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Full Length Research Paper

Mycorrhizal Symbiosis in the Roots of Anoectochilus elatus

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A cytochemical, cytoenzymological and biochemical studies were investigated on the mycorrhizal fungus associated with the terrestrial orchid, *ANOECTOCHILUS ELATUS.* The results suggested that fungal invasion and digestion caused many changes in the orchid cells.

Key words: Orchid mycorrhiza, Peloton, lysis, enzyme activity

INTRODUCTION

Orchidaceae belongs to monocotyledons family with great variety of floral morphology, pollination relationships and diversity of ecological preferences (Douzery et al 1999). The roots of orchids join with mycorrhizas which plays a critical role in the orchid development. Mycorrhizal fungal associations with orchids are of particular interest because of their essential role in seed germination and protocorm development and their similarly in many respects to biotrophic fungal infections. The majority of orchids are photosynthetic at maturity. However, more than 100 species of orchid are completely dependent on their fungal partners throughout their lifetime (Dearnaley,2007). These mycorrhizal fungi are characterised by forming fungal coil formation, the pelotons which is to be digested by the host cells resulting in a degenerated hyphal mass surrounded by host plasma membrane (Senthilkumar et al 2000). However, studies on the enzyme and other activities are still little developed in the epiphytic orchids. The available information did not contribute substantially new to our understanding on Indian orchid mycorrhizal system. However, many features are still unclear on the epiphytic system. The aim of this study was to investigate the biochemical, cytochemical and cytoenzymological studies on the epiphytic orchid, Anoectochilus elatus.

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MATERIAL AND METHODS

Collection and processing of the material

The epiphytic orchid, Anoectochilus elatus was collected from the Manjolai Forest of Western Ghat's, from Thirunelveli in Tamil Nadu, South India. The orchid contains the natural inoculum of the mycorrhizal fungus of the genus Rhizoctonia. The tubers were washed to remove the debris, and the roots were cut into 1 cm long bits and fixed in formation-acetic acid-alcohol (FAA). The root bits were dehydrated in ethanol-xylol series, embedded in paraffin, and cut into 15 m thin sections were subjected to several cytochemical and histoenzymological staining procedures (Tables 1 and 2) to understand the changes in lysing pelotons as well as in the host cells (Berlyn and Miksche 1976). Photomicrographs were taken using Nikon Eclipse (E400 Japan) photomicroscope.

Estimation of Lipids

About 60 g of sample was oven-dried at 138o C. Later the sample was homogenized and dissolved in 50 ml of neutral solvent containing a few drops of phenolphthalein. The contents were titrated against 0.1 N potassium hydroxide with continuous shaking until pink colour developed (Cox and Peterson 1962).

Table 1 – Cytochemical procedures

Stains used, Treatment duration	Substances localized, Colour obtained
Acid fuchsin (1% in distilled water)	Proteins, Reddish pink (Robinow and Marak 1966)
Acridine orange (0.05% AO in Acetate buffer pH 4.2)	DNA, yellow fluorescence RNA, red fluorescence (Gahan 1984)
Alcian blue 8GX (3% solution in 0.1 N sodium acetate buffer, pH 5.6)	Carboxylated polysaccharides, blue (Gahan 1984)
Aniline blue(aqueous solution of aniline blue 0.005%)	Callose, blue (Johansen 1940)
Azure B (0.25 mg/ml in Mcllavine buffer, pH 4.2) 2 hr	Lignin, blue green. DNA, blue green (Jacqmard *et al* 1972)
Methyl green (0.15% methyl green in 100 ml acetate buffer, pH 4.7, 1% n-butanol) 1-2 min	DNA, blue (Gahan 1984)
Orange G-Aniline blue (2 g orange G and 0.5 gm aniline blue in 100 ml 0.1 m citrate buffer, pH 3.0)	Phospholipids, yellow; Nucleo protein, blue (Lacour *et al*. 1958)
Sudan black E(Saturated sol. in 70% alcohol)	Lipids, black (Krishnamurthy 1999)
Toluidine blue 0 (0.05% in acetate buffer, pH 4.4)	Carboxylated polysaccharides, pink to reddish pink (McCully 1966)

Table 2 – Histoenzymology Procedures

Enzyme	Procedure	Duration and pH of Reaction	Control
Acid phosphatase E.C. 3.1.3.2	Precipitation method (Gomari 1950) Colour : Brownish black	30-40 min 5.0	Substrate omitted
Esterase E.C. 3.1.1.2	Simultaneous azo coupling method (Malik and Singh 1980) Colour : Purple red to brown	1.5 min 7.0	-naphthyl acetate omitted
Glutamate dehydrogenase E.C. 1.4.1.2	Tetrazolium method (Malik and Singh 1980) Colour: Blue violet	15-20 min 7.0	Sodium glutamate omitted
Peroxidase E.C. 1.11.1.7	Benzidine method (Dejong *et al* 1967) Colour : Brownish	15 min 7.0	H_2O_2 omitted

Estimation of Proteins

Fresh material (500 g) was homogenzied with ice cold phosphate buffer (pH 7.2) and filtered through muslin cloth. The filtrate was centrifuged at 10000 rpm (- 4^o C) for 10 min and equal volume of 10% TCA was added and left for 30 min (- 4^o C). The extract (1 ml) was added to 5 ml of alkaline solution with 0.5 ml of folin-ciocalteau reagent. After 30 min optical density was read at 660 nm using a Spectrophotometer 21 D (USA) (Lowry *et al* 1951).

Estimation of Phenols

Root sample (1 g) in 80% ethanol was homogenized in mortar and pestle and the homogenate was centrifuged at 10000 rpm for 20 min. After adding 0.5 ml folin – ciocalteau and Na_2CO_3 (20%), the alcoholic extract was mixed thoroughly. After boiling the mixture for 1 min. optical density was measured at 650 nm using a Spectrophotometer 21 D (USA) (Malik and Singh 1980).

Determination of Enzyme Activities

Malate Dehydrogenase

Root sample (1 g) was homogenized in 1 ml of 50 mM Tris HCl (pH 8.0), 50 mM $MgCl_2$, 5 mM mercaptoethanol and 1 mM EDTA and centrifuged at 10000 rpm (- 4^o C) for 20 min; 0.5 ml of oxaloacetic acid, 0.5 ml of magnesium chloride, 1.3 ml of Tris HCl (pH 7.8), 0.5 ml of NaOH and 0.2 ml of enzyme extracts were mixed. After mixing, the enzyme activity was determined at 340 nm in a Spectrophotometer 21 D (USA).

Acid Phosphatase

Root sample (1 g) was homogenized with 10 ml of ice cold 5 mM citrate buffer (pH 5.3). The homogenate was filtered and centrifuged at 10000 rpm (- 4^o C) for 10 min; 3 ml of the substrate solution was incubated at 37^o C for 5 min and 0.5 ml enzyme extract was added. After mixing 0.05 ml of the above, it was mixed with 9.5 ml of 0.085 N sodium hydroxide. After incubation (37^o C for 15 min) the

Table 3 – Phenols, proteins and lipid levels in the presence and absence of mycorrhizal colonization

Phenols mg/g		Proteins mg/g		Lipids mg/gm	
Colonised	Uncolonised	Colonised	Uncolonised	Colonised	Uncolonised
0.14 ± 0.00089*	0.1± 0.0014*	0.2± 0.0038*	0.2± 0.0013*	0.8± 0.0054*	0.6± 0.001*

* Significant at 5% level

enzyme activity was measured at 405 nm using a Spectrophotometer 21 D (USA) (Lowry *et al* 1951).

Peroxidase

Root sample (1 g) was added to 3 ml of 0.1 M phosphate buffer (pH 7.0) and centrifuged at 18000 rpm (-10^{o} C) for 30 min; 0.1 ml of enzyme extract was mixed with 3 ml buffer solution, 0.05 ml guaiacol solution and 0.03 ml hydrogen peroxide. After mixing, the enzyme activity was measured at 436 nm using a Spectrophotometer 21 D (USA) (Putter 1974; Malik and Singh 1980).

RESULT AND DISCUSSION

The cortex proper consists of twenty five layers of cells. In which pelotons were seen. The pelotons were settled down in the middle part of cortex and followed by outer most regions. The pelotons could be also observed even in the inner layer of the cortex but never seen in the endodermis.

The fresh peloton get stained to corboxylated acidic polysaccharides, proteins, fairly good amount of lipids with more neutral lipids and good DNA content with methyl green. The biochemical comparison of protein, phenol and lipid values are presented in Table-3. During fungal lysis a definite increase of protein was observed and it is proved through cytochemical and biochemical studies. A similar response has been reported with reference to vesicular arbuscular mycorrhizal fungi that are the former are the major contributors to an abundant protein. Many reports have demonstrated that cytoskeleton proteins are crucial in plant-fungal associations (McLusky *et al* 1999). Due to the close interactions between the plasma membrane and proteins of the cytoskeleton, these latter will be regarded as components of the interface in mycorrhizas (Bonfante 2001). Hybrophobins are the fungal proteins widely distributed in the fungal kingdom which play crucial role in fungal morphogenesis and pathogenesis. A clear picture of related proteins in other mycorrhizal systems and analysis of their functional significance will be useful to know the specific wall proteins. The total phenol content of the colonised cells and high when compared to uncolonised cells might be the presence of phenols in the fungal structures. Histochemical studies of the mycorrhizas showed higher phenol content when compared to cortical cells devoid of the fungus. The higher amount of phenol might be one of the factor responsible for increased disease resistance found in mycorrhizal plants (Krishna and Bagyaraj 1984). The phenolic substances like alexins, orchinol, hircinol and loroglossol have been isolated and identified in the European orchids during the process of infection (Fish *et al* 1973). A controlled balance may exist between these two partners is responsible for the maintenance of the association between *Rhizoctonia* hyphae and orchid cells for the prevention of parasitism. The protocorms of *Orchis morio*, the low concentration of orchinol, a phytoalexin, increased slightly in the presence of symbiotic fungus was observed by Uetake *et al*. (1997).

A thorough cytochemical studies have been carried out on the lysing pelotons by a number of investigators (Senthilkumar and Krishnamurthy 1998; Senthilkumar *et al* 1998; Peterson and Currah 1990). A fairly good amount of lipids particularly acidic lipids was noticed in the cytoplasm during lytic stages and there could not be detected in late stages. The formers were seen in the cytoplasm of host cells indicating that they are released into host cells by the fungal partner (Peterson and Currah 1990). Further, Richardson *et al* (1992) have stated that lipids and phosphates were observed during peloton degradation in *Platanthera*.

Williamson (1973) reported a marked increase in acid phosphatase activity in the lysing pelotons while esterase was active during intact stage of the peloton especially at hyphal tips. It was recorded that there is a indensive activity of peroxidase and esterase in the fresh pelotons while glutamate dehydrogenase was observed only during the lysis (Table 4). However, Senthilkumar (1996) observed the activity of acid phosphatase continued to be moderate while esterase could not be detected in the ground orchid, *Spathoglottis plicata*. Further we have observed a very high activity of malate dehydrogenase, starch phosphorylase, succinate dehydrogenase, glucose-6-phosphatase and -glucosidase were observed in the cytoplasm of fresh pelotons; the activity of these enzymes were gradually lost during lysis. Symbiosis between the fungus and the plant results in the reciprocal exchange of substances, mainly salts, metal ions and nitrogen, as ammonium or nitrates, from the fungal hyphae to the plant roots and carbon compounds from the plant to the fungus (France and

Table 4 – Acid phosphatase, malate dehydrogenase and peroxidase levels are expressed in the presence and absence of mycorrhizal colonization

Acid phosphatase per min/mg P		Malate dehydrogenase per min/mg P		Peroxidase per min/mg P	
Colonised	Uncolonised	Colonised	Uncolonised	Colonised	Uncolonised
0.02 + 0.0012	0.71+ 0.0011	0.8+ 0.0001*	0.41+ 0.004*	1.13+ 0.004*	0.32+ 0.028*

* Significant at 5% level

Reid 1983). Hyphae penetrate the first few layers of the cortex and the enzymes are expressed here, suggesting some important role of its presence where the molecular exchanges occur between the symbionts. It would be of interest to observe the interaction of these enzymes with various orchid mycorrhizal partners under natural conditions.

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