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Occurrence and influence of arbuscular mycorrhizae on chemical and enzymatic parameters of soil grown with different crops

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Abstract
In the present system of agriculture, chemical fertilizers are used indiscriminately throughout the world. However, the cost of these fertilizers has been increasing, exponentially and at the same time polluting soil and ground water. In this scenario, the use of eco-friendly bio fertilizers, particularly arbuscular mycorrhizae (AM) is one of the promising alternatives to increase the productivity of agricultural crops. An attempt was made to study the chemical and enzymatic parameters of soil with the occurrence and prevalence of AM fungi in different agricultural crops of Andhra Pradesh. Root and rhizosphere soils of four major crops such as cotton, sugarcane, rice and black gram were collected at different growth stages of the crops and assayed for AM fungi colonization and spore density. Also, the soil chemical and enzymatic parameters such as organic carbon, alkaline phosphatase, glomalin were assayed from these soil samples. Our results showed that the four crop roots colonized by AM fungi. The percent root colonization by AM fungi was maximum in cotton (82.3%) followed by black gram (70%) and rice (35.6%). Whereas the spore number was highest in black gram (42/100g of soil) followed by cotton (39.2/100g of soil) and rice (26.6/100 g of soil). The chemical and enzymatic parameters measured such as organic carbon was maximum in rice (0.79%), followed by sugarcane and cotton (0.73%). Alkaline phosphatase was highest in sugarcane (32.73 p-nitro phenol μg/g wt. of soil/hour) followed by black gram (23.93 p-nitro phenol μg/g wt. of soil/hour) and glomalin was highest in sugarcane (7.96 mg/g) followed by black gram (5.49 mg/g). Our results showed that there was a positive correlation between chemical and enzymatic factors measured with the AM fungi prevalence in the roots and rhizosphere soils of the crops evaluated.

Keywords: AM fungi, abundance, host crop, root colonization, spore number, glomalin

Introduction

Arbuscular mycorrhizal fungi (AMF) are among the most ecologically significant organisms on the planet (Fitter *et al.* 2011) [6]. Since they act as a living connection between soil and plants, influencing soil fertility and plant nutrition, diversity and productivity (Smith and Read 2008) [19]. The natural abundance of mycorrhizal fungi in the agricultural soils and their shifts in the composition and function in developing ecosystem is poorly understood. Most studies of arbuscular mycorrhizal fungi (AMF) during ecosystem development focus on abundance, the extent of root colonization, with few studies characterizing community composition. In this symbiosis, the host plant provides the fungus with soluble carbon sources, at the same time the fungus enhances the uptake by plants of certain nutrients, particularly phosphate (Jayachandran and Shetty 2003) [9], Stabilize soil aggregates (Miller & Jastrow 1990) [12] by producing the glycoprotein Glomalin, modify root morphology and protects against plant pathogens (Wright and Upadhyaya 1998) [22]. Agricultural practices like tillage (Anderson *et al.* 1987) [2] and irrigation also had impact on AMF diversity. But the major factors affecting the diversity, abundance and distribution of Arbuscular Mycorrhizal Fungi in agricultural soils are soil physicochemical properties along with host plant species (Abbot and Robson, 1991) [1]. These factors could also affect the crop production in different in agro ecosystems (Porras-Soriano *et al.* 2009) [14]. So there is need to understand and develop, AMF management strategies applicable for sustainable low input but reasonably productive performance. In the present study we have evaluated the natural abundance of AM fungi in different agricultural crops grown in various ecological Zones of Andhra Pradesh in relation to soil chemical properties.

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Materials and Methods
Rhizosphere Soil Sample Collection
The present study was undertaken to investigate occurrence of AM fungi in four major crops distributed in five different agro-climatic Zones. A critical benchmark location has been fixed to collect soil samples, for determining the occurrence and abundance of arbuscular mycorrhizal fungi in existing field crops. Soil samples were collected to a depth of 0-25 cm of the root Zone by using soil auger. A total of thirteen random spots were taken in each bench mark location. These thirteen samples were pooled together as one sample, transferred it to a clean polythene bag, labeled and brought to the laboratory and stored in a refrigerator around 4ºC until further process.

Colonization of Am Fungi
The fine roots were carefully removed, washed free of soil in running tap water without damaging the external mycelium and cut into 1-2 cm long segments. The roots were cleared with 8% of KOH at 90ºC for 10 minutes and roots were then acidified with 1% HCl and root segments were treated with 0.05% Trypan blue kept it for overnight. Before observing root bits destaining was done with lacto-glycerol solution (Philips and Hayman, 1970) [13]. The stained root bits were placed on a glass slide, covered with a cover slip, and examined under microscope to detect the presence or absence of colonization by AM fungi. Only those segments containing mycorrhizal hyphae and either vesicles or arbuscules were counted as colonized. The number of root segment colonized was expressed as a percentage of total root segments in the sample, and the percentage of mycorrhizal infection was calculated as follows:

$$\text{Percent of AM colonization} = \frac{\text{Total no. of root segments colonized}}{\text{Total No of root segments examined}} \times 100$$

Collection of Am Fungal Spores
Air dried soil sample of 100 g was subjected to wet sieving and decanting technique (Gerdemann and Nicolson, 1963) [7] for enumeration AMF spores. In order to separate spores from organic debris, the sieved sample was centrifuged with 55% sucrose solution at 1750 rpm for 5 min. Spore counting was done by preparing a grid of thin layer of spore suspension was spread on this and counted under a stereoscopic binocular microscope.

Soil Analysis
The chemical and enzymatic properties of the soil such as Organic Carbon, Alkaline phosphatase and Glomalin were analyzed. Soil organic-carbon was estimated by the following method described by Walkley and Black (1934) [21]. The organic carbon in the soil gets oxidized by chromic acid (Potassium dichromate plus conc. Sulphuric acid) utilizing the heat of dilution of Sulphuric acid. One gram of soil was placed in a 500 ml conical flask and added 10 ml of 1N Potassium dichromate ($K_2Cr_2O_7$) and swirl a little then added 20 ml of Conc. Sulphuric acid and swirl again two or three times. The flask is allowed to stand for 30 minutes preferably in darkness. Add 200 ml of distilled water, 10 ml of Ortho Phosphoric acid 1ml of Diphenyl amine indicator and titrate the contents with ferrous ammonium sulphate solution, till the colour changes from blue-violet to green.

Alkaline phosphatase activity was estimated by (Eivazi and Tabatabai 1977) [5] taking one gram of the soil sample (<2.0 mm) in a wide mouth test tube and added 0.2 ml of Toluene plus 4 ml of modified universal buffer (pH 11) for assay of Alkaline phosphatase, then one ml of p-nitrophenyl phosphate solution prepared in the same buffer was added. Tubes were swirled for a few seconds to mix the contents. Tubes were then placed with stoppers and placed in incubator at 37 ºC. After one hour stoppers were removed and 1ml of 0.5M $CaCl_2$ plus 4ml of 0.5M NaOH were added. Again swirl the flask and filter through Whatman no. 1 filter paper, and the intensity of yellow colour of the supernatant was measured using spectrophotometer at 420 nm wavelength. The p-nitrophenol content of the filtrate was obtained by reference to a calibration graph plotted from the results obtained with standards containing 0, 10, 20, 30, 40, and 50µg of p-nitro phenol. The control was performed with each soil analyzed to allow for color not derived from p-nitro phenol released by phosphatase activity. Alkaline phosphatase is expressed in ug p-nitrophenol/g soil/hour.

Glomalin extraction was performed as described by Wright and Upadhyaya (1998) [22], Glomalin protein was estimated by Bradford protein assay method as described.

Bradford reagent
Dissolve100 mg of G 250 Coomassie Brilliant Blue in 100 ml of absolute ethanol in on a shaker for 60 minutes. Add 100 ml of 85% of ortho-phosphoric acid, mix well and make the volume to 500 ml with distilled water. Filter through whatman No.1. Paper and dilute 1:1 with water.

Sample Extract
One gram of soil sample was taken in autoclavable centrifuge tube and 50 mM sodium citrate was added. These centrifuge tubes are autoclaved for 1 hour at 121 ºC at 15 lbs pressure. The supernatant was removed carefully and it is stored in refrigerator. The supernatant which was stored was taken and centrifuged for 15 min at 10,000 rpm. The clear solution was taken as sample. One ml of sample solution was taken then 4.5 ml of Bradford reagent was added to all the tubes mix gently leave the tubes for 5 minutes. The absorbance was recorded at 595 nm against the blank. Taking bovine serum albumin as the standard, a standard curve of A_{595} versus μg of protein was plotted. Glycoprotein content was determined in the sample extract from the standard curve and glomalin was expressed in mg/g soil.

Statistical Analysis
Analysis of variance (Anova) was performed using Mstatc to determine the differences in root colonization, spore population, soil chemical and enzymatic parameters such as organic carbon, alkaline phosphatase and glomalin.

Results
Occurrence of Am Fungi in Different Field Crops of Andhra Pradesh
Natural occurrence of AM fungi in four major agricultural crops was thoroughly assessed in the present investigation. It was observed that four major agricultural crops of Andhra Pradesh namely Cotton, Sugarcane, Paddy and Blackgram were taken for extensive study for the presence of AM fungi at all stages of crop periods. All the four crops were studied across the crop growth periods. There was a gradual increase in root colonization from vegetative stage to 50% flowering stage and attained maximum at harvesting stage (Table 1). During vegetative stage sugarcane harbored maximum colonization of AM fungi (28%) followed by cotton crop (26.6%). Whereas at 50% flowering stage, the per cent

colonization of AM fungi was significantly higher in cotton crop (80%), followed by sugarcane crop (68.3%) and the lowest root colonization was recorded in paddy (27.3%) (Table1). During harvesting stage also, the cotton crop retained highest root colonization (82.3%) of AM fungi followed by blackgram (70%) and lowest root colonization was recorded in paddy (35.6%). The over-all root colonization of AM fungi across different crop species was in Fig. 1. Among four crop species cotton crop supported highest root colonization (63%) followed by sugarcane (55.3%) and lowest reported in paddy (25.7%). AMF spore abundance in rhizosphere soils of various agricultural crops grown in different agro-climatic Zones of Andhra Pradesh was monitored. Spore population was increased gradually from initial stage of the crop and attained maximum at harvesting stage in all crops (Table 2). At initial stage the spore number in paddy rhizosphere is significantly lower (11.3/100g) than the other crops. Similar trend was recorded in vegetative stage of four crops. Gradually the spore number has been enhanced in 50% flowering and harvesting stages of the crops. Cotton crop rhizosphere soil has supported highest spore production (33.3) at 50% flowering stage followed by black gram crop rhizosphere soil. Whereas the trend has been changed when the crops attained harvesting stage, Black gram crop rhizosphere soil has a buildup of 42 spores per 100g soil and was significantly higher than other crops except cotton (Table 2). The cumulative count of AM fungal spores per 100 g rhizosphere soil recorded highest in black gram crop rhizosphere followed by cotton and sugarcane and lowest was reported in paddy rhizosphere soil (Fig 2). The spore numbers in the rhizosphere of paddy is significantly lower than rest of the crops taken in the present investigation.

Influence of Am Fungi on Different Soil Parameters with Different Host Crops
Organic carbon in the rhizosphere soil of four field crops was analyzed across the growth stages. All the field samples showed increased behavior of soil organic carbon from initial to vegetative state except in blackgram field (Table 3). The increasing trend was continued from vegetative to 50% flowering stage in all field crops rhizosphere soil except in cotton crop. During harvesting stage paddy attained highest organic carbon (0.79%) except paddy all the crops was declined slightly during harvesting stage. Alkaline phosphatase activity in the rhizosphere soils of four crops was analysed and found that an increasing trend from initial stage to 50% flowering stage and declined at harvesting stage(Table 4). The maximum activity was recorded at 50% flowering stage in all the crops. At initial stage of measurement cotton crop rhizosphere soil has maximum alkaline phosphatase activity (13.63 μg p-nitrophenol g^{-1} soil hr^{-1}) followed by sugarcane (13.03 μg p-nitrophenol g^{-1} soil hr^{-1}) and lowest was recorded in black gram. At vegetative stage sugarcane crop rhizosphere soil has maximum alkaline phosphatatse activity (36.50 μg p-nitrophenol g^{-1} soil hr^{-1}) followed by blackgram (27.40 μg p-nitrophenol g^{-1} soil hr^{-1}). Similar trend was continued at 50% flowering and harvesting stages in all the crops. And paddy rhizosphere soils exhibited same levels of alkaline phosphatase activities and they are significantly lower than sugancane and black gram crops (Fig. 3). Among four crop species sugarcane crop has highest alkaline phosphatase (67.33μg p-nitrophenol g^{-1} soil hr^{-1})), followed by blackgram (39.10 μg p-nitrophenol g^{-1} soil hr^{-1}) irrespective of the crop growth stage.

All the four major agricultural crops rhizosphere soils were extensively studied for the presence of glomalin, at all crop growth stages. There was a gradual increase in glomalin from initial stage to vegetative stage and attained maximum at harvesting stage as the trend observed with alkaline phosphatase (Table 5). At initial stage, sugarcane crop rhizosphere has accumulated maximum glomalin content (1.15 mg/g) followed by blackgram (1.07 mg/g) and the trend was continued at vegetative stage and 50% flowering stage including harvesting stage. The paddy crop rhizosphere has accumulated lowest glomalin content in rhizospahere (Table 5) when compared to other crops. Among four crop species sugarcane crop showed highest glomalin content (7.96 mg/g) accumulation at harvesting stage followed by blackgram (5.49 mg/g) under field conditions of Andhra Pradesh.

Discussion
In the present study the per cent AM fungal colonization in four agricultural crops grown in different agro-climatic zones of Andhra Pradesh indicated that AM fungi is ubiquitous in nature. Root colonization varied among different field crops as affected by host plant species. This shows that there is a host species preference for AM fungi in colonization and infected heavily on cotton host followed by black gram. These results are in support of the work of Tahat *et al.*, (2008) [20] who reported the inoculation of Glomus mosseae gave highest percent of root colonization on maize crop compared to all other crops. Safi and Khan (1997) [17] also reported that host preference of AM fungi is good with cereals when compared to other field crops. The over-all root colonization was significantly higher in cotton crop (63%) as compared to paddy crop (25.7%) which is could be the influence of host physiological characters (Fig 1) and soil environment. And the establishment of association between host plant and AM fungi may be mediated by the interaction between host plant environment and fungi as reported by Correnho *et al.*, (2002). [3] As the AM fungus known to prefer aerobic conditions, it could not colonize much on paddy crop which was generally grown in water logged situations in Andhra Pradesh. Cotton has got good root system and penetrate into deeper layers of the soil when compared to sugarcane that could be the reason to record maximum root colonization in cotton. The spore abundance is high in black gram rhizosphere soil. Black gram which grows much swallow depth to a maximum of soil depth of 30 to 40 cm might have provided the more infective sites to occupy next to cotton for root colonization status. This is in support of finding of Lekberg *et al.* (2011) [11] that the communities of AMF respond strongly to soil properties. Sinegani and Sharifi (2007) [18] also who reported, higher AMF spores in the rhizosphere with corn crop and expressed that the host type is the most important factor for spore production and multiplication as well as growth period in the host is critical for more colonization as recorded in the blackgram. Further the investigation indicates that spore production in the top soil indirectly depends on the root density present in the particular soil layer along with other soil traits like soil organic carbon and bulk density etc. Gianinazi *et al.* (2010) [8] reported the ecosystem services are more dependent on AM fungi and soil characters which plays a key role in enhancing crop growth. The changes in soil organic carbon against host plant species depends on the root exudates deposited in the rhizosphere soil by mycorrhizal fungi as reported by Ryan and Graham (2002) [16]. The raise of soil organic carbon in paddy rhizosphere soil (0.79%) may be because of several factors present in the ecosystem.

Mycorrhizal fungi, together with plant roots, soil microorganisms and decaying organic matter, release organic acids, acidifying the soil and increasing mineral weathering could be reason for enhanced organic carbon levels in the soil as reported by Chadwick and Chorover (2001) [4]. The buildup of organic carbon is may be due to another important factor that the release of macromolecules by microsymbiont is most likely a glycoprotein called as glomalin. The glomalin is a recalcitrant molecule and remains as a black diamond contributes for organic carbon values in the rhizosphere soil. The increase of alkaline phosphatase activity is because of release from dying soil organisms or desorption and reactivation of previously accumulated phosphatase from organic and inorganic soil components as reported by Kramer and Green (2000) [10]. The present behavior of alkaline phosphatase clearly indicating that soil bacteria and fungi population might have stimulated by the natural abundance of mycorrhizal colonization in the host rhizosphere and ultimately the buildup was noticed in the rhizosphere of sugarcane (Fig 3). The increase was up to a period of 50% flowering stage where we generally observe highest microbial population at this particular stage of all crops. As the crop senescence starts at harvesting stage and the alkaline phosphatase also decreased in all crops (Table 4).

The glomalin which is a major fraction of organic carbon in the rhizhosphere soil is found to be high in sugarcane rhizosphere (Table 5) and it is positively correlated with the production of alkaline phosphatase (Fig 4). Similar observation was reported by Rilling, *et al.* (2001) [15] where several soil enzymes activity has been enhanced in relation to accumulation of glomalin. In the present investigation accumulation of glomalin is further correlated with percent AM fungal colonization of sugarcane roots. Wright & Upadyaya (1998) [22] reported percent AM root colonization enhanced accumulation of glomalin and helped in improving soil quality and aggregates stability in the soil. The glomalin accumulation not only promotes the soil enzyme activities but also stimulate biogeochemical cycling which could benefit plant nutrient uptake. Occurrence of AMF communities in the rhizosphere region of agricultural crops was revealed that selectivity of AM associations and greater dependence of resulting functions on particular host/fungus combinations than previously suggested. Host plant in a field could provide a heterogeneous environment favouring certain naturally occurring AM fungi. The effects of specific host and AM fungi combinations ranges several beneficial effects in maintaining soil health and ultimately crop yields. Since large scale application of AM fungi has several draw backs one can manipulate the naturally occurring indigenous AM fungi for obtaining good crop yield.

Table 1: Root colonization of AM fungi across growth stages of different crops.

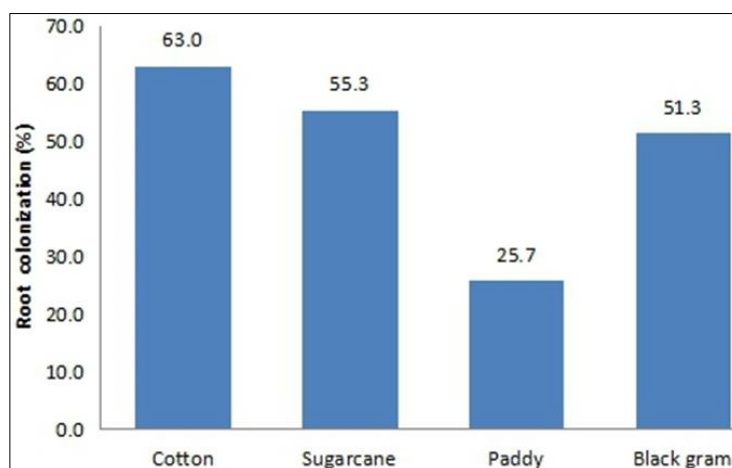| Crop	Mycorrhizal Root Colonization (%)		
	Vegetative	50% Flowering	Harvesting
Cotton	26.6	80	82.3
Sugarcane	28	68.3	69.6
Paddy	14.3	27.3	35.6
Black gram	18.6	65.3	70
SEM	3.3	2.6	2.6
CD at 5%	NS	8.5	8.7

Fig: 1 over-all AMF root colonization in various agricultural crops of AP.

Table 2: Effect of different host crops on AM Fungal Spore production at different growth stages.

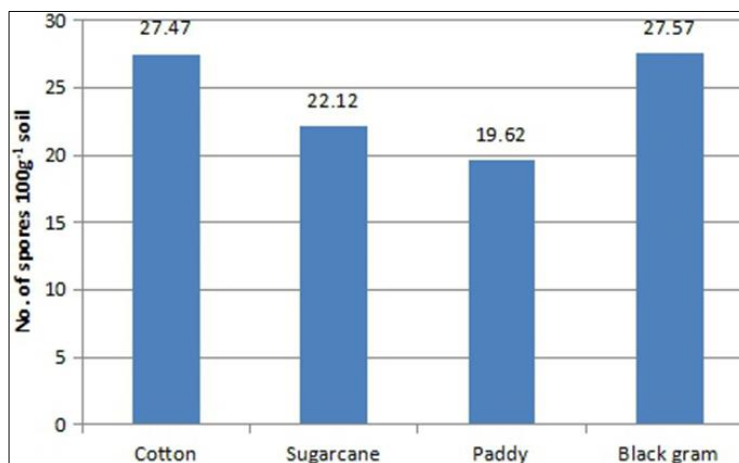| Crop	No. of spores /100g soil			
	Initial	Vegetative	50% Flowering	Harvesting
Cotton	15.3	22	33.3	39.3
Sugarcane	14.3	18.6	26.3	29.3
Paddy	11.3	16.6	24	26.6
Black gram	15	21.3	32	42
SEM	0.52	0.87	0.61	0.67
CD at 5%	1.3	1.4	3.2	4.1

Fig 2: Cumulative number of AM fungal spores in different crops of A.P

Table 3: Effect of different crops on soil organic carbon at different growth stages

Crop	Organic Carbon (%)			
	Initial	Vegetative	50% Flowering	Harvesting
Cotton	0.61	0.72	0.66	0.47
Sugarcane	0.55	0.62	0.78	0.73
Paddy	0.41	0.60	0.66	0.79
Black gram	0.64	0.43	0.65	0.73
SEM	0.01	0.01	0.01	0.01
CD at 5%	0.04	0.05	1.92	3.55

Table 4: Effect of different crops on soil alkaline phosphatase at different growth stages

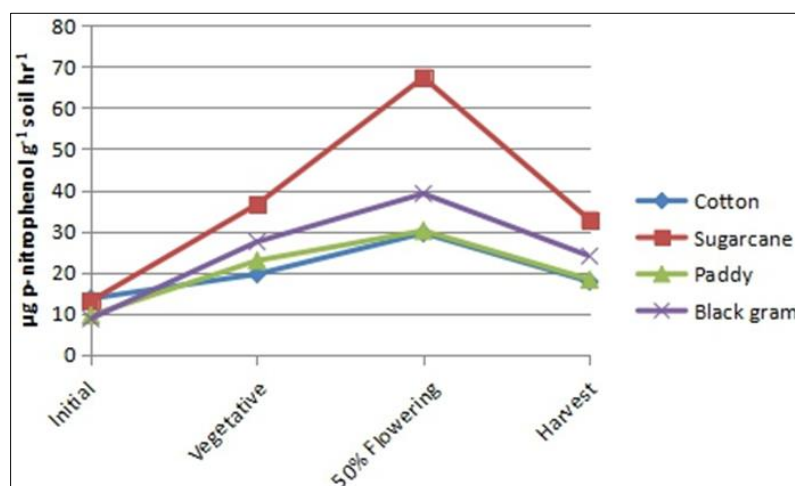| Crop	Alkaline phosphatase (μg p-nitro phenol g^{-1} soil hr^{-1})			
	Initial	Vegetative	50% Flowering	Harvesting
Cotton	13.63	19.53	29.50	17.73
Sugarcane	13.03	36.50	67.33	32.73
Paddy	9.53	22.83	30.10	18.26
Black gram	8.73	27.40	39.10	23.93
SEM	0.83	0.62	1.36	0.74
CD at 5%	2.74	2.05	4.50	2.44

Fig 3: Soil alkaline phosphatase as influenced by natural AMF against different host crops.

Table 5: Effect of host plant associated with natural AMF on glomalin content in the rhizosphere soil

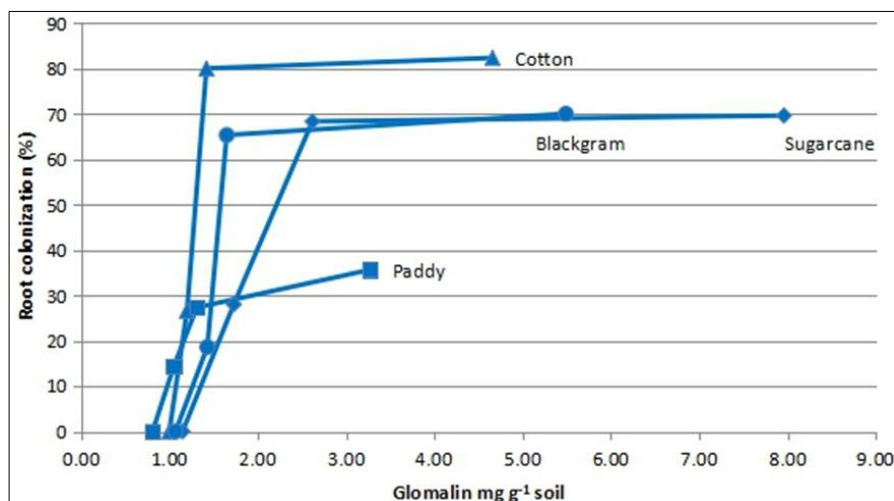| Crop	Glomalin (Mg/G)			
	Initial	Vegetative	50% Flowering	Harvesting
Cotton	1.00	1.20	1.42	4.66
Sugarcane	1.15	1.73	2.62	7.96
Paddy	0.81	1.05	1.31	3.27
Blackgram	1.07	1.43	1.65	5.49
SEM	0.049	0.045	0.044	0.098
CD at 5%	0.161	0.148	4.333	0.325

Fig 4: Glomalin accumulation as influenced by per cent root colonization in different host crops.

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