Chapter – 3
HISTOCHEMICAL STUDIES ON ARBUSCULAR MYCORRHIZA (AM) INOCULATED FOUR FLORICULTURAL PLANTS

INTRODUCTION

Histochemical technique is one of the useful tool in understanding the basic biology of arbuscular mycorrhizae. The obligate symbiotic characteristic nature of arbuscular mycorrhizal fungal anatomical, morphological and physiological processes could be examined with the help of histochemical techniques. Different histochemical staining procedures aids in the identification and localization of certain chemical substances such as proteins, polysaccharides, polyphosphates, lipids, DNA, RNA and enzymes, cytochrome oxidase, peroxidases etc. which are present in fungal components. Therefore, the ultimate aim of histochemistry helps in understanding mycorrhizae and their structural composition.

The visualization of fine structural components and chemical substances within the mycorrhizal system and host tissues considered being the most important. Many workers have detected varied chemical constituents in different components of mycorrhiza. Recent studies suggested that VAM infection might change the biochemical composition of the host plant. Presently no literature is available with histochemical work on floricultural plants colonized with AM fungi. Only a few studies have been undertaken other than floricultural plants by different workers. The present work deals with localization of carbohydrates, proteins, lipids and enzymes. This will enable to
understand the passive physiological and biochemical changes that occurred between host and mycorrhizal fungi.

**REVIEW OF LITERATURE**

Histochemical protocols have been integral components of investigation on VAM and their host plants and are important basic techniques, which give first hand information to understand the various metabolites and their reaction within the fungal components, even when these fungi are directly associated with host plants. Galloud (1905) for the first time explained how fungal components could be stained by using cotton blue. O'Brein and McNaughton (1928) stained fungal roots by using lactic acid and acid fuschin with picric acid. Daft and Nicolson (1966) used methylene blue and later on examined by tearing mycorrhizal roots. However, staining of mycorrhizal roots were gradually improved with additional new staining techniques proposed by different workers (Furlan et al., 1973; Mosse and Hepper, 1975; Davis and Menge, 1981). Philips and Hayman (1970) had given modified and improved staining procedure for rapid assessment of infection of root with vesicular mycorrhizae. Kessler and Blank, (1972) demonstrated the localization of phenolic substances and polyphosphates in Eucalyptus roots colonized by ecto and endo mycorrhiza. Many workers are of the opinion that the localization of different chemical substances formed during physiological process are mainly due to VAM symbiosis (Mosse, 1973; Cox and Sanders 1974; Kinden and Brown; 1975; Bonfante and Fosola and Scannerini, 1977; Dexheimer et al., 1979; Ling Lee et al., 1977; McDonald and Lewis, 1978; Gianinazzi et al.,
1979; Bonfante-Fasolo 1988; Ronald and Söderhall, 1985; Gianinazzi-Pearson et al., 1996; Daft and Okusanya, 1973). However, staining of mycorrhizal roots was gradually improved with the development of new staining techniques proposed by different workers (Furlan et al., 1973; Mosse and Hepper, 1975; Davis and Menge 1981). Nemec (1981) demonstrated histochemical characteristics on Lemon (Citrus) fibrous roots, which were infected with Glomus etunicatus. In a histological study, the root of Arnthogalum umbrellatum infected with Glomus fasciculatum hyphae and vesicles found to be accumulated with fat, polysaccharides and proteins (Banfonte-Fasolo and Scanserini, 1977). McDonald and Lewis (1978) reported the occurrence of some acid phosphatases and sodium dehydrogenase activity in onion roots colonized with Glomus mosseae. Gianinazzi et al., (1979) have also reported the acid phosphatase and alkaline Phosphatase activity in onion roots colonized with Glomus species. Lakshman 1996; Senthlikumar and Krishnamurthy, (1999) have studied the peroxidase and acid Phosphatase activities in ground orchid (Spathoglottis plicata) with special reference to mycorrhizae. Solaiman et al., (1999) detected polyphosphates in intraradical and extraradical hyphae of Gigaspora margarita. Bago et al., (2002) have investigated the translocation of fungal storage lipids in mycorrhiza colonized roots.
MATERIALS AND METHODS

Seeds of experimental plants were sown in earthen pots already filled with 3 kg of sterilized garden soil (3:1). When the seedlings were one week old they were inoculated with 50 g inoculum of *Glomus fasciculatum*. All the pots were arranged in triplicate sets and they were maintained in green house. After 30 days, VAM colonization was examined in all the experimental plants. Other half of the roots were washed thoroughly with distilled water and some selected root bits were fixed in Carnoy’s fluid (6 parts of ethyl alcohol + 3 parts of chloroform + 1 part of acetic acid). The fixed root bits were dehydrated through series of alcohol grades for two-hour interval between each grades. (50% alcohol, 70% alcohol, 80% alcohol, 90% alcohol, 100% alcohol, 3:1 (alcohol + butanol), 100% alcohol + butanol (1:1), 100% alcohol + butanol (1:3), Pure butanol-2 times). Then these root bits were subjected to infiltration, embedding for slide preparation.

**Infiltration, embedding and slide preparation.**

The dehydrated root pieces were transferred to small vials containing pure butanol, then pieces of paraffin were added until the solvent (butanol) become saturated under room temperature then kept under table lamp (45 w) for 24 hrs. For each sample 4-6 changes were given at 4 hrs interval. Then the vials were transferred to temperature-controlled oven kept at 60°C to eliminate last traces of butanol. Six to eight changes were given for all samples with an interval of 3 hrs were maintained between each paraffin change. Root bits were
embedded in paraffin kept in paper boat on hot plate. Then the paraffin blocks containing root bits were prepared and were fixed to the wooden blocks. The series of sections of 6μm thicknesses were taken using Leica rotary microtome. The paraffin ribbons (with serial sections) were cut into convenient size with blade and were arranged on slides flooded with gelatin adhesive. (Adhesive = 1 g of granular gelatin + 100ml of warm distilled water + pinch of potassium dichromate). These slides with ribbons were kept on hot plate so that the sections were stretched to their original size. All the slides were labelled properly and kept in dust free boxes for drying for about 7 days. Then after drying slides are subjected for staining process.

Before staining, the slides were deparafinized by immersing in xylene. And the slides were treated with different stain for localization of proteins, polysaccharides and RNA. The histochemical studies and staining of mycorrhiza infected roots adopted according to the methods proposed by (Johansen, 1940; Feder and O’Brein and 1968 Pearse, 1972).

Preparation of reagent

Amido black 10B (For proteins):

10 g of mercuric chloride, 100 mg of bromophenol blue powder was mixed in 100 ml of absolute alcohol, stirred well till all the mixture dissolved properly, and then was stored in freezer.

Staining procedure.

i. Hydrated sections were incubated for 15 minutes in 0.05% amido black 10B at room temperature.

ii. Sections were rinsed in 7% acetic acid for 1 minute, air dried, cleared in xylol and mounted with DPX.

Colour indication:

Proteins appear blue.

Results: Blue colouration indicates the presence of total proteins in mycorrhizal roots.

Control test: Deparaffinized sections were incubated for 1 hr at 37° C in 0.05 N phosphate buffer at pH 8.9 containing 1mg/ml of pure trypsin, washed in water and dehydrated in 95% alcohol and stained with mercuric bromophenol blue. Sites of proteins do not show blue colouration in non-mycorrhizal root bits.
Test for polysaccharides:

Total insoluble polysaccharides are localized by employing Periodic Acid Schiff’s (PAS) method. In this method periodic acid (HIO₄) is used to oxidise 1,2-glycol groups of polysaccharides into aldehyde groups. Aldehyde groups thus formed, react with leucobasic-fuschin of Schiff’s reagent to produce a visible magenta-red colour. Periodic Acid Schiff’s method is highly recommended for the localization of total insoluble polysaccharides because of following characteristics.

i. The reaction does not cause the breakage of polysaccharide chains.

ii. It is specific to polysaccharides.

iii. It offers least interference and gives no false localization.

iv. It results in intense and stable colour complex.

Preparation of reagent

Periodic acid 0.5%:

500 g of periodic acid powder was dissolved in 10 ml of distilled water.

Schiff’s reagent:

2 g of basic fuschin was dissolved in 200 ml of distilled water and boiled. The solution was shaked well for 5 minutes and allowed to cool, then filtered with Watman filter paper No. 44 when temperature was at 50°C, to which 20 ml of N-hydrochloric acid was added. Stored in dark over night, then 2 g of activated charcoal was added and shaken well for 5 min, filtered and stored in dark bottle at 4°C.
**Bleach:** 5 ml of 10% aqueous Sodium or Potassium metabisulphite was mixed with 5 ml of HCl and 90 ml of distilled water.

**Staining procedure**

i. Hydrated sections were incubated in 0.5% periodic acid for 15 minutes at room temperature.

ii. Sections were rinsed with water and incubated in Schiff’s reagent for 30 minutes.

iii. After incubation sections were rinsed with water and was bleached in 2% sodium bisulfite for a minute, air dried, cleared in xylol and mounted with DPX.

**Results:** The magenta red colouration indicates the presence of insoluble polysaccharides in infected mycorrhizal roots.

**Control test:** Hydrated sections were directly incubated in Schiff’s reagent without pre-treatment with periodic acid.

**Test for RNA: Toluidine blue method** (Chayen et al., 1973).

**Preparation of reagent (RNA)**

i. 0.50 mg toluidine blue is dissolved in 100 ml of 0.05 M Citrate phosphate buffer at pH 4.4:

**Stock solutions:**

A: 0.1M solution of Citric acid (19.21 gms in 1000 ml)
B: 0.2M solution of disodium phosphate (53.65g of Na$_2$HPO$_4$ 7H$_2$O or Na$_2$HPO$_4$, 12H$_2$O in 1000 ml).

C: Citrate phosphate buffer 27.8 ml of A+22.2 ml of B, diluted to a total of 100 ml with distilled water.

**Staining procedure:**

i. Deparaffinized and hydrated sections were immersed in 0.05% toluidine blue for 5 minutes.

ii. Sections were rinsed in distilled water, air dried, cleared in xylol and mounted with DPX.

**Colour indication:**

RNA stains purple and DNA blue green.

**Control test for RNA:**

*Ribonuclease extraction method (Pearse, 1960):*

Deparaffinized and hydrated sections were incubated for 1 hour at 37° C in 0.1 mg/ml solution of ribonuclease in distilled water. After washing in water, the sections were stained with azure B. Sites of RNA do not stain.
Test for lipids:

Preparation of reagent.

Sudan Black-B:

0.7 g of Sudan black-B powder was completely dissolved in 100 ml of Ethylene glycol, while dissolving the dye, ethylene glycol was heated to 90° C. Then solution was filtered, cooled and stored in freezer.

Glycerin jelly:

10 g of Gelatin powder was dissolved in 60 ml of distilled water with 70 ml of glycerol and Phenol crystals (250 mg).

Staining procedure:

VAM colonized fresh roots were selected and thoroughly washed with tap water and distilled water. Then the roots were subjected to free hand cut sections and maceration with KOH and distilled water. Then the roots were treated with ethylene glycol for five minutes, excess ethylene glycol was blotted out and treated with Sudan black-B for 10 minutes, slides were washed 3-4 times in distilled water and sections were mounted on cleaned slides with glycerin jelly.

Results: Black colour indicates presence of lipids.

Control test: Fresh free hand cut sections were treated with heat cold alcohol and kept in reaction mixture for 5 minutes.
**Test for polyphosphate:**

The fresh VAM infected roots were used to localize polyphosphates. Sections were passed into 0.1% Toluidine blue at pH 1-2 at room temperature and mounted on clean glass slides with glycerin jelly.

**Toluidene blue:**

0.05% Toluidene blue in citrate phosphate buffer of pH 4.4.

**Results:** Red coloured granular depositions indicate the presence of polyphosphates.

**Control test:** Sections were treated with warmed and cooled alcohol in the reaction mixture.

**Enzymatic studies**

**Test for Peroxidase:**

Sections were inoculated at room temperature in stock solution. The stock solution was freshly prepared, which consists of one part NH₄Cl, one part of EDTA 5% solution, 6 parts of saturated benzidine and one part of H₂O₂3%.

**Results:** Blue-brown coloration in the arbuscules indicates the presence of peroxidases.

**Control test:** The non-mycorrhizal fresh root bits have been treated in heat cold alcohol and immersed in reaction mixture arbuscules do not showed blue/brown colour.
RESULTS AND OBSERVATIONS

The fungal components and cells of the host tissues were generally well preserved in both fixation and embedding procedures adopted in present study. Recent upsurge of interest and research on mycorrhiza associated plants, exhibited various biochemical substances in their roots. It was noticed that translocation of materials takes place from host to fungus and vice versa. Specific observations such as localization of different chemical constituents in different components of VAM were made in the present study. The specific observations were made on different components of VAM and localization of different chemical constituents were recorded. Freshly colonized roots were used to localize lipids, polyphosphates and peroxidase (Plate IX).

However, detection of polysaccharides, nucleic acids and proteins was detected by paraffinized technique. In the present study, it was seen that the mature arbuscules stained very dark, but proteins were not detected in vesicles shown in (Plate IX; Fig. A). The inter/intracellular hyphae and mature arbuscules showed positive test for PAS indicating high amount of insoluble polysaccharide (Plate IX; Fig. C). The localization of RNA was seen only in the arbuscules (Plate IX; Fig. B). Accumulation of lipids in VAM localized roots imparts black colour, which had strong affinity for the general lipid stain called Sudan black-B. Accumulation of lipids in the form of very thick dark droplets was observed in hyphae and vesicles of mycorrhizal roots (Plate IX; Fig. D). Arbuscules shows the localization of peroxidase and Protein (Plate IX; Fig E and F) respectively and remains absent in vesicles.
DISCUSSION

The results clearly demonstrated the translocation of materials from host to fungus and vice-versa. Basic proteins appeared to be most common type detected in immature arbuscules, vesicles and hyphal cytoplasm; these results are in conformaty with that of Nemec, (1981). The periodic acid schiffs (PAS) reaction of polyphsaccharides in the arbuscular walls shows osmophilic and acidic properties and suggests that the wall is primarily made up of glycolipid in composition. The absence of starch grains in arbuscules and presence of starch grains in host cells lead to the conclusion that the carbohydrate materials has been taken by arbuscules, but not in the form of starch. Similar findings were also reported by earlier workers in *Eucalypts* tree (Ling Lee *et al.*, 1977; Weete, 1974). The present study clearly demonstrates that the mycorrhizal roots were considerably large and cells were densely stained with PAS than non-mycorrhizal roots, this indicates that they are rich in polysaccharides (Junifer and Roberts, 1966; Harris and Northcote, 1970; Scannerini and Bonafonte-fasalo, 1979; Lakshman 1996; Inchal 2002; Firoz, 2002). It has been pointed out that, VAM fungal hyphae and vesicles are rich in osmophillic substances (Bevenge *et al.*, 1975; Cox *et al.*, 1975; Harley, 1975; Kinden and Brown, 1975; Holley and Peterson, 1979). The accumulation of lipids in hyphae, vesicles and arbuscules has been observed in apple and Ericaceae members (Mosse, 1957). The present investigation supports the work of earlier workers that the vesicles and hyphae contain rich lipids probably of
triglyceride. The vesicles acts as storage sink of lipids for the host plant (Cox et al., 1975, Brannan and Losel, 1978). More accumulation of lipid in vesicles resulted in the transformation into triglycerides, monoglycerides, fats, sterols, etc. The potential role of polyphosphate uptake in hyphae is well documented by Callow et al., (1978). The peroxidase activity was detected only in senescing arbuscules of all the experimental plants. It seems that mycorrhizal fungi colonize the host tissue by combination of mechanical interaction and enzymatic mechanism (Gianinazzi-Pearson, et al., 1996). Thus, VAM fungi can play an important role in developing the pathogen resistance host plant. The peroxidase activity was detected only in senescing arbuscules in the present study and similar to the reports of Nemec, (1981); Lakshman, (1996). Thus the study revealed that different chemical substances were accumulated and transformed to the host tissue. The arbuscules tend to be the barriers for exchange of nutrients between the fungus and the host.
Plate-IX

A. Presence of insoluble polysaccharides in arbuscules (Ar) of *Chrysanthemum morifollum* (X500).

B. *Chrysanthemum morifollum* showing arbuscules rich in RNA (Ar) (X450).

C. *Callistephus chinesis* roots showing presence of insoluble polysaccharides in hyphae (HY) (X450).

D. *Callistephus chinesis* vesicles showing large amount of lipids (V) (X450).

E. Peroxidase (Po) in arbuscules (Ar) of infected roots *Solidago virgaurea* (450).

F. Arbuscles (Ar) of *Bellis perennis* showing rich in protein (X450).