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# Histochemical localization of polyphosphate (Poly P) granules and lipid bodies in intraradical mycelium of two Arbuscular Mycorrhizal (AM) fungi

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## ABSTRACT

Pure cultures of two AM fungal species *viz.*, *Gigaspora albida* Schenck & Smith and *Glomus clarum* Nicolson & Schenck were used to study the localization of polyP granules and lipid bodies respectively using histochemical stains. Accumulation of polyP granules was located in the intercellular hyphae in roots inoculated with *Gi. albida* which stained pinkish purple in Toluidine blue O at pH 1. In *G. clarum*, accumulation of lipid bodies was observed in the form of droplets of varying sizes in the intraradical hyphae and in spores, which stained bluish black in Sudan Black. Trypan blue staining was also carried out to check AM fungal root colonization. Besides hyphae, arbuscules and vesicles, intraradical spores also showed staining with trypan blue.

## INTRODUCTION

Plants and their Arbuscular mycorrhizal (AM) fungal symbionts have coexisted for more than 400 million years (Redecker *et al.*, 2000) and the association is widespread in terrestrial ecosystems. Arbuscular Mycorrhizal symbiosis provides multiple benefits for the plants not only in the form of enhanced P and nitrogen nutrition but also reduced pathogen and abiotic stresses (Smith *et al.*, 2003). Nutrition in arbuscular mycorrhizas is based on the acquisition of soil nutrients by the fungus (Jakobsen, 1999) and fixation of atmospheric carbon by the plant (Ho & Trappe 1973) and on the exchange of these nutrients at specially adapted symbiotic interfaces (Gianinazzi-Pearson *et al.*, 1991).

Much attention has been found in the mechanisms involving the two transport processes *viz.*, nutrient uptake and nutrient transfer (Ferrol *et al.*, 2002), but little is known about other two key classes of transport processes that take place within the AM fungi, which are intracellular *viz.*, the transport of nutrients into and out of the organelles where these get metabolized or stored, and the bidirectional translocation of nutrients along the coenocytic AM fungal hyphae.

Inorganic polyphosphate (polyP) is a linear polymer of phosphate linked by high-energy bonds and a wide range of microorganisms store P by accumulation of poly P (Kornberg *et al.* 1999). It has been suggested that AM fungi accumulate poly P for long distance translocation along hyphae (Callow *et al.* 1978; Solaiman *et al.* 1999). High affinity type Pi-transporter genes have been isolated from AM fungi, and the gene products are responsible for uptake of Pi from the soil solution and into the fungal cytosol (Maldonodo-Mendoza *et al.*, 2001).

Arbuscular mycorrhizal fungi are oleogenic fungi and store large amounts of lipids as triacylglycerides (TAGs) a main type of neutral lipid found in large amounts in AM fungal spores and vesicles (Beilby & Kidby, 1980). The synthesis of TAG is a substantial sink for carbon in the intraradical hyphae (Bago *et al.*, 2000). The major fluxes of carbon in the intraradical mycelium thus appear to be: efficient uptake of host derived hexose, conversion to trehalose and glycogen as interim storage forms and the synthesis of large amount of storage lipids. Bago *et al.* (2000) suggested that these lipid bodies also play key roles in fungal morphogenic and reproductive events such as sporulation.

In recent years, attention has been given in particular to the mechanism of P uptake and activity of selected enzyme in this symbiotic interaction. Callow *et al.* (1978) suggested that AM fungi synthesized polyphosphate vacuolar granules from soil phosphate and that these granules are broken down in the arbuscules to inorganic phosphate for release to the host. Lipid staining of AM fungus colonized roots has demonstrated abundant lipids in the vesicles and intercellular and external hyphae. Synthesis of lipids by the fungal endophyte has been suggested as an alternate storage sink for the plant photosynthates (Cox *et al.* 1975).

The present study reports histochemical staining of polyphosphate granules and lipid bodies in the intraradical mycelium of *Gigaspora albida* and *Glomus clarum.*

## MATERIALS AND METHODS

Spores from two AM fungal species *viz.*, *Gigaspora albida* Schenck & Smith and *Glomus clarum* Nicolson & Schenck isolated (Gerdemann &

Nicolson, 1963) from pure cultures using *Coleus* sp. as a host were used for the study. Intact spores mounted in PVLG (Poly-vinyl Lacto Glycerol) (Koske & Tessier, 1983) with or without Melzers reagent were examined under Leica compound microscope and identified based on spore morphology and subcellular characters and were compared with original descriptions (Redecker *et al.*, 2000; Schenck & Perez, 1990; Almeida & Schenck, 1990; Morton & Redecker, 2000). Spore morphology was also compared with the culture data established by International Collection of Vesicular Arbuscular Fungi (INVAM) (http://invam.cag,wvu.edu).

The inoculum (spores) was mixed with autoclaved soil: sand in a ratio of 1:3 and filled in 15 cm diameter pots. Pots were planted with *Coleus* cuttings, maintained in the glasshouse at 28°C and were watered regularly with sterile distilled water. Hoagland solution (Hoagland & Arnon, 1938) (minus P) was added after every 15 days. From $40^{th}$ to $45^{th}$ day, the plants were stopped watering to induce stress. On the $46^{th}$ day, the roots of *Coleus* sp. were checked for colonization using 0.5% trypan blue (Koske & Gemma, 1989).

*Coleus* root (46 days old) bits were stained with Toluidine blue O (TBO) (Kumble & Kornberg, 1996), and Sudan Black (McGee-Russell & Smale, 1963) to check for polyphosphate granules and lipid bodies respectively.

Preparation of Toluidine blue O: 1 g of Toluidine blue in D.W and 1N HCl was added to adjust the pH to 1. For Polyphosphate staining, roots were gently washed in tap water and cut in 1cm root bits, cleared in 2% KOH at 90°C for 30-45min, thoroughly rinsed in water, acidified with 5N HCl (Koske & Gemma, 1989) and were stained with Toluidine blue for 20 minutes and were then rinsed with distilled water.

*Preparation of Sudan Black:* For staining of lipid droplets 100 mg of Sudan black in 70% saturated solution of 10ml of alcohol was prepared and then filtered before staining (Baker, 1960). Root bits were stained in Sudan black for 1-5 minutes after 1N HCL treatment (Koske & Gemma, 1989). Photomicrographs of the stained root bits under different magnifications were taken using Olympus DP12 -Olympus BX41 compound microscope. Staining reactions were examined in the hyphae, arbuscules and vesicles of AM fungi.

## RESULTS

Mycorrhizal colonizations were observed in all the root bits of *Coleus* sp. stained with trypan blue inoculated separately with *Gi. albida* and *G. clarum*. Arbuscules, vesicles, hyphal structures and extraradical spores were stained dark blue in the roots **(Plate 1a-c).** In *Coleus* sp., *Gi. albida* arbuscules develop by repeated dichotomous branching and grow until they fill the cortical cell. At some point after maturity, the arbuscules collapse degenerate and die. Lipid droplets of variable size were found in vesicles, spores and intercellular hyphae of *G. clarum*, which also stained blue with trypan blue.

Localization of polyP granules was observed in the roots of *Coleus* sp. inoculated with *Gi. albida* stained with TBO at pH 1, which showed the accumulation of polyphosphate granules of variable size in the intercellular hyphae, stained pinkish purple **(Plate I d)**. PolyP granules stained strongly whereas the arbuscules showed weak staining, consistent with the hydrolysis and export of Pi to the plant. It was also observed that the P granules that occurred in the young arbuscules had disappeared during the degeneration of arbuscules.

In *G. clarum*, vesicles developing into intraradical spores in the root cortex were observed which stained bluish black. Vesicles formed were oval to elliptical when formed intercellularly whose walls thicken to produce spores were also observed in the roots colonized by *G. clarum* (Plate I e-h). It was also observed that the lipid bodies accumulated in the spores through intercellular hyphae. In these spores, lipid bodies were present in the form of droplets of varying sizes that stained bluish black. Lipid droplets were not observed in arbuscules. Some vesicles appeared to be completely full of lipids. The movement of such lipid bodies appeared to be carried out possibly by cytoplasmic streaming.

## DISCUSSION

The results of the present study confirm the presence of arbuscules and vesicles, and arbuscules in roots of *Coleus* sp. inoculated with *Gi. albida and G. clarum* respectively, stained in trypan blue. Details of arbuscular structures are easier to observe in cells containing mature arbuscules. It was possible to visualize the network of fine hyphae that comprise the arbuscule whereas matured arbuscules are harder to observe because the host cell was often occluded by arbuscular branches. The mechanisms underlying arbuscule turnover are unknown but do not involve plant cell death. Each invaded plant cell remains alive throughout the life of the arbuscule and can host successive arbuscules (Bonfante-fasolo, 1984; Gianinazzi-Pearson & Gianinazzi, 1988). Studies on arbuscule lifespan are limited, but where examined,

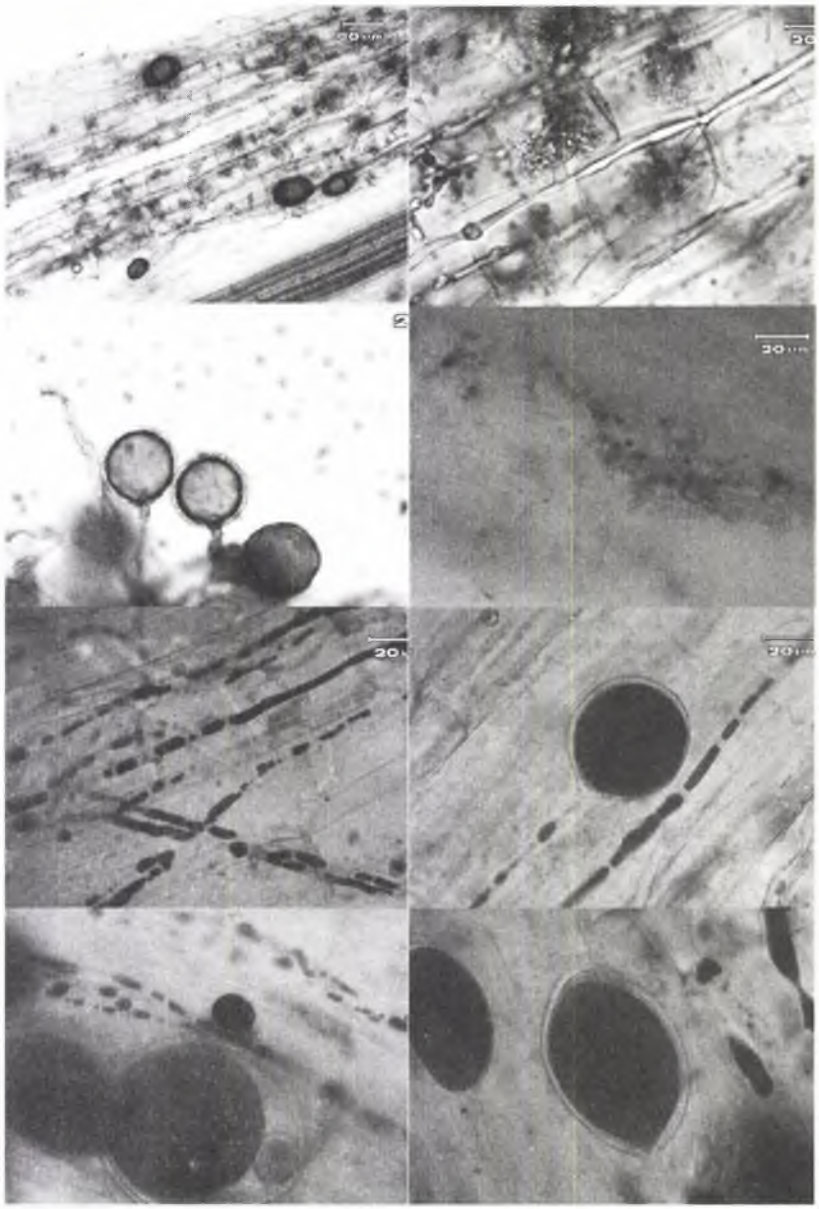

PLATE I: a. Vesicular and arbuscular colonization in *Glomus clarum*. b. Arbuscular colonization in *Gigaspora albida*. c. Extraradical spores of *Glomus clarum* stained in trypan blue (100x). d. Polyphosphate granules stained pinkish purple with TBO in *Gigaspora albida*. e. Lipid bodies in intraradical mycelium of *Glomus clarum*. f - h. Accumulation of lipids during spore formation in *Glomus clarum*. (Arrow showing wall layers of the spores).

the life span is suggested to be ~8.5 days (Alexander *et al.*, 1989).

The occurrence of polyphosphate granules in the intercellular hyphae of *Gi. albida* suggests that they are of fundamental importance in mycorrhizal P nutrition. Histochemical observation of polyphosphate granules in the intercellular hyphae stained with TBO supports the histological observations that arbuscular mycorrhizal hyphae contain small granules, which react metachromatically with Toluidine Blue (Cox *et al.*, 1975). PolyP granules in the intraradical mycelium stained with TBO have been observed also in roots of *Medicago truncatula* inoculated with *Glomus versiforme* (Javot *et al.*, 2007). Cox *et al.* (1975) reported the cytochemical identification, by light and electron microscopy of polyphosphate granules in individual small vacuoles within inter- and intracellular hyphae of *Glomus mosseae* Gerd. & Trappe (Nicol. & Gerd) forming vesicular arbuscular mycorrhizae with onion roots. The degeneration of phosphate granules takes place when the host cytoplasm show metabolic activity and enzymes are probably responsible for the disappearance of granules. It appears that the granules have an essential role of

transitory storage of P in the fungus vacuole before its transfer to the host plant (Strullu *et al.*, 1981). Cox *et al.* (1980) reported that polyphosphate granules occupy a total volume fraction of 0.008 of the fungal structures within the root.

Bowen *et al.* (1975) have shown that unlike ectomycorrhizas, arbuscular mycorrhizas store relatively little of the absorbed phosphate, but there is a rapid transfer to the host plant. Nutrients taken up by the fungus are then transferred to the host at high P fluxes, which has been calculated experimentally (Pearson & Tinker, 1975). Such high rates of translocation require mechanisms based upon bulk flow and cytoplasmic streaming (Tinker, 1975) and it has been suggested that P may be translocated in a condensed form as polyphosphate granules by cyclosis (Cox *et al.*, 1975).

According to Uetake *et al.* (2002) Pi as polyphosphate (poly P) accumulated in the vacuolar compartment of extraradical hyphae is translocated into the intraradical hyphae possibly *via* cytoplasmic streaming or along a motile tubular vacuolar system. Poly P then is hydrolysed in the arbuscule where it is broken down by polyphosphate kinase type or polyphosphatase (Harold, 1966) and Pi is then exported to the periarbuscular space (Kohjima & Saito, 2004) from where it is available to the plant, which is mediated by plant transporters (Javot *et al.*, 2007).

The presence of lipid droplets in vesicles, spores and intercellular hyphae of *G. clarum*, which stained bluish black in the root cortex, confirms the earlier observations of Mosse & Bowen (1968), Ho & Trappe (1973) that oil droplets are frequently observed in the hyphae and spores of AM fungi. The present study also observed the formation of spores from vesicles by accumulation of lipid droplets from the intercellular hyphae in the root cortex. Similar observations were made earlier (Bago *et al.*, 2002). Some intracellular vesicles appeared to be almost completely filled by large lipid droplets. Accumulation of lipids by the AM fungi in colonized roots is a significant carbon cost to the plant (Peng *et al.*, 1993). Formation of spores developing from vesicles in *G. clarum* in the present study is supported by Dodd *et al.* (2000), who reported the formation of intraradical spores from vesicles in *G. intraradices* and *G. clarum*.

Electron microscopic examination of mycorrhizal roots for lipids revealed lipid droplets in vesicles of AM fungi and in their intercellular and external hyphae (Cox & Sanders, 1974). Cooper & Losel (1978) were the first to quantify lipids in mycorrhizal roots of onion, clover and ryegrass. They found that mycorrhizal roots contained significantly more total lipids, triglycerides and phospholipids than did non-infected roots. It has been pointed out that AM fungal hyphae and vesicles are rich in osmiophilic and lipid material (Cox *et al.*, 1975; Harley, 1975), mainly neutral lipids as triglycerides and fatty acids (Gaspar *et al.*, 1994; Jabaji-Hare, 1988) in the form of vesicles and spores (Bago *et al.*, 2002) as stored energy. Jabaji-hare *et al.* (1984) reported that the high amount of polyunsaturated fatty acid of neutral lipids in the vesicles of AM fungi helps to maintain in their viability in nature.

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