Research in Biotechnology, 1: 06-14, 2010 **ISSN: 2229-791X**
www.researchinbiotechnology.com

Regular Article

Biochemical changes in cotton plants by Arbuscular Mycorrhizal colonization

Damodaran, P. N.[1, 2*], Udaiyan, K.[2] and Jee H. J.[1]
[1]Division of Plant Nutrition, NIAST, RDA, Suwon 441-707 Korea
[2]Department of Botany, Bharathiar University, Tamil Nadu 641 046, India

Biochemical changes in ten cultivars of cotton (*Gossypium hirsutum* L. Merr.) in relation with arbuscular mycorrhizal (AM) colonization was studied. Proteins, sugars, amino acids and phenols in both leaves and roots were quantified and compared with the mycorrhization levels of the cultivars. Cultivars showed varying levels of mycorrization which correlated with the biochemical levels of the plants. It is concluded that cultivars which are more mycorrhizal do show increased levels of phenols and proteins which in turn help them to resist pathogenesis.

Keywords: Amino acids, Arbuscular mycorrhizal fungi (AMF), *Gossypium hirsutum*, Sugars, Phenols.

Soil microorganisms are paramount in the biogeochemical cycling of both inorganic and organic nutrients in the soil and in the maintenance of soil quality. In particular, microbial activity in the rhizosphere is a major factor that determines the availability of nutrients to plants and has a significant influence on plant health and productivity.

It has been well discussed that arbuscular mycorrhizal (AM) association as agents in biological control will be acting by more than one mechanism. The activation of specific plant defence mechanisms as response to AM colonization is an obvious basis for the protective capacity of AMF. The elicitation by an AM symbiosis of specific plant defence reactions could predispose the plant to an early response to attack by a root pathogen (Gianinazzi-Pearson *et al*., 1994). During their life cycle, plants evolve a number of defence responses elicited by various signals, including those associated with pathogen attack.

Among the compounds involved in plant defence studied in relationship to AM formation are phytoalexins, enzymes of the phenylpropanoid pathway, β-1,3-glucanases, chitinases, peroxidases, pathogenesis-related (PR) proteins, callose, hydroxyl-proline-rich glycoproteins (HRGP) and phenolics (Bowles, 1990; Gianinazzi-Pearson *et al*., 1994, Hare Krishna *et al*., 2005). Proteins associated with mycorrhizal associations have been well studied and showed that mycorrhization increases protein production in the roots (Benabdellah *et al*., 1998; Dassi *et al*., 1999). Symbiotic association between AMF and plants is based on the exchange of carbohydrates and other nutrients between both the partners. Plants roots become a strong sink for sugars during mycorrhization which in turn increases the photosynthethic ability of the phototroph to compensate this usage of sugars (Wright *et al*., 1998). There are many works showing that amino acid concentration is more in mycorrhizal than non mycorrhizal plants.

*Corresponding author : pndamodaran@gmail.com

Baltruschat and Schonbeck (1975) showed increase in both arginine and citrulline in mycorrhizal plants over non-mycorrhizal ones, which inhibits the propagation of *Thielaviopsis basicola*. There are many other reports mentioning the change in amino acid concentration due to AMF colonization (Abdel-Fattah and Mohamedin, 2000; Sood, 2003). Phenols are important components functioning as defence mechanisms against pathogen attack. Phenols occur naturally in plants and they do have antimicrobial properties which prevent fungal spore germination and toxin production (Vidhyasekaran, 1973). New polypeptides are synthesized during AM colonization (Garcia-Garrido *et al*., 1993; Dumas-Gaudot *et al*., 1994). Considering these facts, we have conducted experiment to study the biochemical changes associated with mycorrhization in ten cotton cultivars.

Materials and Methods

Raising of experimental seedlings

Acid-delinted seeds of seven varieties LRA 5166, LRK 516, PAIYUR-1, MCU-7, SUVIN, MCU 5 VT, SUPRIYA and three hybrids, NHH 44, SAVITHA, SURYA of cotton were used for the study. The seeds were procured from Central Institute for Cotton Research (CICR) Regional Station, Coimbatore, Tamil Nadu.

Six cotton seeds were sown in each polybag (30 x 11 cm) containing *ca*. 1.5 kg black soil having natural microflora. The soil had an initial pH of 7.8 and an electric conductivity of 47.35 mS cm^{-1}, having 0.135 mg kg^{-1} of total Nitrogen, 0.017 mg kg^{-1} of available Phosphorus and 0.10 mg kg^{-1} of exchangeable Potassium and 4.01 % Organic carbon. Immediately after emergence, the seedlings were thinned to one seedling per polybag.

Non-mycorrhizal treatments were established for comparative study. For this, the soil was autoclaved at 120 psi for 1 h for three subsequent days and incubated for 10 days. In order to reintroduce natural microflora other than AMF, a 500 ml of soil filtrate prepared by suspending 1.5 kg soil in 5 L of sterile water and passing it through 38 µm sieve was added to polybags containing sterilized soil.

The polybags were arranged in a completely randomized block design. Each treatment was replicated five times. Plants were watered as and when necessary, throughout the duration of the experiment. The positions of the polybags were altered once in every 15 days to expose seedlings to uniform conditions.

Sampling

Rhizosphere soil samples and root samples were collected every 30 days upto 120 days after emergence (DAE) of seedlings. Rhizosphere soil was collected by removing the loose soil attached to the roots. Root samples were fixed in FAA and analyzed later.

Preparation of roots for AM assessment

Roots fixed in FAA were washed thoroughly to remove FAA and observed under dissection microscope (X 20) to examine AM fungal spores attached to them. After examination, the roots were cut into 1 cm bits, cleared in 2.5% KOH (Koske and Gemma, 1989), acidified with 5 N HCl and stained with trypan blue (0.05% in lactoglycerol) by keeping overnight immersed in the stain. One hundred root bits were used for examination. The stained root bits were examined with a compound microscope (X 200 – 400) for AM fungal structures developed inside and the percentage of root length colonization was estimated according to magnified intersection method of McGonigle *et al*. (1990).

Isolation and identification of AMF spores

One hundred gram soil was dispersed in 1L water and the suspension was decanted through a series of 710- to 38-µm sieves. The residues in the sieves were

washed into beakers. The sievates were dispersed in water and filtered through gridded filter papers. Intact spores were transferred using a wet needle to polyvinyl alcohol-lactoglycerol with or without Melzer's reagent on a glass slide for identification. Spores were identified based on their morphology and sub-cellular characters and compared with original descriptions of Schenck and Perez (1987). Spore morphology was also compared with the INVAM culture database (http://invam.caf.wvu.edu).

Biochemical studies

Proteins

Proteins were extracted from 1 gram of the plant tissue in 0.02 M phosphate buffer (pH 7.0) and quantified using folin-phenol reagent (Lowry *et al*., 1951) after mixing with alkaline copper solution (containing 2% sodium carbonate in 1 ml of 0.1 N NaOH).

Sugars

Reducing sugar was estimated by extracting 1 gram of the plant material using alcohol and heating it with 1 ml alkaline copper mixture. Using arsenomolybdate reagent, the intensity of the extract was measured at 500 nm using UV-VIS spectrophotometer (Spectronic D20) (Nelson, 1944).

Total soluble sugar was analyzed by heating 1 ml alcohol extract with 0.2% anthrone reagent and reading the intensity at 625 nm using UV-VIS spectrophotometer (Spectronic D20) (Mahadevan and Sridhar, 1986).

Amino acids

Amino acid was estimated by using ninhydrin reagent. One ml of the alcohol extract was added with 1 ml ninhydrin, heated and the intensity of colour developed was read at 625 nm in a UV-VIS spectrophotometer (Spectronic D20) using glycine standard graph (Mahadevan and Sridhar, 1986).

Phenols

Total phenol was estimated according to Bray and Thorpe (1954). One ml of the tissue alcohol extract was mixed with 1 ml Folin-ciocalteu reagent followed by 2 ml sodium carbonate, heated and colour intensity was read at 650 nm using UV-VIS spectrophotometer (Spectronic D20). Phenols were estimated from a standard graph using catechol standard.

Statistical analysis

All data were subjected to Analysis of Variance (ANOVA) and the means were separated using Duncan's Multiple Range Test (DMRT). Pearson's correlation analysis was used to assess the relationships between various parameters (Zar, 1984) using SPSS software (SPSS 9.05 for Windows).

Results

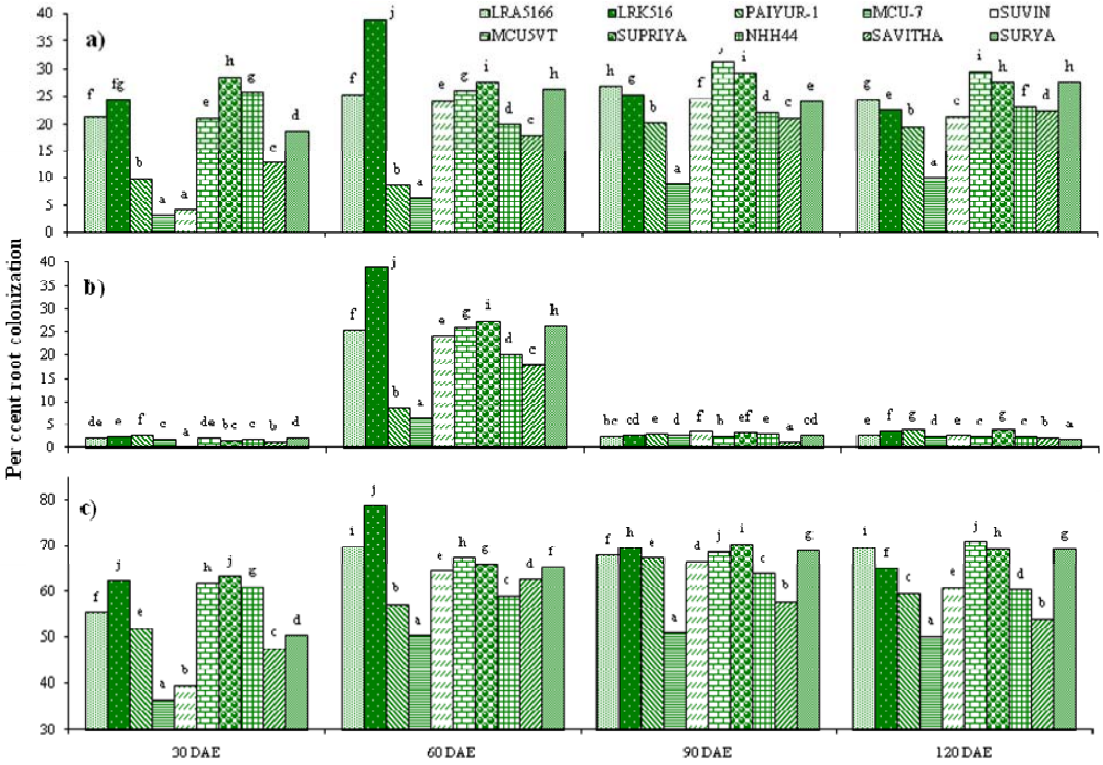
Mycorrhizal abundance

The AM fungal species isolated from the rhizosphere of the 10 cultivars were *Glomus geosporum* (Nicol. & Gerd.) Walker, *G. fasciculatum* **(**Thaxter**)** Gerd. & Trappe emend. Walker & Koske, *G. taiwanensis* (Wu & Chen) Almeida & Schenck, *G. macrocarpum* Tul & Tul., *G. intraradices* Schenck & Smith, *Gigaspora margarita* Becker & Hall, *Scutellospora gregaria* (Schenck & Nicol.) Walker & Sanders and *Acaulospora scrobiculata* Trappe. Spores of all these species were present in the rhizosphere soil of all the cotton cultivars.

Mycorrhizal colonization

Mycorrhizal colonization was assessed in terms of per cent root length with hyphae (%RLH), vesicles (%RLV), arbuscules (%RLA) produced inside the roots and total root colonization (%RLC) which is the cumulative of the three (%RLH + %RLV + %RLA). Results are given in Figure 1.

Fig. 1. Per cent root length with vesicles (a), arbuscules (b) and total root colonization (c) in different cotton cultivars at 30 to 120 days after emergence (DAE).



Bars bearing same latter(s) do not differ significantly according to DMRT (P<0.05).

Biochemical changes

Reducing sugars

Reducing sugar contents of leaf and root showed significant variation among the cultivars. Maximum reducing sugar content in leaves was recorded in MCU-7 followed by Savitha. Minimum reducing sugar content in leaves was exhibited by MCU 5 VT followed by Supriya. MCU 7 had the maximum root reducing sugar content followed by Savitha and minimum in MCU 5 VT and LRA 5166 (Table 1).

Total sugars

Total sugar contents of leaf and root also varied significantly among the cultivars. Total sugar content in leaves was maximum in MCU-7 followed by Savitha. Minimum total sugar content in leaves was exhibited by MCU 5 VT followed by LRA 5166. Total sugar content in roots was maximum in MCU 7 followed by Savitha. Minimum root sugar content was exhibited by MCU 5 VT and Supriya (Table 1).

Protein

Leaf protein was maximum in Supriya followed by Surya and minimum in Paiyur-1 followed by NHH 44. Root protein was maximum in Supriya followed by LRK 516 and minimum in Paiyur-1 followed by NHH 44 (Table 1).

Amino acids

Amino acids of both leaves and roots showed significant variation among the cultivars. Leaf amino acid content was maximum in MCU 5 VT followed by LRA 5166. Minimum leaf amino acid content was exhibited by Savitha and MCU -7 (Table 1). The amino acid content in root was maximum in LRA 5166 followed by Supriya.

Minimum amino acid content in root was found in MCU-7 followed by Savitha (Table 1).

Phenols

Total phenol content in the leaves were maximum in LRK 5166 and MCU-5V T followed by Supriya. Minimum phenolic content in leaves was recorded in NHH 44 followed by Paiyur-1. Root phenol content was maximum in Suvin followed by Paiyur -1. Minimum phenol content in roots was found in Savitha followed by NHH 44 (Table 1).

Discussion

The present study indicates significant variation in the extent of AM association among different cultivars of cotton. Even though AM fungi are considered to be less host-specific, the results show some critical differences in the association. The cotton cultivars exhibited differential mycorrhizal colonization ranging from 36.52 % to 73.05 % over the four harvests of 30 through 120 DAE under the same environmental conditions. Similar results were reported for cowpea (Mercy *et al*., 1990) and pearl millet (Krishna *et al*., 1985). Studies on maize (Toth *et al*., 1990), bean (Sutton, 1973) and wheat (Bertheau *et al*., 1980) inbreds showed that the differences in host genome can control the degree of mycorrhizal colonization. The two cotton cultivars, MCU-9 and MCU-7 varied in their mycorrhizal colonization levels (62.06 % and 67.6 % respectively) when inoculated with *Glomus geosporum* (Jaganathan, 1996).

Table 1. Biochemical concentrations in mycorrhizal (M) and non-mycorrhizal (NM) cotton cultivars at 120 days after emergence (DAE)

Cultivar	Reducing sugar (mg g^{-1})				Total sugars (mg g^{-1})				Protein (mg g^{-1})			
	Leaf		Root		Leaf		Root		Leaf		Root	
	M	NM	M	NM	M	NM	M	NM	M	NM	M	NM
LRA5166	2.75b	1.78 ab	1.83 a	1.43 bc	6.9 b	4.8 bcd	1.77 b	0.95 a	5.9 d	4.8 efg	6.1 e	4.7 c
LRK516	2.9 b	1.69 a	2.12 c	1.57 cde	7.2 bc	4.9 cd	1.85 b	1.21 c	5.6 c	4.5 d	5.95 de	4.37 b
PAIYUR-1	3.4 c	2.1 cd	2.34 d	1.68 de	7.9 e	5.1 de	2.2 c	1.36 ef	4.98 a	4.95 g	5.2 a	4.9 d
MCU-7	3.78 e	1.85 ab	2.89 f	1.49 cd	8.5 g	5.3 e	2.78 e	1.11 b	5.1 a	4.76 ef	5.35 ab	5.05 d
SUVIN	3.5 cd	1.95 bc	2.18 c	1.19 a	7.6 d	4.45 b	2.17 c	1.34 de	5.37 b	3.76 a	5.46 b	4.95 d
MCU5VT	2.12 a	2.1 cd	1.76 a	1.6 cde	5.4 a	1.66 a	1.58 a	1.13 b	5.63 c	4.31 c	5.71 c	4.65 c
SUPRIYA	2.2 a	1.8 ab	1.95 b	1.55 cde	7.1 bc	4.91 cd	1.76 b	1.39 f	6.1 d	4.09 b	6.33 f	4.38 b
NHH44	3.67 de	1.98 bc	2.3 d	1.64 de	7.4 cd	5.38 e	2.3 c	1.32 d	5.35 b	4.78 ef	5.38 ab	4.09 a
SAVITHA	3.7 de	2.2 d	2.54 e	1.3 ab	8.2 f	4.6 bc	2.6 d	1.56 g	5.1 a	4.69 e	5.9 d	4.91 d
SURYA	2.95 b	1.81 ab	1.98 b	1.7 e	7.05d	4.38 bc	1.77 b	1.59 g	5.95 d	4.87 fg	5.85 cd	4.35 b

Table 1. cont.

Cultivar	Amino acids (mg g^{-1})				Phenols (mg g^{-1})			
	Leaf		Root		Leaf		Root	
	M	NM	M	NM	M	NM	M	NM
LRA5166	128.7 h	106.4 e	57.2 h	38.5 f	195 bc	178 c	68.5 ef	52 ab
LRK516	124.6 f	99.5 a	54.6 f	39.4 g	201 e	169 a	68.8 f	57 b
PAIYUR-1	120.5 cd	100.5 b	49.8 d	40.1 h	193 b	181 cd	69 f	56.4 b
MCU-7	118.5 b	108.6 f	40.5 a	36.6 e	198 cde	183 de	63.8 c	53 ab
SUVIN	121.4 de	103.4 c	48.6 c	35.4 d	217 f	186 ef	71 g	55.9 b
MCU5VT	136.5 i	110.8 g	59.5 i	37.9 f	201 e	183 d	64.6 c	52 a
SUPRIYA	127.5 g	112.6 i	56.4 g	38.2 f	199 de	187 f	67.2 de	56 b
NHH44	119.8 c	113.5 j	52.5 e	33.6 b	189 a	184 d-f	59.4 b	53 ab
SAVITHA	117 a	104.6 d	46.8 b	31.9 a	196 bcd	182 d	57.2 a	49.8 ab
SURYA	121.9 e	111.9 h	49.5 d	34.8 c	199 de	173 b	66.3 d	52.6 ab

Means followed by a common letter(s) are not significantly different at 5% level according to DMRT.

Table 2 . Correlation between mycorrhizal colonization, plant biomass, tissue nutrients and biochemical changes

	% RLA	% RLV	% RLC	Leaf amino acids	Root amino acids	Leaf proteins	Root proteins	Leaf phenols	Root phenols	Leaf Sugars	Root Sugars
% RLA											
% RLV	-	-									
% RLC	-	0.871**									
Leaf amino acids	-	-	0.840**								
Root amino acids	-	-	0.885**	0.844*							
Leaf proteins	-	0.710*	0.839**	-	0.654*						
Root proteins	-	-	0.797**		-	-					
Leaf phenol	-	-	-	-	-	-	-				
Root phenol	0.686*	-	-	-	-	-	-	-			
Leaf Sugars	-0.842*	-	-	-	-	-	-	-	-		
Root Sugars	-0.840*	-	-0.820**	-	-	-	-	-	-	-	

* Correlation is significant at the 0.05 level (2-tailed);

** Correlation is significant at the 0.01 level (2-tailed);

- Not significant.

AM fungal penetration and establishment in the host roots involves a complex sequence of events and intracellular modifications (Bonfante-Fasolo and Perotto, 1992). The compatibility between plant roots and AM fungi implies a clear and selective recognition by the plant host that recognizes the beneficial feature of AM fungi. AM colonization always serves as a strong sink for sugars, both reducing and total sugars (Muthukumar and Udaiyan, 2000). As a result there is possibility for a decrease in sugar content in the host with increased mycorrhizal colonization levels. In the present study, reducing sugar and total sugar contents of both leaves and roots were significantly and negatively correlated with mycorrhizal colonization and arbuscule formation which could be due to the utilization of sugars by mycorrhizal fungi present in the roots.

A qualitative and quantitative difference in the expression of proteins has been found in AM roots (Arines *et al.,* 1994). Mycorrhizal formation is known to increase the expression of low molecular weight proteins as reported in tobacco (Dumas *et al.,* 1990) and soybean (Arines *et al.,* 1994). In the present study both leaf and root protein contents were positively correlated with the extent of mycorrhizal colonization among the cultivars (Table 2). This is in accordance with the results of Abdel-Fattah and Mohamedin (2000) and Vazquez *et al.* (2000) where the total soluble protein content of *Sorghum bicolor* and *Medicago sativa* respectively was significantly higher in mycorrhizal than non-mycorrhizal plants, which could be due to the induction if *Ltp* (lipid transfer protein) gene expression (Blilou *et al.,* 2000).

In the present study, both leaf and root amino acid contents were higher in highly mycotrophic than in less mycotrophic cultivars which may be due to the enhanced production and/ or transport of amino acids. Many authors have already reported higher amino acid content (up to 3-7 times) and a greater predominance of arginine and citrulline in mycorrhizal roots compared to non-mycorrhizal roots (Baltruschat and Schonbeck, 1975; Dehne *et al.,* 1978).

Plant phenolics, particularly flavonoids and isoflavonoids are the most widespread classes of secondary metabolites known to be involved in the plant-microbe interactions (Morandi, 1996). In the present study, increased concentration of total phenol in roots and leaves were found in the cultivars with higher colonization levels than in cultivars with low colonization levels. A similar increased total soluble phenol content in response to mycorrhizal association has been reported for *G. mosseae* - tomato (Dehne and Schonbeck, 1979) and *G. fasciculatum* - *Arachis hypogaea* (Krishna and Bagyaraj, 1984). Glyceollin, a phenol, has been shown to accumulate in soybean roots when mycorrhizal colonization is mature and well-developed (Morandi *et al.,* 1984). An increased level of total phenols among cultivars with high mycorrhizal colonization levels could be due to reaction of the cultivars against mycorrhizal colonization.

The results showed that there exist some host-dependant variations in AMF colonization among the cultivars of cotton. This variations in turn attributes to the biochemical changes in the plants, like phenols and sugars which are important for the plant health.

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