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RESEARCH ARTICLE

SEED GERMINATION STUDIES ON CALANTHE MASUCA(D.DON) LINDL. USING MYCORRHIZAL FUNGI

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ABSTRACT

Symbiotic ex-situ germination of terrestrial orchid, *Calanthe masuca* D.Don Lindl. was investigated using seven isolates recovered from the roots of five terrestrial orchids of Western Ghats, Karnataka, India (*C.masuca, Malaxis versicolor, Pecteilis susanne, Satyrium nepalense*, *Nervilia crociformis*). Molecular and phylogenetic analysis of the fungal isolates was done based on the alignment of internal transcribed spacer regions of nuclear ribosomal DNA. *Calanthe* seeds collected from the natural habitats were used for germination studies along with isolated fungal cultures under laboratory conditions. Seed germination was found with *Tulasnella*, an isolate from *Malaxis* rather from *Gliocladium*, an isolate from *Calanthe*.

INTRODUCTION

Orchidaceae is one of the largest families of flowering plants worldwide with over 30,000 species in 750 genera and they grow as epiphytes, terrestrials, even as saprophytes. Richness of orchid species occur in the tropical zone and the diversity decreases with increasing distance from the Equator (Brundett *et al.,* 2001). Orchids are represented by 125 genera and 1500 species in India of which 70 genera and 250 species are recorded from South India (Abraham and Vatsala, 1981). Orchids are commercially exploited worldwide for their beautiful flowers. Though the orchid flora have been explored taxonomically, only a few orchids have been investigated for mycorrrhizal colonization. Orchid seeds are very minute, dust like, lack endosperm, which cannot be propagated like any other angiosperm seed. In nature, orchid seeds need to be infected by a suitable symbiotic mycorrhizal fungi for normal growth and development. There is an acceleration in growth and development of protocorm after symbiotic germination when compared to asymbiotic germination but unfortunately only a few orchid seeds out of the millions produced germinate symbiotically in nature (Arditti, 1992). Protocorm of most of the terrestrial orchids are achlorophyllous and depend on mycorrhiza for all its resources like carbon and phosphorus, which thereby increases its tolerance to environmental stress. The presence of mycorrhizal fungi in the roots of young seedlings may protect the seedling against pathogens.

Germination of orchid seed *in vitro*, is done using asymbiotic protocols while symbiotic germination is done using appropriate suitable fungi. The symbiotic methods of seed germination was pioneered by Bernard (1909), developed by Warcup (1971) and Clements (1986) would appear to provide a method that might closely resemble that in nature, enabling plants with their appropriate mycorrhizal fungus to be transferred to pot culture and eventually to the wild. Asymbiotic methods of seed germination are tedious, laborious and not all orchids can be germinate asymbiotically (Arditti 1992). The present work involves the inoculation of orchid seeds with a suitable mycorrhizal fungus to achieve symbiotic germination. *Calanthe masuca* is a terrestrial orchid distributed in the Western Ghats of India. Stem pseudobulbous annulate. Leaves - many tapering to sheath, ribbed, elliptic-ovate to lanceolate, acuminate upto 50cm long, 5cm broad, sessile. Spikes upto 90cm long, crowded with small flowers. Flowers puberulous, purplish mauve in colour on long raceme, white with purple tip, sepals acute. Flowering and fruiting in the month of November-December (Ananda Rao, 1988).

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MATERIALS AND METHODS

Collection of plant and soil samples

The underground plant parts and rhizosphere soil samples of this terrestrial orchid were collected from Shola vegetation of Kemmannugundi, Chichmagalur district, Karnataka, India. (Fig.1a)

Isolation of mycorrhizal fungi

Potato dextrose agar (PDA) medium was used for fungal isolation from the roots. The root samples were washed and surface sterilized using 0.1% mercuric chloride, rinsed several times in sterile distilled water, cut into pieces of 0.5-1cm length and plated aseptically in petriplates containing PDA and incubated at room temperature (28^{o}C). The plates were observed periodically for mycorrhizal fungal growth from the cut ends. The fungal cultures were purified by hyphal tip method and were maintained on PDA slants in refrigerator for further studies.

Fungal identification

The fungal isolates were preliminarily identified using colony characters and morphological characteristics of hyphae described by Barnett and Hunter (1987). Molecular identification of fungal isolates were done by analyzing the internal transcribed spacer (ITS) region of nuclear ribosomal DNA of fungal hyphae. It was then amplified by

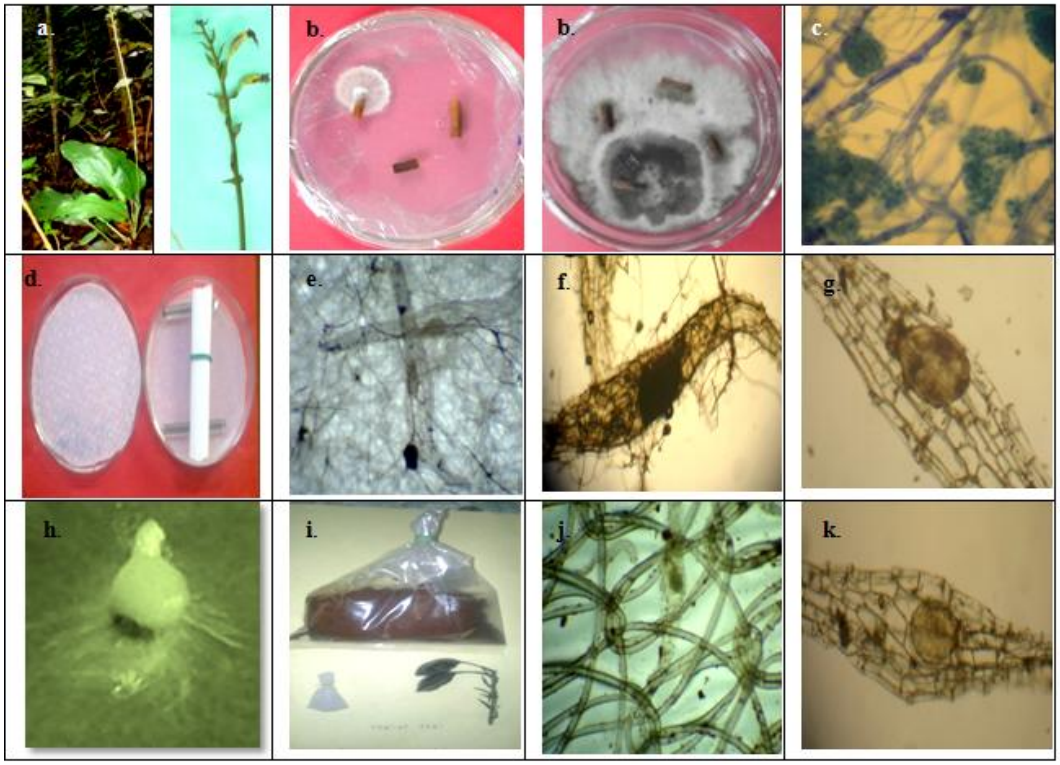

Figure 1. a. Habit of *Calanthe masuca* with capsule b. Isolation of the fungus on PDA c. *Gliocladium* conidiophores with conidia (450x). d. *Ex-situ* germination by filter paper method e. Seeds and fungal hyphae on the filter paper f. Entry of fungal hyphae into the seed g. Development of protocorm like body h. Protocorm *i. Ex-situ* germination by seed bait method along with seed packet and capsules j. Seeds on nylon mesh k. Seed showing swollen embryo

polymerase chain reaction (PCR) using primers ITS1 and ITS4 under thermal conditions.

Seed germination by filter paper and baiting technique

Mature capsules of *Calanthe masuca* were collected from the natural habitats during November and were used for germination studies. Seed viability test was performed using triphenyl tetrazolium chloride (TTC) (Lakon, 1949). The TTC test gives an indication of the ability of the seeds to germinate. Seeds were soaked in 1% solution of TTC for 24 hours (pH 6.8) at 30^{o} C. Non-viable seeds remain unstained and viable seeds show pink colour under the light microscope. Unopened fruit capsule was surface sterilized for a minute in 90% ethanol and carefully twisted using sterilized forceps to release the seeds. The seeds were spread on the surface of a adequately wet filter paper (Whatmann No. 4) along with a mycelial plug of 10mm diameter. The filter paper was then rolled and placed over two glass rods within a petridish containing sterile distilled water (Fig.1d). Petridish with seeds on filter paper and individual fungal isolate was maintained in triplicates. Control was also maintained without fungal isolate. The plates were sealed, incubated at room temperature (28^{o}c) under 12h dark and light conditions. The plates were examined periodically under the stereomicroscope for seed germination. *Ex situ* seed germination experiments were carried out using mature unopened fruits and the soil collected from the natural habitats. After surface sterilization the seeds were sown in 2x2cm packets of nylon mesh whose pore size was chosen such that it retains the seeds, but allows fungal hyphae to pass through (Fig.1j). Seed packets were buried in fresh polythene bags having native soil (Fig.1i). Adequate moisture content was maintained using sterile distilled water and incubated in a glass hood under natural day light conditions.

RESULT AND DISCUSSION

Fungal isolates

Calanthe masuca yielded one fungal isolate on PDA media from the root (Fig1b,c). It was identified as *Gliocladium* using molecular biology technique. The other fungal isolates used were *Colletotrichum dematium, Psathyrella candolleana, Tulasnella*, *Macrophomia phaseolina* which were isolated from four different terrestrial orchids (*Satyrium nepalense*, *Nervilia crociformis, Pecteilis susanne, Malaxis versicolor*) from Western Ghats region of Karnataka. The *Gliocladium* isolate of *Calanthe* appeared as a white colony with wool like surface becoming grayish in the centre. The mycelial growth was observed in the range of 8-10cm in 7-14 days of incubation. Microscopic observation revealed septate mycelium producing conidiophores with conidial mass. Conidia one celled, smooth walled, hyaline, shortly cylindrical with obtuse to slightly rounded ends.

Seed germination

The seed viability test revealed that the intensity of staining with TTC varied with the number of living cells. Non-viable seeds remained unstained while the viable seeds stained pink when observed under the light microscope. In the viable seeds the embryo was robust and ovoid. The seeds placed on the filter paper along with the fungal isolates were opened at intervals of 15 days incubation and the different stages of seed germination was observed under the stereomicroscope. The first visual evidence of germination was observed in the form of marked increase in the translucence of the embryo within the intact testa After four weeks of incubation the process of fungal colonization at the base of the embryo was noted with many hyphae entering into the testa (Fig.1 e,f). This stage was followed by breakage of seed coat and release of the embryo. The embryo failed to show further development into protocorm like bodies with *Gliocladium*, the isolate of *Calanthe.* However protocorm developed with the other isolate *Tulasnella* obtained from *Malaxis versicolor* (Fig.1g,h). The failure of germination with *Gliocladium* may be due to secondary colonization by this fungus at a later stage of plant development. Seeds without the fungal isolates (control) showed imbibition but failed to germinate. Earlier workers like Chutima *et al.* (2011), Wang *et al.* (2011) have successfully used Oat meal agar for seed germination in terrestrial orchids and have observed germination

process up to seedling stage. In the present experiment no nutrient medium was provided to emulate symbiotic seed germination under natural environmental conditions. The *ex situ* germination provide a condition closer to natural situations than occurs in sterile conditions. Seed germination using baiting technique was observed at 30 days intervals. It was observed that there was an increase in the mean volume of the seeds. The embryo appeared swollen and green in colour (Fig.1k). The proximity and colonization of a suitable fungal symbiont which is a pre-requisite for further germination was not observed even after 6 months of incubation. This may be due to the absence or inadequacy of suitable fungal symbiont or spatial variability of the fungal hyphae in the collected soil.

Conclusion

The present work is an attempt to imitate symbiotic orchid seed germination close to natural condition. Suitable mycorrhizal partner is necessary to initiate seed germination. Of the two techniques used in the present study, filter paper technique was found to be more effective and has wide applicability in orchid mycorrhizal studies.

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