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A study on mycorhizal associations in an economically important orchid

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Abstract

The present work is based on mycorhizal association in *Aerides multiflora* (an orchid) with emphasis on extent of infusion, fate of fungal partner, isolation and identification of fungi. Mycorrhizal association plays an important role in growth and development in the orchids. It was observed that the fungal partner entered to cortex. Fungal entered through roots, mediated through the thin walled 'passage cells. The fungal entophytes were identified as anamorphs of *Rhizoctonia* species, based on their morphological (bi- to multinucleate condition, fungal clamps, mycelial loops, and formation of 'moniliods') and growth characteristics on different media. MS and PDA followed by Mitra media found to be more perfect for growth and extraction of fungus. PDA is found to be most suitable for isolation generally.

Keywords: epiphytes, fungi, media, mycorrhiza, orchids, *Rhizoctonia*

1. Introduction

Orchid plant are known for their myriad of shapes, colors, size and embody an order of aristocracy among flowering plants, therefore, justifying its position as amongst top ten floriculturally important flowering plants in international market. The numerical strength has been assessed at 24,500, in terms of species (Dressler RL., 2006) [15] and 25316 (world checklist of selected Plant Families (Kew., 2006) [25] with 736 genera distributed throughout the world except in Polar Regions and deserts (Chase *et al*., 2015) [8]. The orchids account for 8-10% of all the flowering plant species and one-third of all the monocotyledons (Arditti, J., 1992) [2]. Nearly 73% species are epiphytic which are distributed in tropical and subtropical climates.

Fungi, in orchid roots, were first reported by Heinrick Friedrich Link in 1840 with the graphic evidence of *Goodyera procera* protocorm, but he failed to identify the associated fungus (Arditti, J., 1992) [2]. Term 'mycorrhiza' was first pointed in 1885 by Albert Bernhard Frank to describe the root-fungus combination. He hypothesized that mycorrhiza represent a pervasive mutualistic symbiosis in which fungus and host nutritionally rely on each other; the fungus extracts nutrients from both mineral soil and humus; translocates them to the host plant and the fungus gets 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 (Trappe and Frank., 2005) [46]. 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 (Yeh *et al.*, 2019) [51].

Symbiotic germination using mycorrhizal fungi is considered for very effective for higher germination rate, by contributing nutrients for growth and development. Orchid seeds under natural conditions are unable to germinate without a supply of C, mineral nutrients as well as vitamins from their symbiotic partners (Dearnaley., 2016; Smith and Read, 2008) [14, 40]. Additionally, mycorrhizal fungi also contribute to supplying and retaining water for orchid species during germination (Dearnaley., 2007) [13]. Under the co-existence with mycorrhizal fungi, orchid seeds can germinate and develop into unique seedling structures called protocorms that consist of parenchyma cells (Yeung., 2017) [52]. Then, 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 (Peterson and Farquhar., 1994) [33].

Further, most of these fungi fall under the category of basidiomycetes in the *Rhizoctonia* complex (Zettler *et al*., 2017; Zettler and Correy., 2017) [54, 55] a group that persists mostly as free-living saprophytes (Moore, 1996) [29], but also pathogens, microparasites, and orchid symbionts (Héctor Herrera., 2020; Swarts and Dixon., 2017) [20, 44]. During the past 30+ years, much has been published on orchid endophytes recovered from temperate terrestrials (Currah *et al*., 1987; Warcup., 1991; Zelmer *et al*., 1997) [11, 49, 53] and more recently the tropical epiphytes recommended that peloton extraction take place the same day of root collection, and indeed many studies have adopted this protocol (Chen *et al*., 2012; Hoang *et al*., 2017) [9, 21].

The technique involves selection and treatment of roots, preparation of pelotons, treatment of pelotons, culture of pelotons so that fungal hyphae grow out and strain purification. The technique is considered better because this technique helps to solve the problem of fungal and bacterial contamination.

Considering the above views, the present study was designed to isolate the fungal endophytes and observe its infestation in

the roots of *Aerides multiflora* Roxb. (Table 1). An attempt was also made to compare growth rate of fungal endophytes on nine different media (OMA, WA, PCA, MEAB, MS, DPA, DPA, MOM. and CMA). Overall objectives of the study were to isolation of fungal endophyte from the roots, morphological Identification of the fungal isolates and the extent of infestation of fungus in the roots.

2. Materials and methods

Aerdies multiflora had been collected from their natural habitat and to ensure the ready availability of materials during experiments, the species maintained the orchid house, Botanical garden, Panjab University, Chandigarh, India.

Important to mention that the glassware used were sterilized properly before using. Chemically defined MOM and MS (Mitra., 1976; Murashige and Skoog., 1962) [27, 31] and undefined [PDA (Potato Dextrose Agar), OMA (Oat Meal Agar), DPA (Dextrose Peptone Agar), WA (Water Agar), PCA(Potato Carrot Agar), CMA (Corn Meal Agar) MEAB (Malt Extract Agar Base) media were used as source of nutrition *in vitro* (Table 2).

For raising fungal cultures, the autoclaved media were poured in the sterile Petri plates in the 'clean air' laminar air flow cabinet, with ca. 25m1 in each plate and after gelling of media. They covered with lid followed by sealing with parafilm. They were stored in the position at 4^0C until use.

For isolation, the roots of actively growing plants were used to isolate the fungal endophytes. For the purpose, the soil substratum sticking to the roots were carefully removed and roots rinsed with running tap water prior to surface sterilized by dipping successively into 70% ethanol for 1 minute, 100% sodium hypochlorite for 3 minutes and finally rinsed three times in sterilized distilled water segmented into 10-12 mm long pieces under artificial conditions, and with the help of forceps transferred the piece of roots in Petri plates and add 2-3 drops of sterile distilled water. With the help of sterilized blade or scalpel, roots were teased off and then poured the MOM, PDA, MEAB, PCA, MS, OMA, WA, CMA, and DPA media into the Petri plates. The cultures were incubated in the dark at 25°C until hyphae emerged from the inoculated segments and grew onto the medium. Pure culture was the obtained by transferring hyphae onto PDA medium in dark.

Identification was done by measurements of fungal hyphal diameter; dimensions of monoploid cells were made by examination of the mycelium mounted in methanol blue on microscopic slides (Shan *et al*., 2002) [38]. Growth rates were determined according to the technique of Currah *et al*., (1987) [11]. A small fragment of mycelium (approximately 1mm^2) was inoculated on the middle of petriplates containing PDA media. Radial increments in colony diameter were measured in two directions, every 48 hours over a two-week period. Growth rates were represented by averages based on three replicates. The nuclei number in vegetative cells of fungal endophytes of the presently studied species was studied by modifying the procedure as follows (Sneh *et al*., 1991) [42]; hyphae growing on the dialysis membrane were fixed in 2%formaldehyde for 2 minutes and rinsed in distilled water for 1 minute. Hyphae were then stained with 5 mg/mI of 4, 6-diamidino-2-phenylindole (DAPI) for 10 minutes, and de-stained with water for 2 minutes. 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 (Athipunyakom *et al*., 2004) [3].

Explants and sterilization

Green capsules of *Aerides multiflora* were collected were washed under running tap water and teepol. The capsules were than surface sterilized by submerging in 100% bleach (NaOCI) for 30 minutes with occasional agitation followed by a dip in ethanol for 5-10 seconds. The sterilized capsules were then flamed to remove the excess alcohol and split longitudinally 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 minutes 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.

The data was recorded on daily basis as well as o weekly basis as per requirement. The responses were recorded on the basis of visual observations. Each set of experiments was initiated with a minimum of 8 replicates and repeated at least twice. Effect of different treatments were quantified on the basis of the percentage of cultures showing the response and degree of response/culture which include number of proliferative loci/explants, time taken in weeks for initiation, multiplication and development, and number of seedlings/plantlets with 2-3 leaves and 1-2 roots were obtained after specific time.

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 sclerotia initiation using low power microscopy.

3. Results and discussion

Investigations under present studies were presently conducted in *A. multiflora,* and commercially ornamental and threatened orchids 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.

It is observed that the roots in contact with the tree bark were infected in present species in accord with similar earlier findings that the aerial roots in epiphytic species are generally devoid of infection but those in contact with the substrate are extensively infected (Goh *et al*., 1992) [18]. The meristematic root tips were observed to be free of fungal infection in present species as reported earlier (Katiyar *et al*., 1986) [22]. It is the rhizoplane that is the zone of maximum microbial activity in

epiphytic orchids and not the rhizosphere as in other plants (Tsavkelova *et al*., 2004) [45]. Incidentally, the mycorrhizal associations, though an important component of epiphytic orchids are less consistent in epiphytic orchid (Benzing and Friedman., 1981) [6].

In epiphytic orchids, the entry was directly through the epiblema cells. A perusal of literature reveals that the initial fungal infection of some orchid species is through the epiblema hairs (Sathiyadasha., 2012) [37], epiblema cells and through epiblema cells and hairs (Vij and Sharma., 1983) [47]. In the current work, the fungal hyphae gained entry into the cortex through thin walled exodermal cells (passage cells) in accord with similar earlier reports *Dendrobium* kin gianu, *Epidendrum radicans*, and *Stanhopea tigrina* (Esnault and Masuhara., 1994) [17] and for *Vanilla planifolia* (Madhaiyan *et al*., 2003) [26] and others (Murugan *et al.,* 2010) [32]. The penetration of the passage cells of the exodermis by mycorrhizal fungi in angiosperms could indicate 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 (Smith and Read., 1991) [40] 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 (Esnault and Masuhara., 1994) [17]. The fungal hyphae were observed to be present in the passage cells of old roots in present taxa, corroborating almost similar suggestion by earlier workers (Smith and Read., 2008) [41]. The extent of infection and peloton (coils of fungal hyphae in the host/cortical cells) formation in the root cortex varies with the species.

Presently, it was observed that the fungus although entered in deeper but failed to invade all the root cells, may be related to the development of certain inhibitory factors and loss of vitality of the hyphae during their cell to cell passage (Mollison., 1943) [28]. Activation of antioxidant enzymes is coincident with digestion of pelotons in the mycorrhizal protocorms of orchids (Blakeman., 1976) [5].

Interestingly, the sclerotial masses formed as a result of aggregation of fungal moniliods were observed in aerial surface in presently studies taxon, probably developing its penetrating structure so as to resist unfavorable conditions (Fig 1 D). The fungus subsequently entered inner cells to form pelotons in outer layers of cortex (Fig 1 C). 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 to contain defensive compounds that exclude mycorrhizal fungi and according to chlrophyllous tissue is not susceptible to infection (Hadley and Pegg., 1989) [19].

The formation of fungal pelotons in the cortex of orchid roots is the characterstic of Orchidioid mycorrhizal (Peterson and Massicotte, 2004) [34] and the present taxa under observation were no exception. Incidentally, the younger pelotons were loosely coiled and older ones tightly interwoven in the line with similar earlier observations (Warcup and Talbot., 1967) [50]. The pelotons in the orchid root cortex are believed to act as an interface for nutrient exchange between the two partners (Rasmussen., 2002) [35].

In species under study, fungal endophyte was isolated from infected roots. Failure of most of these endophytes to sexual reproduction bodies and distinct conidia indicates their affinity group 'Mycelia sterilia'. The fungus emerged as a whitish outgrowth the inoculum within 5- 10 days. Based on their hyphal characters, part moniliod appearance and occasional formation of sclerotia like structure strongly suggest that the isolates belong to the genus *Rhizoctonia* (Figure 1 D). Previous literature reveals that orchid roots are easily infected by various saprophytic fungi (Dearnaley and Cameron., 2017) [12]. A wide variety of genera including *Armillaria, Corticium, Fomes, Hypochnus, Marasmius, Rhizoctoni Xerotus* have been reported to form mycorrhizal associations with orchids most of the orchid symbionts bear an overall and superficial resemble the anamorphs of *Thanatephorus cucumeris (*A. B. Frank) Dc *Ceratobaslidium cornigerum* (Bourd.) D.P. Rogers i.e. *Rhizoctonia* sen (Currah and Sherbourne., 1992, Esposito *et al.*, 2016) [10, 16].

In present work also, the fungal isolate was identified according to the previous workers (Saksena and Vaartaja., 1960) [36]. On PDA medium, the fungus proliferates as profusely milky-white aerial hyphae. The vegetative hyphae were non-granular which underwent branching at acute to the right angles at the distal end near the septum. No sexual reproductive bodies were observed (Tables 1, Fig 2A-I). Almost same growth pattern, but comparatively lesser growth was seen on other medias viz. DPAand CMA, MEAB but mat and aerial growth in Mitra. The fungus appeared white on PCA. On CDM medium, colony appeared white with lime centre and granulated hyphae. The growth of fungus in all the media comparatively slower than on PDA (Table 1: Fig 2 (A-I).

Moreover, the present species were found to harbor an endotrophic intracellular fungus in the root tissues regardless of their habit and habitat in accord with the generalization that the orchids are almost always associated with fungal mycelia at some period of their history under natural conditions. The aerial plant parts (floral parts, inflorescence axis, leaves, stem etc. in almost all the taxa under study, were invariably free of fungal infection. It was observed fungal endophytes even in the leaf tissues of seven epiphytic and lithophytic species of Lepanthes and observed these to be different from that present in the roots (Bayman., 1997) [4]: this complexity and variability of the endophytes (Lodge *et al.*, 1996) [25]. Such interactions may affect not only the host plant but also plant's interaction with herbivores and other organisms.

A variety of patterns, of fungal entry, was observed in the terrestrial and epiphytic orchids. A perusal of literature reveals that the initial fungal infection of some orchid species is through the epiblema hairs, epiblema cells and through both the epiblema cells and root hairs (Kumar and Krishnamurthy, 1995) [24], similar functioning have been observed in the species under consideration.

But on contrary, in present studies, the fungal hyphae gained entry into through the thin walled exodermal cells (passage cells) in accord with similar earlier reports for *Dendrobium* sp, *Epidendrum radicans*, and *Stanhopea* and for *Vanilla planifolia*. Fungal mycelium eludes the thick wailed exodermal in present work. The penetration of the passage cells exodermis by mycorrhizal fungi in angiosperms could indicate metabolic controlled process by both the partners. Incidentally, the fungal hyphae in the present study do not settle down to form coils in these cells, indicating thereby that the meant only as channels for hyphal entry into the cortex.

It was observed that the extent of infection and pelotons (coils of fungal hyphae in the host/cortical cells) formation in the root cortex, varies 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 (Vij *et al*., 2002) [48]. However, the infection extent was same irrespective of the level of maturity of the roots in presently studied species. It may be speculated that the host cortical cells in fresh roots have better repositories of photosynthetic assimilates which are initially offered by these cells to lure the fungus, on the other hand in older roots such repositories may be lass available on account of vasculature (which has to translocate the photosynthates into the cortical cells of these aging roots).

Consequently to the establishment of mycorrhiza, the invaded root tissues in the present species could be differentiated into host-cell and digestion-cell zones which differ from each other in the form and sizes of their cells, nuclei, and the starch contents. While the host cell zone comprises of the outer few cortical layers. Though the cells of host and digestion zones are infected by the continuously growing hyphae, the fungus differ markedly appearance in these layers. When the infection spreads from young to mature tissues the hyphal penetration follows accumulation of starch (Burgeff, 1959) [7]. The storage process in the fungal mycelium is believed to favor continuous and fresh supply of nutrients from the mycelia net in the soil to the host.

Notably, in present work, the fungal hyphae were subjected digestion in the invaded cortical cells; during digestion, the hyphal coils lost their individuality, coalesced together into fungal clumps, and lost their contents; these fungal clumps of adjacent cells appeared interconnected by hyphal threads (Fig 1D). They subsequently, diminished in size and released the enmeshed host nucleus. The host cell nucleus 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 (Kumar and Krishanmurthy., 1995) [24]. The mycorrhizal fungi (upon its digestion) are believed to transfer soluble carbohydrates, essential ions, and water into orchid seedlings during their development (Suetsugu *et al*., 2017) [43].

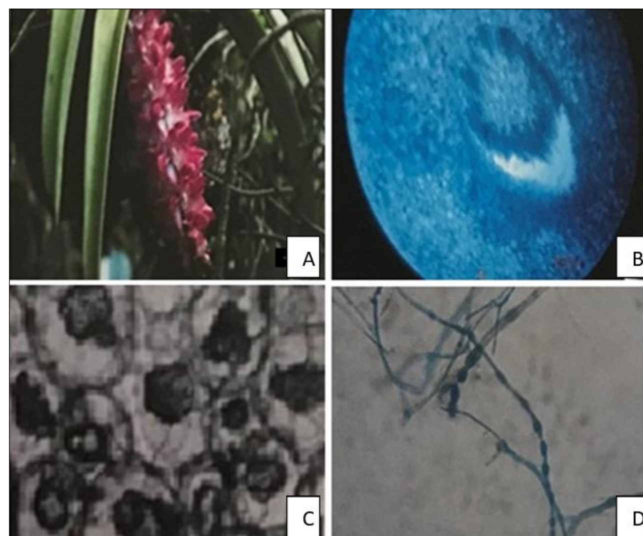

Fig 1: A, *Aerides multifloa* (Roxb); B, *Digested hyphae*; C, pelotons formation; D, single Moniloid

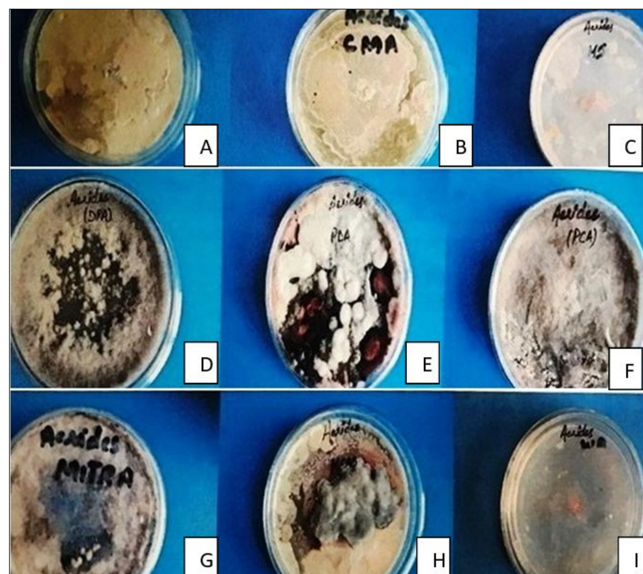

Fig 2 (A-I): *A. multiflora* (Roxb.); A, OMA media with no growth; B, CMA media with no growth; C, MS media with no growth; D, DPA media with aerial hyphae growth; E, PDA media with Profusely aerial growth; F, PCA media with growth; G, Mitra media with mat and aerial growth; H MEAB media with aerial growth; I, WA media with no growth

Table 1: Fungal isolates from *Aerides multiflora*: Hyphal characters (Fig 2 (A-I)

Nutrient Media	Growth behavior	Mycelia growth rate		
		Colony growth	Extent of growth	Color
PDA	Mat & Aerial	++++++	Non granular, Branched	Milky white
MEAB	Mat & Aerial	+++	Non granular	White
MOM	Mat & Aerial	++++	Non granular	White
DPA	Mat & Aerial	+++	Non granular	White
PCA	Aerial	+++	Non granular	Whitish Brown
CMA	-	-	-	-
OMA	-	-	-	-
WA	-	-	-	-
MS	-	-	-	-

4. Conclusion

Conclusively, presently investigated orchid invariably possesses an intracellular and entophytic fungus in their roots and fungal entry into roots was mediated through the thin walled ‘passage cells. The fungal entophytes were identified on the basis of their morphological and growth characteristics on different media. It is invariably, anamorphs of *Rhizoctonia* species based upon branching pattern from right to acute angles, bi- to multinucleate condition, fungal clamps, mycelial loops, and formation of ‘moniliods’ and their aggregations forming ‘sclerotia. PDA is found to be most suitable for isolation for all the species under study, generally.

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