Research Article

Studies on Mycorrhizal Associations in an Orchid

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ABSTRACT

Background: Known for their myriad of shapes, size and colors, orchids embody an order of aristocracy among flowering plants and are amongst top ten floriculturally important flowering plants in international market. Worth to note that for growth and development, the Mycorrhizal association plays an important part in orchids; therefore, we want to trace the structure, pattern of infestation, growth pattern and genus involved in mycorrhiza. **Aim:** The present work is based on mycorrhizal association in *Cymbidium pendulum* (Roxb.), an orchid plant, with a stress on an extent of infusion, fate, isolation and identification of fungi partner. **Materials and Methods:** *Cymbidium pendulum,* an epiphytic, is an important Medicinal orchid. It had been collected from their natural habitat, Kanchenjunga hills and was maintained in the orchid house, Botanical Garden, Panjab University, Chandigarh, India for further use. After section cutting, observations recorded. Chemically defined MOM and MS and undefined PDA, DPA PCACMA and MEAB media were used as source of nutrition *in vitro*. Fungal cultures were raised using standard methods and stored at 4°C until use. **Results:** After sectioning, it was observed that the fungal partner was established to enter in the cortex. Entry of Fungal partner in the course of roots is mediated through the thin walled 'passage cells. Further, the fungal symbiont was observed to anamorphs of *Rhizoctonia* species, when processed with cotton blue, after characterization based on their morphological and growth characteristics on different media. PDA and MS followed by Mitra media were studied to be more fruitful for fungal growth. **Conclusion:** The fungal endophytes have been isolated from the different isolated medium and their pure cultures were obtained on PDA medium. The fungal partner was established to enter in the cortex. The access of fungal symbiont through roots was mediated via the thin walled 'passage cells. The fungal entophyte was identified on the basis of morphological and its growth characteristics, it was found to belong the genus *Rhizoctonia*.

Keywords: Media, Mycorrhiza, Orchids, *Rhizoctonia.*

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INTRODUCTION

Orchids, a group of bizarre plants are justifying its position amongst top ten floriculturally important flowering plants in international market due to their innumerable shapes, colors and size. The numerical strength has been assessed at 24,500, in terms of species[1] and 25316 (world checklist of selected Plant Families[2] with 736 genera distributed throughout the world except in polar regions and deserts.[3] The orchids account for 8-10% of all the flowering plant species and one- third of all the monocotyledons.[4] Nearly 73% species are epiphytic which are distributed in tropical and subtropical climates. Orchids have outmaneuvered their counterparts in adaptive connotation and morphological appearances and still continue to be in an active evolutionary flux due to poorly developed barriers of reproductive isolation, which promote gene flow across taxonomic limits, high survival frequency across the taxonomic limits and high survival frequency of neotypes.[5]

Heinrick Friedrich Link in 1840, first reported Fungi present in roots of orchid with the graphic evidence of *Goodyera procera* protocorm section, but he failed to identify the fungus. Worth to mention here that Albert

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Bernhard Frank first coined the term 'mycorrhiza' in 1885 to describe the root-fungus combination. According to him, mycorrhiza embodies a pervasive mutualistic symbiosis where fungus and host nutritionally relied on each other; the fungus extracted nutrients from mineral soil and humus; translocated them to the host plant and the fungus got support in return. Nevertheless, the revolution in thinking about plant and fungal evolution, ecology and physiology generated by Frank and Bernard is still in the process of acceptance and some 21,000 scientific papers have been published since the term 'mycorrhiza' was coined.[6] The most important and perceptive observation of the role of fungus in orchid seed germination in which both fungus and host are benefited by each other was made by Noel Bernard in 1899.[7]

The germination of orchid seeds (especially, the early stages of orchid reproduction and development), are influenced to great extent by the relationship with their appropriate mycorrhizal fungi as well as their surrounding environments. Orchid seeds are incredibly tiny like dust particles, ranging in weigh from 0.3 to 14 ug (micrograms), and each seed capsule may contain 1300 to 4 million seeds that are adapted for wind dispersal.[8] These characteristics feature including reduced size of embryo and the absence of endosperm, cause difficulty in germination. Mycorrhizal fungi associated symbiotic seed germination is considered to be very effective for higher germination rate, probably by contributing nutrients for growth and development. Orchid seeds under natural conditions are unable to germinate without a supply of C (carbon), mineral nutrients as well as vitamins from their symbiotic partners.[9,10] Additionally, mycorrhizal fungi also contribute to supplying and retaining water for orchid species during germination.[11]

Under the co-existence with mycorrhizal fungi, orchid seeds can germinate and develop into unique seedling structures called protocorms that consist of parenchyma cells.[12] Further development of the protocorm into plant is also supported by their mycorrhizal fungi. In protocorms of orchids, the entered fungal hyphae form coiled complexes called pelotons. Orchids can obtain nutrients by digesting them.[13]

Many environmental factors affect not only orchid seeds but also the success in symbiotic association between orchids and mycorrhizal fungi.[14] Future studies exploring mechanisms of orchid seed germination must need to pay attention on the effects of abiotic and biotic factors other than mycorrhizal fungi.

Most of these fungi fall under the category of basidiomycetes in the Rhizoctonia complex[15,16] a group that persists mostly as free-living saprophytes,[17] but also pathogens, microparasites, and orchid symbionts.[18,19] During the past 30+ years, much has been published on orchid endophytes recovered from temperate terrestrials[20,21] and more recently the tropical epiphytes recommended that peloton extraction takes place the same day of root collection, and indeed many studies have adopted this protocol.[22]

Considering the above, the present study was designed to isolate the fungal endophytes, morphological identification of the fungal isolates and to observe the infestation in the roots of *Cymbidium pendulum* (Roxb.) (Figure 1A). An attempt was also made to compare growth rate of fungal endophytes on different media namely OMA, WA, PCA, MEAB, MS, DPA, DPA, MOM and CMA.

MATERIALS AND METHODS

Cymbidium pendulum, an important Medicinal and ornamental orchid, after collecting from their natural habitat, had been maintained in the orchid house, Botanical Garden, Botany Department, Panjab University, Chandigarh, India for further use.

Chemically defined MOM and MS[23,24] and undefined [PDA (Potato Dextrose Agar), DPA (Dextrose Peptone Agar), PCA(Potato Carrot Agar), CMA (Corn Meal

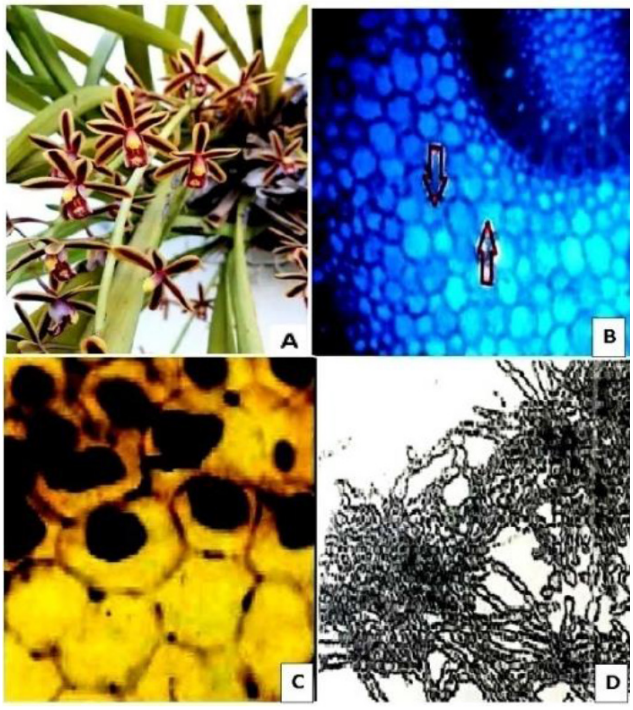

Figure 1: A- Plant *Cymbidium pendulum,* B- TS of roots showing behavior of fungal infection showing digested fungal hyphe, C- peloton formation, D- Morphological features of fungal endophyte showing Moniloid aggregation form sclerotial masses.

Agar) and MEAB (Malt Extract Agar Base)] media were used as source of nutrition *in vitro*. For raising fungal cultures, the autoclaved media were poured in the sterile petriplates in the 'clean air' laminar air flow cabinet, with ca. 25mL in each plate and after gelling of media. After covering with lid, they were sealed with parafiilm. They were stored in the position at 4°C until use.

Isolation

Actively growing roots of plants were used to isolate the fungal endophytes. The roots were washed carefully under with running tap water prior to surface sterilization by dipping successively into 70% ethanol for 1 min, followed by treatment with 100% sodium hypochlorite for 3 min and finally dipped three times in sterilized distilled water. The treated roots were segmented into 10-12 mm long pieces under artificial conditions, and with the help of forceps transferred the piece of roots in petriplates and added 2-3 drops of sterile distilled water. With the help of sterilized blade or scalpel, roots were teased off and then PDA, MEAB, PCA, CMA, and DPA media were poured into the petriplates. The cultures were incubated at 25°C in the dark till the hyphae emerged from the inoculated segments and its growth onto the medium. The pure culture, therefore, obtained by transferring hyphae on PDA medium.

Identification

The identification of fungal partner was done through measuring diameter and dimensions of monilioid cells fungal hyphae. For the purpose, the fungal mycelium was mounted on methanol blue and observed under microscope.[25] Growth rates were determined according to the technique where a small fragment of mycelium (approximately 1mm^2) was inoculated PDA media on the middle. Every 48 hr, over period a two-week, a radial increment in colony diameter appeared and were measured in two directions, The growth rates were characterized by considering averages of three replicates.[26]

In the presently studied species, the number of nuclei in vegetative cells of fungal endophytes was noted by modifying the procedure as follows;[27] hyphae growing on the dialysis membrane were fixed in 2% formaldehyde for 2 min and rinsed in distilled water for 1 min. Hyphae were then stained with 5 mg/mL of 4, 6-diamidino-2-phenylindole (DAPI) for 10 min, and destained with water for 2 min. The material was finally placed in a drop of 50% glycerin on a microscope slide. A cover slip was used without pressing down onto the hyphae. The fungal isolates were identified according to the previous workers.[28]

Explants and Sterilization

The capsule of *C. pendulum* (green), were harvested from the Orchid house, Department of Botany, Panjab University, Chandigarh and washed thoroughly under running tap water and teepol to remove impurities. The capsules were then surface sterilized by immersing in 100% bleach (NaOCI) for 30 min with sporadic agitation; It was followed by a dip in ethanol for 5-10 sec. The sterilized capsules thereafter were flamed to evaporate the excess alcohol followed by a longitudinally split with a sterile surgical blade. The powdery seeds were inoculated on the surface of agar gelled nutrient medium.

The fungal isolates were extracted from the root segments and pure culture were raised in PDA petriplates and then used for further experiments.

Inoculations and Culture Conditions

All inoculations were performed in the Laminar Air Flow chamber. To ensure complete sterilization of the chamber, it was copiously sprayed with methanol or swabbed with ethyl alcohol and the glassware's, petriplates or culture vessels and surgical instruments were subjected to 30 min treatment with U.V. rays using "Philips" brand UV tubes (30w). The rims of the tubes and flasks/surgical instruments were flame sterilized before initiating the inoculation operations. The vessels were properly sealed with parafilm after inoculation of the explants.

Data Recording

The data were recorded out by taking observations on day by day as well as on weekly basis as per requirement based. The responses were also observed on the basis of visual observations. Each set of experiments was initiated with a minimum of 8 replicates and repeated at least twice. Based upon the percentage of cultures showing the response, effect of different treatments was quantified and degree of response/culture including number of proliferative explants, time taken for initiation, multiplication and development, number of seedlings having 2-3 leaves, and 1-2 roots were obtained after periodic observation.

The fungal mycelia mounted on PDA medium on glass slide for microscopic examination. The dimensions of hyphae and the moniliods were measured using micrometer. The minimum and maximum length and width of cells were recorded from more than 10 observations. When cultured in different media (MOM, PDA, MEAB, PCA, MS, OMA, WA, CMA, and DPA) the fungal isolates were observed daily for sclerotial initiation using low power microscopy.

RESULTS

The present work was conducted in *C. pendulum* with a view to isolate fungi from their roots, see the extent of their infestations, fate of fungal partner and to compare the different media for fungal growth (Figure 1 B-D; Figure 2 A-E).

Fungal Infestation

The present species was found to harbor an endotropic and intracellular fungus in the root tissues. The entry was directly through the epiblema cells. The fungal hyphae gained entry into the cortex through thin walled exodermal cells (passage cells) Further, the fungal hyphae do not settle down to form coils in the cells, indicating thereby that these are meant only as channels for hyphal entry into the cortex. Further, it was observed that the fungus although entered deeper but failed to invade all the root cells.

Root- fungal morphological changes

Interestingly, the sclerotial masses formed as a result of aggregation of fungal moniliods were observed in aerial surface in presently studied taxa, probably developing its penetrating structure so as to resist unfavorable conditions. The fungus subsequently entered inner cells to form pelotons in outer layers of cortex (Figure 2 C-D). In fact, these sites promote fungal multiplication as the sclerotia were found to be proliferative and moniliods contained budding therein.

The fungal endophyte was isolated from infected roots. The fungus emerged as a whitish outgrowth to the inoculum within 5- 10 days. Based on their hyphal characters, moniliod appearance and occasional formation of sclerotia like structure, it is strongly suggested that the isolates belonged to the genus *Rhizoctonia* (Figure 1D). On PDA medium, the fungus appeared as milky-white with lime centre when young and turned to yellowish green on maturity having MAT growth on the petriplate with granular hyphae. No sexual reproductive bodies were observed and on CMA media, colony appeared white with lime centre and granulated hyphae. The growth of fungus on CMA was however slower than on PDA (Table 1).

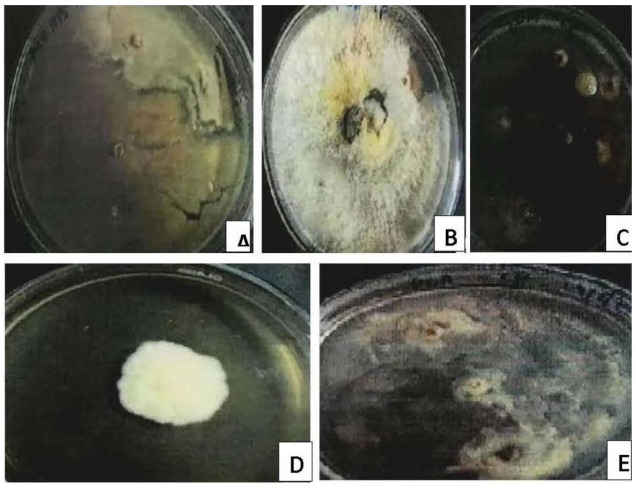

Figure 2: Fungal growth on different media, A-MEAB media no growth, B-PDA media with whitish mat like growth, C-CMA media with white lime Centre growth, D-DPA bounced mat low growth, E- PCA media with slow growth.

Table 1: Fungal isolates from *Cymbidium pendulum*: Hyphal characters.

Nutrient Media	Growth behavior	Mycelia growth rate		
		Colony growth	Extent of growth	Color
MEAB	-	-	-	-
PDA	Mat	+++	Branched	Milky white
PCA	-	-	-	-
CMA	Mat	+	Branched	White with lime Centre
DPA	Mat	+	-	White

Fungal Infection

Presently, it was reported that the roots only in contact with the tree bark were infected. Further the meristematic root tips were observed to be free of fungal infection.

Fungal entry

The fungal hyphae gained entry through the thin walled exodermal cells (passage cells) in the presently studied taxa. The fungal mycelium eluded the thick walled exodermal cells. Moreover, the fungal hyphae do not settle down to form coils in these cells, indicating thereby that they only meant as channels for hyphal entry into the cortex.

Extent of infection

The extent of penetration in the root tissue of currently studied taxon was depicted in inner-cortical layer of the roots. The vascular bundles always remained invariably fungus free.

Digestion

In the taxa under study, the subjected digestion was observed in the infected cortical cells of fungal hyphae, resulting in the deformation in the hyphal coils, the hyphae coalesced together into fungal clumps, and lost their contents; the interconnection of these fungal clumps of adjacent cells through hyphal threads were witnessed during observation. They subsequently,

diminished in size and released the enmeshed host nucleus.

DISCUSSION

The results of present work, conducted in *C. pendulum,* are discussed here. The initial fungal infection is through the epiblema hairs under present work have been studied earlier where the authors studied that the infection is through epiblema cells and hairs.[29,30] The fungal hyphae gained entry into the cortex through thin walled exodermal cells (passage cells) as in *Dendrobium* kin gianu, *Epidendrum radicans*, and *Stanhopea tigrine,*[31] *Vanilla planifolia*[32] and others.[33] The penetration, by mycorrhizal fungi, through the passage cells of the exodermis in angiosperms, could point toward the metabolic control of processes by both the partners. Incidentally, the fungal hyphae in the present species do not settle down to form coils in these cells, indicating thereby that these are meant only as channels for hyphal entry into the cortex.[34] The work on exodermal passage cells stated that the passage cells may not have appropriate physiological conditions necessary for coil formation and subsequent hyphal degradation.

The presence of the fungal hyphae in the passage cells in the presently studied taxa also been suggested by earlier workers. The extent of infection and peloton (coils of fungal hyphae) formation in the root cortex could be varied.

Further, the fungus although entered deeper but failed to invade all the root cells, may be correlated to the involvement of certain inhibitory factors and/or may be loss of vitality of the hyphae during their cell to cell passage.[35] Additionally, as per earlier reports, activation of antioxidant enzymes is also coincide with digestion of pelotons in the mycorrhizal protocorms of orchids.[36] The up-regulation of antioxidant enzymes may cause frequent peloton digestion as in the albino *E. helleborine*, which leads to the favorable promotion of the carbon flux from the fungus to the orchid.[37] The spread of the endophyte in the infected roots can also be checked by some phytoalexins developed in these roots.

The sclerotial masses i.e fungal moniliods, in present taxa, may probably develop its penetrating structure so as to resist unfavorable conditions. In fact, these sites promote fungal multiplication as the sclerotia were found to be proliferative and moniliods contained budding therein. The orchid shoots are in general contain defensive compounds that exclude mycorrhizal fungi and chlrophyllous tissue is not susceptible to infection.[38]

Further, the development of fungal pelotons in the cortex of orchid roots is the characteristic of Orchidioid mycorrhizal[39] as in the present species. Incidentally, the younger pelotons were loosely coiled and older ones tightly interwoven in the line with similar earlier observations.[40] The pelotons in the orchid root cortex are believed to act as an interface for nutrient exchange between the two partners.[41] The mycorrhizal fungi (upon its digestion) are believed to provide carbohydrates, essential ions, and water into orchid seedlings during development.[42,43]

Presently, the fungal endophyte was isolated from infected roots, the failure of most of these endophytes to produce sexual reproduction bodies and distinct conidia indicates their affinity group 'Mycelia sterilia'. A perusal of the earlier literature hinted that orchid roots are easily attached by various saprophytic fungi.[44] A wide variety of genera including *Armillaria, Corticium, Fomes, Hypochnus, Marasmius, Rhizoctoni Xerotus* have been reported to form mycorrhizal associations with orchids and the orchid partners frequently bear a superficial resemble to the anamorphs of *Thanatephorus cucumeris (*A. B. Frank) Dc *Ceratobaslidium cornigerum* (Bourd.) D.P. Rogers i.e., *Rhizoctonia* sen. Therefore, the group is customarily referred to as 'Orchidaceous *Rhizoctonia*s'. The anamorph *Ceratorhiza, Epulorhiza* and Moniliopsis ('Mycelia sterilia', Fungi imp from the Form genus *Rhizoctonia* and the *Ceratorhiza* and *Epulorhiza* are the most common endophytes recovered from the endomycorrhizas of orchids.[45,46] The number of earlier reports regarding diversity of fungi that are in mycorrhizal relationship with orchids have also been available.[47]

The presence of an endotrophic intracellular fungus in the root tissues in present taxa, in accordance with the generalization that almost always the orchids associated with fungal mycelia at some period of their history under natural conditions.

The roots only in contact with the tree bark were infected and the infection free aerial roots in the epiphytic species as reported in earlier findings in Himalayan orchids where the species in contact with the substrate were extensively contaminated.[48,49] The meristematic root tips were observed to be free of fungal infection in *Saccolabiurn papillosum*. Incidentally, the mycorrhizal associations, though an important component of epiphytic habit are less consistent in epiphytic orchids. However, much is still to be learnt about mycorrhizal relationships of epiphytic orchids.

The fungal hyphae entry through the thin walled exodermal cells (passage cells) also reported in *Dendrobium* sp, *Epidendrum radicans*, *Stanhopea* and *Vanilla*

planifolia. The penetration of mycorrhizal fungi through the passage cells exodermis could indicate metabolic controlled process by both the partners.[50] As per current investigations, the fungal partner does not settle down to form coils, indicating, thereby that they only meant as channels for hyphal entry into the cortex; the passage cells may not have appropriate physiological conditions necessary for coil formation and subsequent degradation.

The extent of infection and pelotons (coils of fungal hyphae) configuration in the root cortex, varied with the species. The extent of penetration in the root tissue of currently studied taxon was depicted in inner-cortical layer of the roots. The vascular bundles always remained invariably fungus free. The occasional reports on infection of vascular zone, in *Dendrobium amoenum, Epipactis Jatifolia, Habenaria edgeworthii, H. galeandra* and *Liparis rostrata,* have been documented.[51]

For the establishment of Mycorrhiza, the intruded root tissues could be distinguished into host-cell and digestion—cell zones differing in the form and sizes of their cells, nuclei, and the starch contents. However, the host cell zone comprised of the outer few cortical layers. As there was continued infection by the growing hyphae in the cells of host and digestion zones, thereby the fungus appeared markedly different in these layers. When the infection spreads from young to mature tissues, starch accumulation observed in the hypha.[52]

The subjected digestion, deformation, fungal clumps, followed by lost of their contents in present studies appeared to be metabolically active as it becomes enlarged in size with the large nucleolus within. It thus appears that a large number of enzymes had been released/involved during fungal digestion. Earlier, it observed enhanced activity of peroxidases and acid phosphatases during digestion of pelotons in *Spathoglottis plicata* suggesting thereby that the digestion processes are initiated through enzymatic release.[53] The mycorrhizal fungi (upon its digestion) are believed to transfer soluble carbohydrates, essenstial ions, and water into orchid seedlings during their development).

CONCLUSION

In the currently studied species, the fungal endophytes have been isolated from the different isolated medium and their pure cultures were obtained on PDA medium. The fungal endophyte, obtained from that medium, was identified on the basis of morphological and its growth characteristics. Based upon presently studied parameters and in comparison, with earlier investigations, it was found that these fungal isolates belonged to the genus *Rhizoctonia*.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest.

ABBREVIATIONS

C: Celsius; **hr:** Hours; **min:** Minutes; **sec:** Seconds.

SUMMARY

- From epiblema cells, the fungal hyphae gained entry into the cortex through thin walled exodermal cells (passage cells).
- Although fungus entered deeper but failed to invade all the root cells.
- The fungus formed pelotons in outer layers of cortex.
- The sclerotial masses formed as a result of aggregation of fungal moniliods were observed in aerial surface.
- The fungal endophyte was identified on the basis of morphological characters and its growth characteristics.
- The taxa isolated was found to belonged to the genus *Rhizoctonia*.
- PDA and MS followed by Mitra media were studied to be more suitable for fungal growth.

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