 16h/day under 50µmol m^{-2} s^{-1} light intensity provided by fluorescent lamps. The cultures were perpetuated at 80 rpm were maintained at 25±2°C temperature with a photoperiod of 16h/day under 50µM $m^{-2}s^{-1}$

light intensity supplied by fluorescent lamps and subculturing was done at the end of every 3rd week. Full-grown protocorms were isolated by filtering through a stainless-steel filter (950 µM) and put down in a solid MS basal medium for development. Germinated protocorms were transferred to the MS basal medium or MS medium with BA or Kin for rooting and entrenched saplings were successfully transferred to greenhouse conditions. Protocorms were carefully chosen for histological studies and Cryotome (Leica CM, 1100) sections were taken and stained with 1% safranin. The photographs were taken with the help of an image analyzer (Olympus BX51).

Results and Discussion

Four weeks old mature pods were isolated from the mother plant and disinfected, non – endospermic seeds were inoculated on basal liquid MS medium. It was noticed that these non- endospermic seeds imbibed and showed the sign of swellings after first week of inoculation (Fig.1). At the end of the third week, a green-colored oval or round-shaped embryo was observed in the seed (Fig 2). During the culturing of seeds in a medium, germination happened after permeation, which resulted in swelling of the embryos. The seed coats were burst and germinated embryos emerged, papillae visible on the one end of protocorms (Fig.3). Seeds were found to be germinated and subsequently produce green or pale yellow protocorm after six weeks of inoculation in the same medium after the first subculture (Fig.4). Approximately 90% of germination was noticed in the basal MS medium after six weeks of inoculation. Germinated protocorms were light green colour and tuber shaped with an approximate size of 1-3mm in length. The seeds began abscess after three weeks of inoculation and were inseminated within five weeks in MS medium supplemented with BA (0.1 mg/l). Seeds inoculated on MS medium augmented with BA (0.5, 1.00 & 2.00 mg/l) started imbibition and germination after the third week of inoculation and it was also observed that most of the protocorm remains in the globular stage after the first subculture. MS medium enriched with BA (0.5 mg/l) resulted in seed germination within the third week of inoculation and 98% of germination was noticed. MS medium with Kin (0.1 mg/l) resulted in germination and was started after the 4th week of inoculation. Germinated protocorm observed as green-colored tubular shaped with (1.00-2.00mm) length, formed after seven weeks of inoculation. MS medium augmented with TDZ (1.00mg/l) and (2.00 mg/l) proceed in the advancement of expanded protocorms after the first subculture. Germinated protocorms were visible after the 4th week and 80% of germination was observed. On sprouting, orchid seeds bring about a transient tubercle structure called a protocorm from which shoot and root eventually set apart. Anatomical study of protocorm also revealed the presence of both shoot and root pole in the same axis (Fig.5). Germinated seeds after the third subculture induced the development of shoot and root pole and successive developmental patterns were clearly visible (Fig.6). The stereomicroscopic image revealed the presence of a well-developed shoot along with numerous shoot buds in some protocorms. Fully developed shoots and nascent leaves emerged after the fourth subculture (Fig.7).

Histological studies revealed that at the early developmental stage, pro - vascular tissue occupied the central region of the protocorm, which was observed as round in shape, cells at the apical region continuously divided while the basal part enlarges and was round in shape. Clear vascular connections visible in the central region with well-differentiated xylem and phloem elements were seen intertwist through the cortical region of the protocorm, reaching the layers of the shoot apical meristem (Fig.8). Embryonic cell undergoes continuous cell division, expanded and enlarged into a new pale yellow protocorm. Fungal associations were also noticed in the surface region of the globular protocorm. After the fourth subculture maturation of protocorm noticed and shoot apical meristem was clearly visible during this stage. After the 12th week protocorm displayed fully-developed primordial and young leaves (Fig.9).

In the present study rate of germination of seeds was maximum in MS medium fortified with BA (1.00mg/l) and a slight decrease in the germination frequency was observed in BA (2.00 mg/l). Phytohormones, specifically cytokinins were found crucial for plant growth and development, and for synchronizing mitotic activities in the cell [7]. Shoot up in cytokinin activity coexist with fertilization and initiation of embryo development was also reported earlier[8]. During embryo development the process of cell division in the pericarp also completed and a hike in the cytokinin activity is most remarkably related to the growth and development of embryo. The concentration and nature of plant growth regulators play a prime role during the micropropagation of many orchids. The beneficial impact of BAP for ultimate protocorm multiplication and productiveness of BAP in combination with NAA was reported earlier [9]. This statement is in agreement with some other findings acquired in the micropropagation of orchids [10].

The formation of embryos in terms of occurrence and mass production of vigorous, feasible protocorms in a BAP-containing medium was also reported earlier in orchids. The embryos generally, sprout regularly in the liquid medium and they maintain distended testa which readily consume nutrients from the culture medium, metabolically attentive embryos free from any inertness and impediment factors [11]. The impulse of BA to impair the sprouting reflex and induction and proliferation of protocorms has been reported in a large number of orchids including *Cattleya aurantiaca* [12], *Cyprepedium* spp. [13], *Aerides multiflorum, Dendrobium chrysotoxum, Cymbidium pendulum* [14], *Cymbidium aloifolium*, and *Dendrobium aphyllum* [15].

MS medium augmented with Kinetin (0.1- 2.00 mg/l) initiated seed germination after the third week. Fully developed protocorms were clearly visible after the 4th subculture. Further subculture in the aforesaid hormonal application arouses the development of clumps of protuberance from the emerged protocorm. Seeds cultured on MS basal medium initiated seed germination within one week and fully germinated protocorms were observed in the 3rd week of inoculation. During the time of germination, protocorm was observed as pale yellow and was

rapidly turned into green color. Studies have reported that species-specific media was a prerequisite for seed germination in orchids and the selectivity was reported even within species of the same genus. It was also reported that the rate of seed sprouting was incredibly determined by the nature of growth regulating agents in the medium. MS medium augmented with TDZ (0.1- 1.00 mg/l) enhanced the development of numerous small protuberances on the surface of protocorm after the third subculture.

On germination, orchid seeds give rise to an ephemeral tubercle structure called a protocorm from which a shoot and a root subsequently differentiate [16]. Developmental programs for protocorm development and orientation of shoot and root meristem are established during embryo maturation. Though the protocorm is a post-embryonic structure, it has been considered a perpetuation of embryogeny, an indispensable part of embryogenesis. The process of development of protocorm leading to plantlet formation is a regular advancement process and culminates once a shoot apical meristem is established and then the plantlet stage begins. Succeeding the formation of shoot apical meristem, the developmental pathway may alter and sometimes protocorm can be perverted 'corm-like' structure with different forms [17]. The appearance of roots also differs correspondingly between species. Furthermore, the complete plantlet can abide subterranean for quite some time or elongate immediately, appear from the substratum, and become green [18]. The proposed stages of germination and protocorm development were also previously described [19]. According to [20] five shoot types were reported the ramification of shoots formed by the apical buds of protocorms. Protocorms abundantly generated in later subcultures forming a shoot meristem which finally evolved into an entire plantlet with nascent leaves. Comparable outcome were also previously noticed in *Micropera pallida* [21] *Lycaste aromatica* [22] and in other orchids.

After the distortion of the integument, germination of the embryo resulted in the development of a tapering, light-green colored protocorm. Longitudinal sections of these structures exhibited a layer of parenchyma cells covered by a single-layered epidermis, making it feasible to initiate differences in cell size, volume, and shape. In the region of chalaza, the parenchymatic cells were smaller in size when compared to other parts. In the micropylar region, vacuolated parenchymatic cells and rhizoids were present. A remarkable mitotic activity was also observed in the central region of the protocorm which was found to be associated with pro vascular tissue development. Morphological and anatomical features of protocorms in the present study coincides comparatively with the general account of other orchid protocorms.

Fig 1, 2, 3, 4

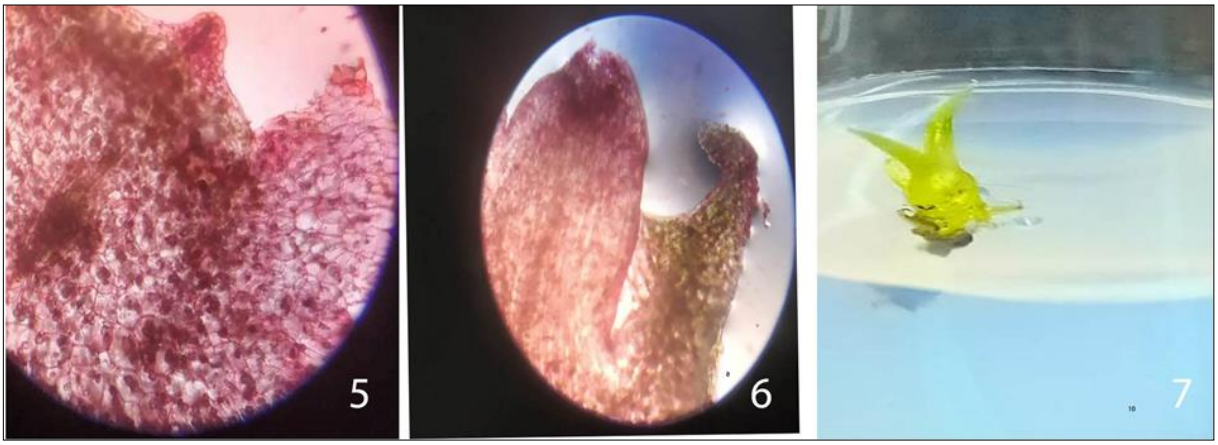

Fig 5, 6, 7

Conclusion

Since the seeds of orchids are non- endospermic it requires specific nutritional and environmental conditions for their germination and development. In nature, generally orchids consume mycorrhizal fungi as an alternate energy source to generate the process of seed germination and subsequent development. In the present study germinating protocorms showed profuse proliferation and maturation. The protocol developed in this work is acceptable for large-scale propagation, conservation, and utilization for developmental and transformation studies. From a developmental and biological point of view, orchid embryos and protocorm serve as a magnificent and appealing exploratory system in order to gain a comprehensive picture of the entire developmental process of embryogeny in monocot plants.

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