A cytochemical study on the mycorrhizae of *Spathoglottis plicata*

S. SENTHILKUMAR* and K.V. KRISHNAMURTHY**

*Department of Microbiology, J.J. College of Arts and Science, Pudukkottai-622 404, India**
*Department of Plant Science, Bharathidasan University, Tiruchirappalli-620 024, India***

Abstract

This paper describes the hitherto unreported aspects of orchid mycorrhizae. The host cells harbour upto 4 generations of fungal pelotons which are formed after each peloton is digested. There are two types of hyphae in a host cell, one forming the pelotons, and the other which lies close to the host cell wall and separated from the former by a callosic wall. The later, called non-pelotonic hyphae form the fresh peloton when the former is digested. Consequently, there are also cytochemical differences between these two types of hyphae.

Additional key words: fungi, orchids, peloton.

Introduction

The mycorrhizal association is a well-known mutual relationship between two organisms, a fungus and the root of a vascular plant. There are different kinds of mycorrhizal association depending on the location of the fungus in relation to the root tissues. The orchids have a special type of endomycorrhizae in which the fungal hyphae produce very characteristic pelotons (coiled balls of fungal hyphae) in the host cortical cells (Peterson and Farquhar 1994). These intracellular fungal bodies get digested by the host cell providing the principle means of nutrition for the orchid (Purves and Hadley 1975, Peterson and Currah 1990). The same host cell very soon gets occupied by another peloton only to be digested again. Upto 4 successive generations of peloton have been reported in the same host cell of a number of orchids (Hadley 1982, Peterson and Currah 1990). Till now, the mechanism of additional peloton formation has not been known. This study, therefore, has been undertaken to investigate the process of additional peloton formation in the mycorrhizae of the common ornamental ground orchid, *Spathoglottis plicata* Blume.

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* Corresponding author, fax. (+091) 04322 22221

Materials and methods

Spathoglottis plicata Blume, an ornamental ground orchid commonly cultivated in several parts of India, was selected as the study material. Its underground bulbs produce several roots which have a mycorrhizal association. The plants were maintained in pots containing compost rich garden soil and natural inoculum of the mycorrhizal fungus, *Epulorhiza repens* (= *Rhizoctonia repens*) (Sneh *et al.* 1991).

The mycorrhizal roots were collected, washed thoroughly in water, cut into small pieces, fixed in formalin - acetic acid - alcohol (FAA), dehydrated, embedded in paraffin and sectioned by microtome (Berlyn and Miksche 1976). In addition, fresh free hand sections as well as cryosections were also made. Both transverse and longitudinal sections were cut and subjected to several cytochemical staining procedures, in order to understand the cytochemical changes noticed in fungal hyphae as well as in the host cells which harbour these hyphae. The host cells containing pelotonic hyphae of various developmental stages were analysed for this purpose. (for detail see Table 1).

Infection density was calculated according to Hadley and Williamson (1972) as a ratio of number of infected cells in cortex to total number of cells in cortex. For calculating this, 100 transections of mycorrhizal roots were randomly selected and used.

Table 1. Cytochemical and cytoenzymological staining procedures followed.

	Procedure employed and treatment time	Substance to be localised - colour to be obtained	Control
1.	aniline blue (aqueous solution of aniline blue 0.005 %)	callose - blue (Johansen 1940)	5 % NaOH (or) saturated $CaCl_2$ (Reynolds and Dashek 1976)
2.	cellufluor HZR New (Sigma) (0.1 % aqueous solution)	cellulose and other cell wall polysaccharides - blue fluorescence (400 - 410 nm) (Gahan 1984, Krishnamurthy 1988)	cuprammonium 5 h treatment before staining (Jensen 1962)
3.	chitin (KOH - iodine - potassium iodide procedure)	chitin - violet or red violet colour (Gahan 1984)	2 % acetic acid treatment after autoclaving
4.	chlorazol black E (Sigma) (in 0.1 % methyl cellosolve) 5 min	cellulose - black to bluish black (Robards and Purvis 1964)	same as 2
5.	coomassie brilliant blue (R 250 - BDH) (0.02 % in Clarke's soln.) 15 min	total proteins - blue (Cawood *et al.* 1978, Eklavya 1979)	pepsin digestion (Krishnamurthy 1988)
6.	fast green FCF (Incubation in 5 % TCA in 0.01 M phosphate buffer, pH 8.0, 30 mg fast green FCF in 50 cm^3 of phosphate buffer) 30 min	basic proteins and histones - green (Gahan 1984)	deamination/acetylation/trypsin extraction

7.	alkaline hydroxylamine hydrochloride (equal volumes of sodium hydroxide and hydroxylamine hydrochloride)	esterified pectins - red (Krishnamurthy 1988)	diazotization (Krishnamurthy 1988)
8.	lacmoid blue (0.17 % in 30 % ethanol)	callose - greenish blue (Reynolds and Dashek 1976)	5 % NaOH 16 h (Reynolds and Dashek 1976)
9.	methyl green - pyronin (BDH) (0.15 % methyl green and 0.25 g pyronin in 100 cm^3 of acetate buffer pH 4.7 - 1 % n-butanol/ tertiary butanol) 1 - 2 min	DNA - blue RNA - red (Gahan 1984)	enzyme digestion (Brachet 1953)
10.	mercuric bromophenol blue (10 g mercuric chloride and 100 mg of bromophenol blue in 100 cm^3 of water)	total proteins - blue (Mazia *et al.* 1953)	deamination/acetylation (Lillie 1954)
11.	nile blue sulphate (*Loba Chemie*) (1 % nile blue sulphate in DM)	neutral lipids - red acidic lipids and basics proteins (Gahan 1984)	lipid extraction by bromine water
12.	periodic acid - Schiff's (PAS) (1 % of periodic acid + Schiff's reagent) 10 min	total insoluble polysaccharides purplish to majenta (Feder and O'Brien 1968)	acetylation
13.	ruthenium red method (1 g in 5000 cm^3 of DM)	non esterified pectins - red to pink (Krishnamurthy 1988)	0.5 % ammonium oxalate treatment, 12 h
14.	acid phosphatase E.C. 3.1.3.2	lead phosphate method/ precipitation method colour - brownish black (Gomari 1950) pH 5.0, 30 - 40 min	omit substrate
15.	ATPase E.C. 3.61.3	precipitation method-brownish black, pH 7.0, 20 - 30 min (Wachstein and Meisel 1957)	omit ATP from reaction medium
16.	glucose-6-phosphatase E.C. 3.1.3.9	precipitation method (Malik and Singh 1980) pH 6.5, 45 min	heat inactivation of tissues
17.	β-glucosidase E.C. 3.2.1.2.1.	simultaneous coupling post coupling methods brownish black (Gahan 1984) pH 5.0, 2 - 3 h	omit 6-bromo-2-naphthyl-β-D-glucopyronoside
18.	malate dehydrogenase E.C. 1.1.1.3.8	tetrazolium method (Gahan 1984) pH 7.6, 10 - 15 min	omit NAD
19.	starch phosphorylase E.C. 2.4.1.1.	blue to black (Godlewski 1960) pH 6.0, 3 min - 2 h	heat inactivation of tissues
20.	succinate dehydrogenase E.C. 1.3.99.1.	simultaneous azo-coupling method - blue (Malik and Singh 1980) pH - 7.2, 30 - 60 min	heat inactivation of tissues

Controlled infection was also studied *1*) to calculate the time taken for a host cell to initiate digestion of its enclosed pelotons, and *2*) to test whether continuous peloton formation in the same host cell is dependent on the continued presence of the mycorrhizal fungus in the soil around the roots. For this purpose, young plants with de-rooted bulbs were grown for just 2 d (time required for fresh uninfected roots to emerge) in the pots containing natural inoculum of the fungus; these were subsequently transplanted to sterilised garden soil (without the inoculum) after carefully washing the underground parts with sterile distilled water. These plants showed infection only in some of the innermost cortical cells of their newly emerged roots, subsequent to which infection totally stopped.

Results and discussion

Infection and peloton formation were noticed firstly in the innermost cortical cells, followed by infections in the more and more outer cortical cells. In the controlled infections for just 2 d, pelotons could be noticed only in some of the innermost cortical cells. However, in plants growing continuously in pots with the natural inocula of the fungus, this temporal distributional specificity was lost soon, leading to a situation where cortical cells, irrespective of their location, contained fresh or old pelotons (Fig. 3).

In transections of the roots, the pelotons of the mycorrhizal fungus appeared as spherical balls (Fig. 1). Initially, the individual hyphae forming the peloton, could be differentiated easily one from another. Further, the peloton almost completely filled up the entire lumen of the host cell and recognition of its individual hyphae was gradually lost, indicating its slow lysis and digestion (Fig. 2). The host cells, where lysis of pelotons takes place, were designated as "digestion cells" (Burgeff 1959). The oldest colonised cells were the first to act as digestion cells followed by subsequently colonised ones. In other words, between the peloton initiation and the onset of digestion, there is a definite interval, which is about 10 d in the taxon studied. This was verified by observing pelotons on the innermost cortical cells of plants subjected to controlled infection for just 2 d; these pelotons showed signs of digestion about 10 d after transplantation to new sterile pots. Purves and Hadley (1975) observed digestion after 2 d of infection while Burges (1939) in other orchid species recorded digestion a month after infection. Since inoculation of cortical cells was random, digestion of pelotons was also randomly observed in the cortex and not in a defined zone of cells, designated as the "digestion layers" or "digestion zone", as has been recognised in some other species (Williamson and Hadley 1970, Hadley and Williamson 1971, Hadley 1982).

Digestion of the first pelotons was not total. Remnants of the undigested first pelotonic material could be often noticed in the centre of the host cell as sperical bodies (Fig. 5), around which hyphae of the fungus organize the second fresh peloton. This process of fresh peloton formation was repeated a maximum three times in the same host cell in this species (see also Hadley *et al.* 1971, Hadley 1982, Peterson and Currah 1990). The formation of fresh pelotons around the digested

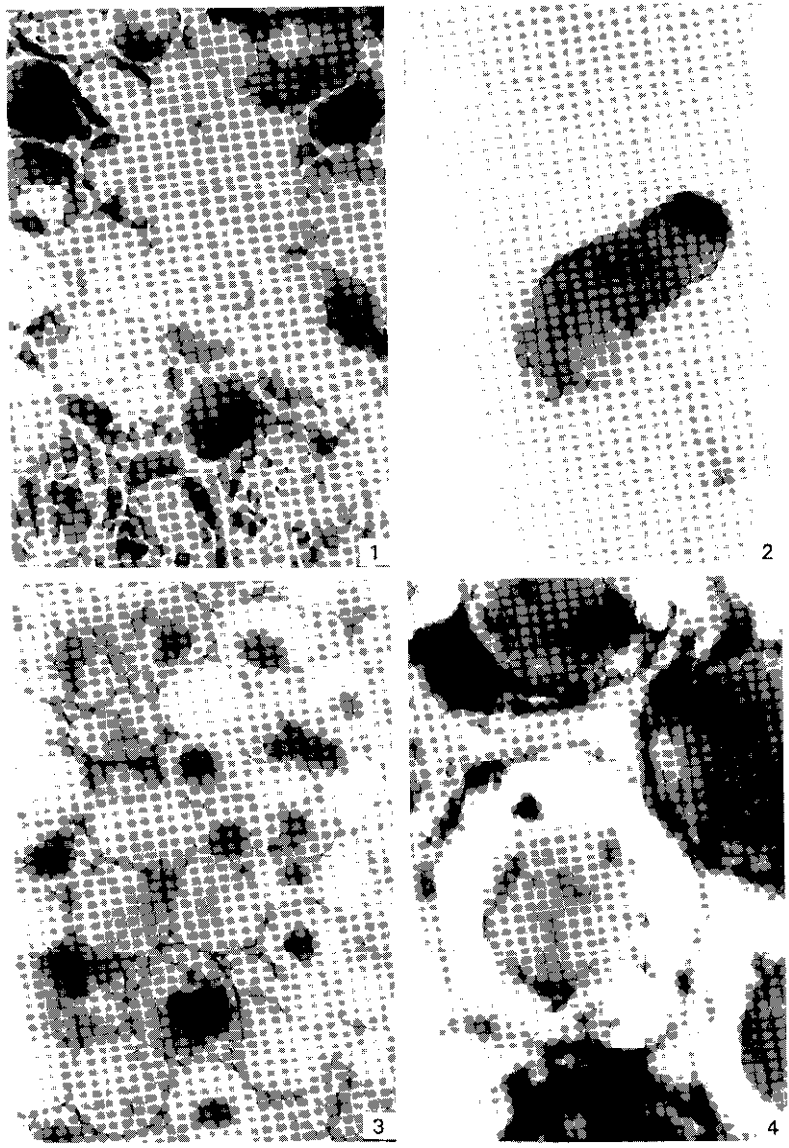


Fig. 1. Transection of root cortex stained with Acridine orange showing loosely arranged hyphae of fresh pelotons with increasing compactness in older pelotons (× 325).

Fig. 2. A peloton undergoing digestion is stained with Alcian blue. Note that the pelotons are surrounded by a layer of Alcian blue positive material (× 650).

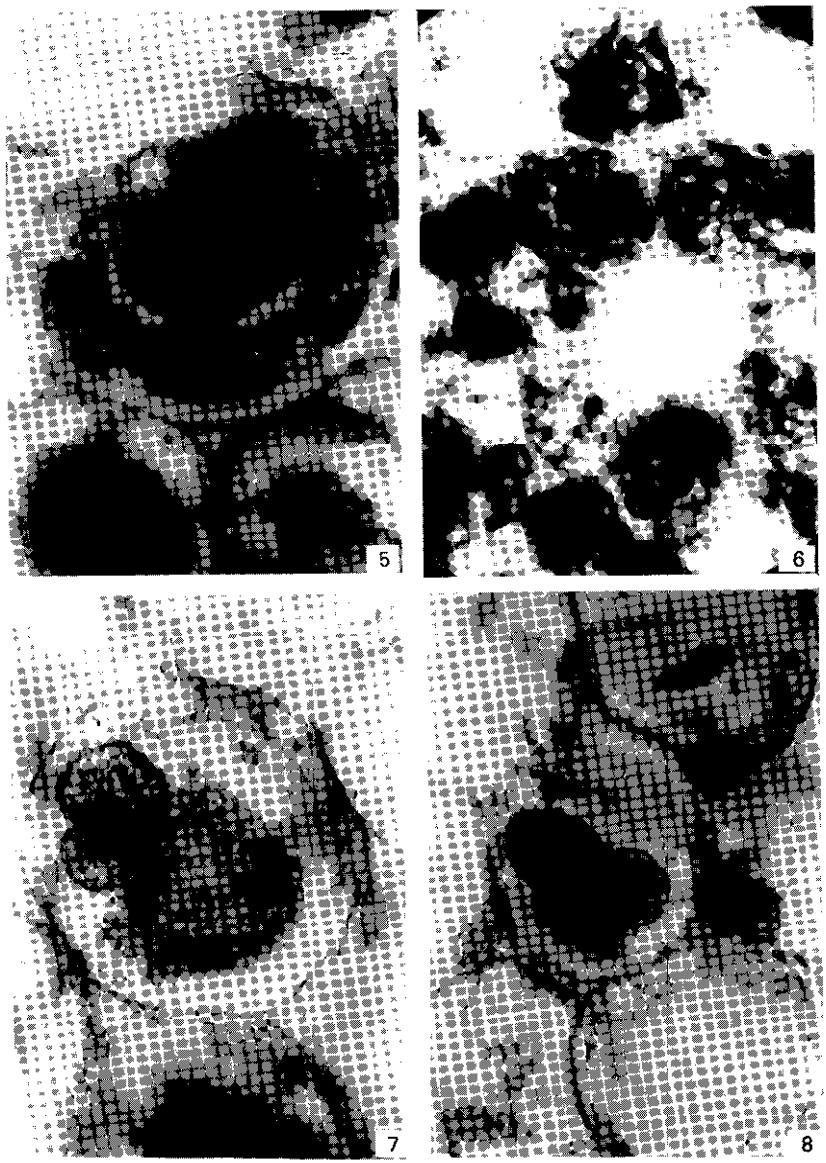
Fig. 3. Pelotons stained with Ruthenium red showing young pelotons are mildly positive for non-esterified pectins where as older pelotons are increasingly positive for the same (× 150).

Fig. 4. Note the distinction of hyphae into pelotonic and non-pelotonic categories, also note the cross connections between these two types of hyphae (× 650).

peloton has not been described till now. A careful examination of infected roots revealed the presence of 2 types of fungal hyphae in the infected host cells, one forming pelotons (= pelotonic hyphae) and the other not participating in peloton organisation (= non-pelotonic hyphae). The latter often remained close to the cell wall of the host cell (Fig. 4), although they might be connected to the former by short hyphal bridges. In certain host cells, some of the peripherally located hyphal filaments of the pelotons get seggregated from the rest of the peloton spatially and became non-pelotonic (Fig. 4). As far the present authors are aware of, such a distinction of hyphae has not been made previously.

The cytoplasm of fresh pelotonic hyphae was rich in RNA, basic proteins, total insoluble polysaccharides and acidic polysaccharides, in lipids (predominantly neutral lipids) and moderately rich in total proteins. The enzymes whose activity could be detected in the pelotonic hyphae were ATPase, malate dehydrogenase, starch phosphorylase, succinate dehydrogenase, glucose 6-phosphatase and β-glucosidase. Two nuclei, which were prominently stained for DNA and nucleophosphates, were distinctly seen in each of the hyphal cells. The walls contained chitin, acidic polysaccharides (including both esterified and non-esterified pectins), and cell wall proteins, but lack wall phenolics. Although the wall was stained with chlorazol black E and cellufluor (previously known as calcofluor), the cell wall did not contain cellulose since these two tests are not specific for cellulose, and the more specific IKI-H_2SO_4 tests was not positive. Therefore, we agree with Peterson and Currah (1990) that cellulose is not part of the wall of pelotonic hyphae (in the host-parasite interface) and that the claim of Barroso and Pais (1985) for the presence of cellulose is questionable. The chitin content gradually declined during the progressive digestion of the pelotons (Peterson and Currah 1990, Werner 1992). During lysis there was also a gradual loss of structural wall proteins. Non-esterified acidic polysaccharides increased during the initial periods of lysis but could not be detected in the very late stages of digestion. This is corroborated by the report of Peterson and Currah (1990) that during lysis the hyphae reacted very strongly with acriflavin-HCl, which indicated the presence of acidic polysaccharides (Krishnamurthy 1988). In the lysing hyphal cytoplasm there was an initial increase in acidic lipids and phospholipids (in contrast to neutral lipids of intact pelotonic hyphae), but these are soon released in to the host cell cytoplasm. Richardson *et al.* (1992) have observed in *Platanthera* that during initial stages of peloton digestion lipids and polyphosphates were observed in the hyphae but they were not sure these were drawn into the host cell cytoplasm. Barroso *et al.* (1986) observed in the infected cell cytoplasm of *Ophrys lutea* a number of steroidal substances which may also perhaps give a similar positivity to lipid stains that was noticed in our material. During lysis the activity of ATPase was considerable, but the activity of other enzymes reported earlier gradually decreased (Williamson 1973).

The non pelotonic hyphae, from the beginning remained intact and had phenolics in their walls. Phenols, probably, help in their staying intact when pelotonic hyphae get digested (Fig. 5). These hyphae initially remained poor in basic proteins, although they had a moderate amount of total proteins. When the digestion process was going on in the original peloton, a fresh peloton was already formed by the rapid growth



Figs. 5 and 6. Successive formation of pelotons in the same host cell stained with Toluidine blue O. (× 325; × 650).

Fig. 7. Cortical cell enclosing a peloton which is to undergo digestion - callose appears in patches (blue colour regions with lacmoid staining) to insulate these peloton from the rest of the host cell cytoplasm. Note the abnormally large highly lobed host nucleus (× 650).

Fig. 8. A peloton undergoing digestion seperated from the rest of the host cell cytoplasm by a fully formed sheet of lacmoid blue positive callose layer. Note also the large host cell nucleus (× 650).

and branching of the non-pelotonic hyphae. In some cells, it was so rapid that even before the primary and secondary pelotons were not fully digested by the host cell, the third peloton was organised (Fig. 6).

The pelotonic and non-pelotonic hyphae get sharply delimited from one another with the initiation of the digestion of the pelotons by a sheet which was formed in patches around the periphery of the pelotons. This sheet gave a very strong positive reaction to callose, a 1-3-β-glucan through all the cytochemical methods employed (Figs. 7 and 8). This sheet of material was first reported by Nieuwdorp (1972), who designated it as "cellulose slime layer", and subsequently by Peterson and Currah (1990). The later two authors reported that this layer was negative to cellufluor, positive to aniline blue fluorescence and electron-luscent in TEM, prompting them to suggest that it was callose. A similar callosic layer was reported in *Platanthera hyperborea* by Richardson *et al.* (1992). Our study also clearly points out that it is callose and that its purpose was to effectively isolate and insulate the lysing pelotons, as callose is already known to be an insulating agent, par excellence (see full literature in Krishnamurthy 1977).

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