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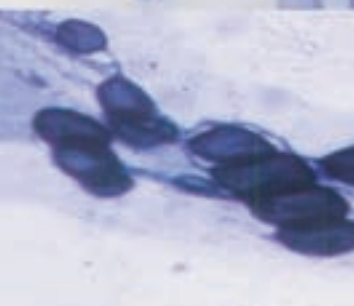

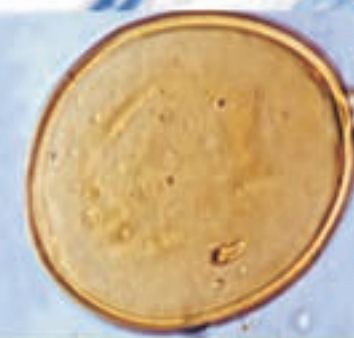

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TERI's Mycorrhiza Network is primarily responsible for establishing the MIC (Mycorrhiza Information Centre), the CMCC (Centre for Mycorrhiza Culture Collection), and publishing Mycorrhiza News. The Network helps scientists carry out research in mycorrhiza and promotes communication among mycorrhiza scientists.

Mycorrhiza News

The Mycorrhiza News provides a forum for dissemination of scientific information on mycorrhiza research and activities; publishes state-of-the-art papers from eminent scientists; notes on important breakthroughs; brief accounts of new approaches and techniques; publishes papers complied from its RIZA database; provides information on forthcoming events on mycorrhiza and related subjects; lists important research references published during the quarter; and highlights the activities of the CMCC.

Contents

Research finding papers

Record of *Scutellospora savannicola* (Herrer and Ferrera) Walker and Sanders from Goa

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Introduction

Scutellospora savannicola (Herrer and Ferrera) Walker and Sanders is known only from Savannah on macarrera soil, i.e., clayey, sandy but not very gravelly soil with high concentrations of Iron (Ferrer and Herrera, 1980). This species of arbuscular mycorrhizal (AM) fungi was earlier placed under genus *Gigaspora*. Later, Walker and Sanders (1986) placed it under *Scutellospora* based on differentiable wall layers and knobby soil borne vesicles or auxillary cells. The spores of *S. savannicola* are reported to be present in soils probably during October to January (Ferrer and Herrera, 1980).

Western Ghats of Goa have been randomly surveyed for the occurrence of AM fungi by Khade (2002). Rhizospheric agro-soils of Collem from Western Ghats of Goa are acidic to near neutral, high in organic carbon and K while, total N and P levels are limiting. Micronutrients, viz., Cu, Zn, Fe, and Mn are present in high levels (Khade, 2003). Therefore, in the present study *Carica papaya* L., from agro-based ecosystem of Collem was surveyed for occurrence of *S. savannicola* post monsoons.

Materials and methods

Root samples and the rhizosphere soil samples of papaya plants were collected during the post monsoons (December 2001) from Collem located in Western Ghats of Goa **(Plate 1 A, B).** Further, freshly collected roots along with root hairs were washed in water, cleared with 10% KOH, acidified with 1N HCl, and stained in 0.05% Trypan Blue in lactoglycerol (Phillps and Hayman, 1970). Spores of AM fungi were extracted from the rhizosphere using wet sieving and decanting method (Gerdemann and Nicolson, 1963). Diagnostic slides with spores were prepared using polyvinyl alcohol lactoglycerol (PVLG) as mountant. Identification of AM fungi was carried out using Manual of identification of AM fungi (Schenck and Perez, 1990).

Results

Scutellospora savannicola (Herrer and Ferrera) Walker and Sanders recorded in the present study is taxonomically described below.

Azygospores formed singly in soil, hyaline to white, turning brown at maturity **(Plate 2A)**, oblong - ellipsoid to broadly ellipsoidal **(Plate 2A)**, and sometimes irregular 300- 360 x 200–300 µm in diam **(Plate 2A)**. Azygospore is borne on a bulbous suspensor **(Plate 2A)** which is globose to pyriform or claviform, 33–51µm in diam at the widest part with thin wall, up to 1.4 µm thick **(Plate 2B)**. Bulbous suspensor attached to a septate hypha **(Plate 2A, Plate 2B)**. Generally lateral hypha extends from the bulbous suspensor and is 3–33 µm long in young

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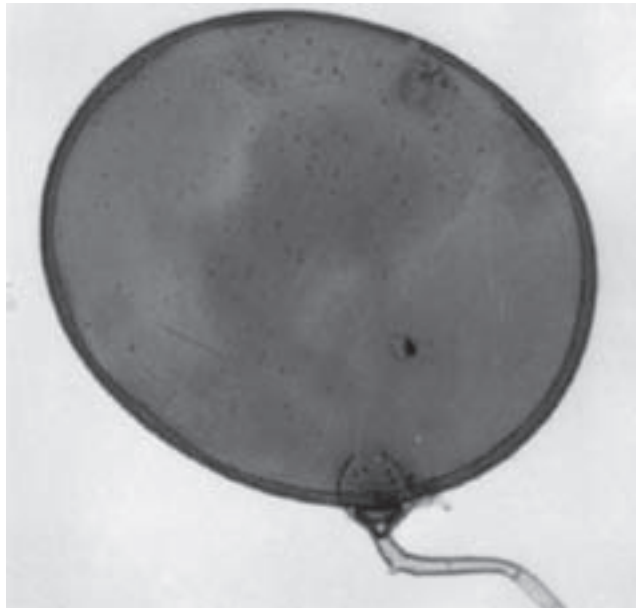
A

B

Plate 1 A) Western Ghats of Collem.
B) Habit of *Carica papaya* L.



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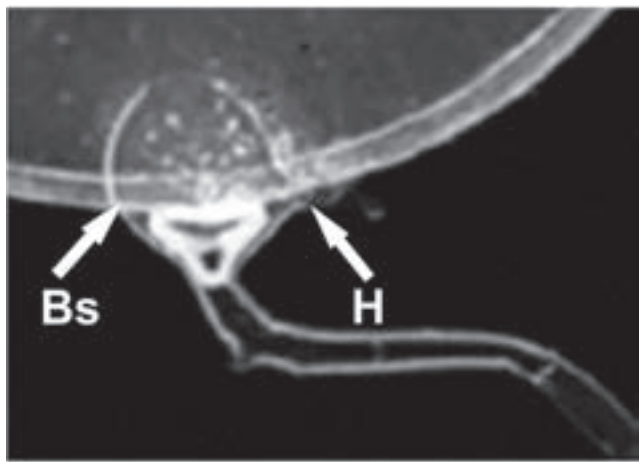


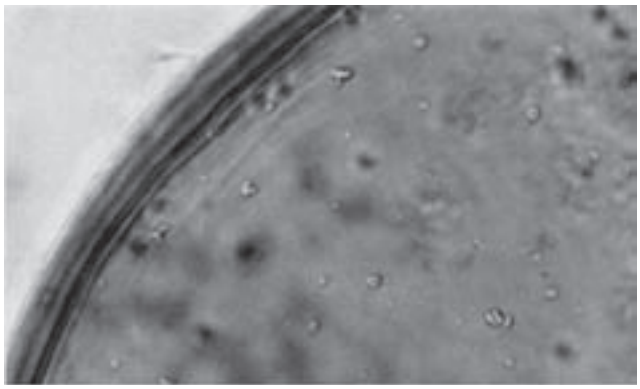
B

Plate 2 (A) A mature spore of *Scutellospora savannicola* borne on bulbous suspensor (x 200).
(B) A portion of spore of *Scutellospora savannicola* showing bulbous suspensor (Bs) and a lateral hypha (H) extending from bulbous suspensor (x 400).

spores and much longer in mature spores **(Plate 2B)**. Spore wall layered and 5–12 µm thick **(Plate 3A)**. Wall composed of an exospore of two wall layers **(Plate 3B)**, a mesospore of two membranes **(Plate 3B)** and a membranous endospore **(Plate 3B).** The external layer of the exospore is up to 3 µm thick and the internal layer upto 2.3 µm thick. The external membrane of the mesospore is up to 1.5 µm and the internal layer upto 1.3 µm thick. The endospore is up to 1 µm thick enclosing reticulate content of the spore. Auxillary cells knobby with blunt ends, borne in groups on a short hypha.

Conclusions

In the present study, the soil was dark brown to reddish brown, well drained gravelly with silt clay loam texture and medium water holding capacity. The present study recorded AM fungal colonization and encountered spores of *S. savannicola* in rhizosphere of



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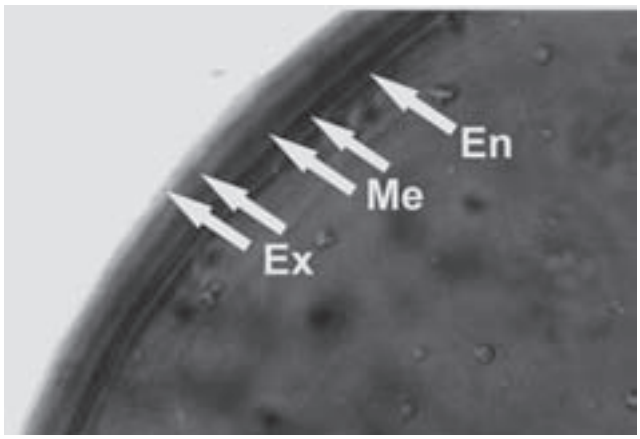

B

Plate 3 (A) A portion of spore of *Scutellospora savannicola* showing wall layers (x 400).
(B) A magnified portion of spore wall of *Scutellospora savannicola* with 5 layers
(Two outer layers of the Exospore [Ex]; Two middle layers of the Mesospore [Me]; and One inner most layer of the endospore [En]). Arrows indicate the spore walls (x 600).

papaya plants from Western Ghats of Goa (Collem) during December. This supports the findings of Khade (2002), Khade *et al.* (2002), and Khade (2003) who reported AM fungal association in papaya plants from Goa and presently the contention that spores of *S. savannicola* are also present in tropical P-deficient and iron rich agro-soils.

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Management of charcoal root-rot caused by *Macrophomina phaseolina* in groundnut by using arbuscular mycorrhizal fungi in field

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Introduction

Chemical pesticides have always been seen as an effective methodology in control of plant pathogens in the past, but greater environment-consciousness is pushing the demand for minimum use of harmful chemical inputs. Moreover, a pesticide does not provide complete or lasting defense against soil-borne plant pathogens besides posing great threat to our lives (Johansson *et al.*, 2004). This has led to advanced research into bio-control methods, which are beneficial for farmers who are not very economically well-off and for sustainable agricultural practices, which will benefit our environment and biodiversity as a whole. The biological control of plant pathogens by using arbuscular mycorrhizal fungi in providing resistance against various plant pathogens have been well-documented (Singh *et al.*, 2000; Declerck *et al.*, 2002; Ludwig-Muller, 2004; Whipps, 2004).

Groundnut (*A. hypogaea* L.) is important as a chief oilseed crop in the Indian subcontinent. Several soil-borne plant pathogens attack groundnut plants during their growth. Among various pathogens, fungal pathogen *M. phaseolina* Tassi (Goid.) causes severe charcoal root-rot disease, which may assume huge economic proportions. Because of being soil-borne in nature, it remains a difficult pathogen to eradicate completely and chemical inputs are already considered both hazardous to the environment as well as costly. *M. phaseolina* is distributed worldwide and prevalent mainly in areas with high temperature and low rainfall (Raut and Bhombe, 1984). The main objective of this study was to evaluate the effectiveness of mycorrhizal inoculation against *M. phaseolina* under field conditions.

Material and methods

Plant material

The groundnut seeds were obtained from Naik Seeds, Pune, Maharashtra, India. The groundnut variety used for this study was of a local cultivar named Phule Pragati (JL-24). The seeds were surface sterilized with 0.02% $HgCl_2$ for five minutes and then washed with distilled water.

Pathogen inoculum

The pure isolate of *M. phaseolina* was provided by Agharkar Research Institute and it was mass multiplied on sorghum grains (250 g), initially soaked overnight in water. About 100 gm of soaked sorghum grains were taken in saline bottles of 500 ml capacity plugged with cotton. The bottles were then sterilized for 20 minutes at 121°C. The sterilized sorghum seeds present in saline bottles were inoculated with 5 mm mycelial disc using a cork borer (5 mm) from the active periphery of a seven-day-old pure culture of *M. phaseolina* in each bottle and the bottles were incubated for three weeks at temperature (28°C ± 2°C) for proper mycelial growth. This sorghum grain culture served as the inoculum. It was applied at the rate of 5 g after 15 days of the groundnut plant's growth.

Isolation, identification, and inoculum preparation of AM

According to the method described by Gerdemann and Nicolson (1963), isolation of AM fungal spores was carried out by wet sieving and decanting methods. Identification of isolated fungal spores was carried out by complying keys recommended by Trappe (1982) and Schenck and Prez (1987). According to Kornerup and Wanscher (1983), determination of colour, shape, and dimensions of arbuscular mycorrhizal (AM) fungal spores was done. Mass multiplication of AM fungi [*G. fasciculatum* (Thaxter Sensu Gerd.) (Gerdemann and Trappe)] was done in pots with different host plants, such as *Sorghum vulgare* and *Panicum maximum* (Jacq.) and were grown on 30 cm earthen pots containing 10–15 kg of sterilized soil and sand in 1:1 proportion. Inoculations were made from this mass multiplied mycorrhizal inoculum after three months to each groundnut plant with 20 g of AM fungi inoculum mixture containing spores, colonized root pieces, and extrametrical mycelium in rhizospheric soil (obtained from the pot culture).

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The mycorrhizal inoculum was placed below each groundnut seeds (about 3–5 cm) under the soil surface before sowing.

Field preparation for experiment

Preparation of field was carried out by ploughing it to a depth of 20 to 25 cm with shape of in-furrow system, ridge height of about 7–10 cm, inter-row distance of 15 cm, and plant-to-plant spacing of 30 cm on rows (16 foot length). The seeds of groundnut were planted by hand. Mycorrhizal (*G. fasciculatum*) inoculations were made with 20 g of inoculum mixture containing spores and colonized root pieces, which were obtained from the pot culture and were placed at about 3–5 cm below the seeds under the soil before sowing. Fungal inoculum of *M. phaseolina* mass multiplied on sorghum grains at the rate of 5 g was applied after 15 days of growth of the groundnut plant. There were four treatments replicated three times, which included: Controls (C); *M. phaseolina* (C+Mp); *G. fasciculatum* (Gf); *G. fasciculatum*; and *M. phaseolina* (Gf+Mp).

Data collection

The plants were randomly chosen as triplicates after 90 days of AM treatment and 75 days of *M. phaseolina* inoculations for determination of various growth responses, viz., leaf number, plant height, pod number, fresh and dry weight, and total biomass content of plants. The total chlorophyll content of the shoot was determined according to Arnon (1949); arbuscule percentage (%) and root colonization (%) was determined according to Phillips and Hayman (1970).

Mycorrhiza dependency

Mycorrhizal dependency was carried out according to Plenchette *et al.* (1983) by measuring the dry weights of the plants after drying the plant material to 70°C for 48 hours.

Percent Mycorrhizal Dependency = [(dry mass mycorrhizal plant − dry non-mass mycorrhizal plant) / dry mass mycorrhizal plant] x 100%

Production loss

Production losses were calculated using the equation given by Teng (1985):
PL = (AY/1.0-PL) – AY
Where:
PL = Production loss
AY = Actual yield

Statistical analysis

The data was examined by one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). Three replications were carried out for each treatment. DMRT was applied as the post hoc test at p= 0.05. Calculations were made by using a Statistical Package for Social Sciences (SPSS) for windows version 9.0 and Microsoft Office Excel 2007 to analyse the data.

Results

Growth responses groundnut plants

The data obtained from field experiment shows that *G. fasciculatum* inoculation on groundnut plant showed significant increase in terms of shoot length, fresh and dry weight, total biomass, total chlorophyll content, and numbers of pods as compared to their counterparts not subjected to mycorrhizal controls. In the mycorrhizal inoculated groundnut plants after 90 days of sowing, the leaf numbers were significantly higher (440.00 in Gf) than their non-mycorrhizal control counterparts (270.00 in Control). The lowest number of leaves (210.67 in C+Mp) was exhibited by diseased non-mycorrhizal groundnut plants after 90 days of sowing. But, when observed in mycorrhizal-infected groundnut plants, it showed higher number of leaves (266.67 in Gf+Mp) as per Table 1.

Table 1 Leaf number, shoot length, fresh weight, dry weight, and mycorrhizal dependency of *A. hypogaea* L. after 90 days of AM inoculation and 75 days of *M. phaseolina* treatment

Treatments	Leafs (no.)	Shoot length (cm)	Fresh weight (gm)	Dry weight (gm)	Mycorrhizal dependency (%)
	90 DAS	90 DAS	90 DAS	90 DAS	90 DAS
C	270.00±97.98b	38.00±4.32ab	43.38±4.35b	31.00±2.02b	0.00
C+Mp	210.67±28.53b	31.33±1.89b	25.84±2.16c	14.26±2.22c	0.00
Gf	440.00±48.99a	51.00±6.48a	92.40±5.39a	47.95±3.37a	35.14±5.17
Gf+Mp	266.67±49.89b	43.33±8.50ab	44.44±3.86b	33.34±1.42b	57.23±6.71

Notes: C: Non-inoculated; C+Mp: Inoculated with *M. phaseolina*; Gf: Inoculated with *G. fasciculatum*; Gf+Mp: Inoculated with *G. fasciculatum* and *M. phaseolina*; values are mean of three replications with standard error; values followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$); DAS = Days after sowing.

There was significant increase in plant height in mycorrhizal-treated groundnut plant (highest by 51.00 in Gf) than non-mycorrhizal groundnut plants (38.00 in Control). The plant height was lowest in non-mycorrhizal groundnut plants infected with *M. phaseolina* (31.33 in C+Mp) as compared to pathogenic mycorrhizal groundnut (43.33 in Gf+Mp). Similarly, the number of pods was significantly higher in mycorrhizal groundnut plants (34.00 in Gf) as compared to pathogen-infected mycorrhizal groundnut plants (27.00 in Gf+Mp). The pod number was observed to be lowest in pathogen-infected groundnut plants (20.33 in C+Mp). The groundnut plant's fresh weight was significantly higher in mycorrhizal groundnut plant (92.40 in Gf) as compared to the non-mycorrhizal control ones (43.38 in Control). The fresh weight appears to be significantly higher in diseased mycorrhizal groundnut plants (44.44 in Gf+Mp) than pathogen-infected non-mycorrhizal groundnut plants (25.84 in C+Mp), which was observed to be the lowest. The dry weight was also significantly higher in mycorrhiza-treated groundnut plants (47.95 in Gf) than non-mycorrhizal control ones (31.00 in Control). The dry weight was higher in plants inoculated with mycorrhiza in pathogenic ones (33.34 in Gf+Mp) than non-mycorrhizal pathogenic groundnut plants, which recorded lowest dry weight (14.26 in C+Mp) (Table 1).

Total biomass

The total biomass obtained from the present results was 140.35 for only mycorrhizal groundnut treatment, which was highest followed by pathogenic mycorrhizal groundnut (77.78). Lowest total biomass was recorded as 40.09 in non-mycorrhizal groundnut plants. Control (C) treatment without any infection or inoculation showed total biomass of 74.38 (Table 2).

Production Loss

The production loss was observed to be 65.50 g/14.864 m^2 for present experiment inoculated with mycorrhizal fungus.

Roots colonization

It was observed that the groundnut plant roots were able to colonize due to vesicles and the maximum colonization was observed in only *G. fasciculatum* treatment (89.00% for Gf) as compared to pathogenic mycorrhizal groundnut plants (45.00% in Gf+Mp) after 90 days of AM inoculation and 75 days after *M. phaseolina* inoculation. The colonization was observed to be negligible in non-mycorrhizal or pathogenic non-mycorrhizal groundnut plants. The percentage of arbuscule was more in only mycorrhizal- treated groundnut plants as compared to mycorrhizal-infected groundnut control ones (Table 2).

Mycorrhizal dependency

The mycorrhizal dependency was recorded to be lower (35.14%) for mycorrhizal treatment without pathogen (*M. phaseolina*) and in presence of pathogen *M. phaseolina* the mycorrhizal dependency was observed to be higher by 57.23% (Table 1).

The data of present experiment revealed that the occurrence of charcoal root-rot decreased due to inoculation of *G. fasciculatum* on seeds of groundnut cultivar (JL-24). AM association significantly reduced disease incidence and disease severity caused by *M. phaseolina* as compared to control non-infected groundnut plants. The disease severity was 32.81%, 39.47%, and 32.60% for non-mycorrhizal pathogen-infected groundnut plants (C+Mp) as compared to mycorrhizal groundnut plants with pathogen (*M. phaseolina*) infection (30.30%, 31.11%, and 29.16%

Table 2 Total biomass, pod number, arbuscule percentage, and root colonization in *A. hypogaea* L. after 90 days of AM inoculation and 75 days of *M. phaseolina* treatment

	Control		Mycorrhizal	
	90 DAS		90 DAS	
Treatments	C	C+Mp	Gf	Gf+Mp
Total plant biomass (gm)	74.38	40.09	140.35	77.78
Pod number (no.)	25.67	20.33	34.00	27.00
Arbuscule percentage (%)	0.00	0.00	42.00	18.00
Root colonization (%)	0.00	0.00	89.00	45.00

Notes: C: Non-inoculated; C+Mp: Inoculated with *M. phaseolina*; Gf: Inoculated with *G. fasciculatum*; Gf+Mp: Inoculated with *G. fasciculatum* and *M. phaseolina*; DAS = Days after sowing; values are mean of three replications.

Table 3 Total chlorophyll content in *A. hypogaea* L. after 30, 60, and 90 days of AM inoculation and 15, 45, and 75 days of *M. phaseolina* inoculation

	Total chlorophyll content mg chl./gm of fresh weight		
Treatments	30 DAS	60 DAS	90 DAS
C	0.72±0.05b	1.08±0.18ab	1.52±0.03b
C+Mp	0.59±0.05b	0.81±0.04b	1.37±0.08b
Gf	1.18±0.05a	1.30±0.17a	1.73±0.15a
Gf+Mp	0.72±0.08b	1.03±0.08ab	1.48±0.06b

Notes: C: Non-inoculated; C+Mp: Inoculated with *M. phaseolina*; Gf: Inoculated with *G. fasciculatum*; Gf+Mp: Inoculated with *G. fasciculatum* and *M. phaseolina*; values are mean of three replications with standard error; values followed by the same letter are not significantly different as determined by Duncan's multiple range test ($P = 0.05$); DAS = Days after sowing.

for Gf+Mp) after 30, 60, and 90 days after AM inoculation and 15, 45, and 75 days after *M. phaseolina* infection (Table 5).

The incidences of root-rot disease caused by *M. phaseolina* were reduced due to inoculation by *G. fasciculatum* as shown in Table 4. The disease incidence after 30, 60, and 90 days of AM inoculation and 15, 45, and 75 days after *M. phaseolina* infection was 53.33%, 63.33%, and 76.67% in pathogenic non-mycorrhizal (C+Mp) and 36.67%, 50.00%, and 53.33 % in mycorrhizal-infected (Gf+Mp) groundnut plants.

Discussion

The present field experiment showed that groundnut plants were able to attain colonization by AM fungus *G. fasciculatum*. The ability of *Glomus* species to colonize the roots of groundnut was shown by Simpson and Daft (1990). The present study showed that due to colonization by *G. fasciculatum*, overall increase in the growth of groundnut plant was observed. The significant growth of groundnut plants may be attributed to mycorrhizal colonization as it is known to improve growth and provide other benefits to host plants during abiotic or biotic stresses (Singh *et al.*, 2000; Ludwig-Muller, 2004). It has been suggested that during colonization there is formation of vesicles and arbuscules in plants cells (Walker, 1992). The phenomenon of competition at the infection level has also been suggested. Even though both pathogen and VAM fungi colonize the same root system, but the development occurs in different cortical cells indicating some sort of competition for space (Azcon-Aguilar and Barea, 1996). So, improved growth of groundnut plants may indicate the potential of mycorrhizal plants in disease resistance.

Table 4 Disease incidence (%) and dead plants in *A. hypogaea* L. after 15, 45, and 75 days of *M. phaseolina* inoculation

	Disease incidence (%)			Dead plants (no.)
Treatments	15 DAI	45 DAI	75 DAI	75 DAI
C	0.00	0.00	0.00	0.00
Gf	0.00	0.00	0.00	0.00
C+Mp	53.33	63.33	76.67	10
Gf+Mp	36.67	50.00	53.33	5

Notes: C: Non-inoculated; C+Mp: Inoculated with *M. phaseolina*; Gf: Inoculated with *G. fasciculatum*; Gf+Mp: Inoculated with *G. fasciculatum* and *M. phaseolina*; DAI: Days after inoculation; values are mean of three replications.

Table 5 Disease severity (%) in *A. hypogaea* L. after 15, 45, and 75 days of *M. phaseolina* inoculation

	Disease severity (%)		
Treatments	15 DAI	45 DAI	75 DAI
C	0.00	0.00	0.00
Gf	0.00	0.00	0.00
C+Mp	32.81	39.47	32.60
Gf+Mp	30.30	31.11	29.16

Notes: C: Non-inoculated; C+Mp: Inoculated with *M. phaseolina*; Gf: Inoculated with *G. fasciculatum*; Gf+Mp: Inoculated with *G. fasciculatum* and *M. phaseolina*; DAI: Days after inoculation; values are mean of three replications.

The content of total chlorophyll was affected by infection when treated with mycorrhiza or pathogen *M. phaseolina*. The total chlorophyll content increased significantly in mycorrhizal groundnut when compared with non-mycorrhizal groundnut, but in the presence of pathogens chlorophyll content was found to decrease considerably, suggesting the role of the pathogens in such decrease. Similarly, degradation of chlorophyll was shown to be prevented by the increase in cytokinin content. Increase in the chlorophyll content has been showed by Allen and Allen (1981).

As nearly 80% of plants on land are mycorrhizal (Smith and Read, 2008) and in the present study inoculation with *G. fasciculatum* showed that colonization was higher in mycorrhizal roots of groundnut when pathogen *M. phaseolina* were not present. But, eventually the root colonization was found to decrease due to the presence of pathogen *M. phaseolina*, which suggests competition between the pathogen and AM fungus *G. fasciculatum*. The substrate for microbial activities is considered to be organic carbon in the rhizosphere or in the rhizoplane released from the roots of plants (Azaizeh *et al.*, 1995). That is why carbon availability in mycorrhizal roots could suggest pathogen (*M. phaseolina*) resistance or decrease in incidence and severity in groundnut plants, which can be correlated with increased mycorrhizal colonization. Even Linderman (1994) and Smith (1987) suggested that both mycorrhizal fungi and root pathogens compete for the carbon compounds reaching the root.

The "dependence" of a plant can be defined as its ability to grow in the presence of colonization (Janos, 2007). Therefore, the incidence of disease and severity was recorded to be lower in pathogenic mycorrhizal groundnut as compared to healthy mycorrhizal groundnut plants. Similar results were obtained by Jaizme Vega *et al.* (1998) who showed increased dependency due to the presence of pathogen and lower dependency due to the absence of pathogen. The marked increase in "mycorrhizal dependency" clearly suggests the efficiency of mycorrhizal colonization by improvement in growth of groundnut plants that occurred during biotic stress exerted by the presence of pathogen *M. phaseolina*.

In conclusion, we may state that mycorrhizal symbiosis is universal and is associated with almost all plant species. The application of AM fungi in a field of groundnut plants infected with pathogen *M. phaseolina* could ensure the good health, growth, and suppression of disease in the plant.

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Glomalin and its association with the rhizosphere soils of some trees in Warangal district, A.P, India

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Introduction

Plant and soil health are dependent upon the interactions of biological, physical, and chemical components of the soil. The symbiotic relationship between roots and Arbuscular Mycorrhiza (AM) fungi may also benefit the formation of soil structure (Hallett *et al.* 2009). Rillig *et al.* (2010) revealed that AM fungal mycelium alone could be sufficient to form and/or maintain water-stable soil macro-aggregates. AM fungi are considered to be the most common and ubiquitous underground endophytic fungi — serving as a crucial link within the plant and soil continuum (Wilson *et al.* 2009) and are treated as a principal functional component in the belowground ecosystem (Smith and Read 2008; Siddiky 2011). The rhizosphere, or root zone, is the location of the greatest flow of energy and minerals among these components (Wright and Millner, 1994). In this highly productive region, a vital symbiotic relationship exists between roots and soil-borne AM fungi (Smith and Read 1997). Mycorrhiza is the most efficient mechanism for Phosphorus (P) acquisition, especially under stress conditions. Although these fungi are not host-specific, host and fungal genotypes and soil abiotic and biotic variables have been shown to influence the nature of the symbiosis (Brundrett, 1991; Gianinazzi *et al.*, 1995; Varma, 1995). Even though AM fungi may be important in natural and managed systems (Bever *et al.*, 2009; Wilson *et al.*, 2009; Klironomos *et al.*, 2011), little is known about the factors that determine their community structure and symbiotic functioning as drivers of plant productivity; as a result, their adaptive evolution is less known (Rosendahl, 2008; Antunes *et al.*, 2011). In addition to improving plant health, mycorrhizal fungi also contribute to soil health. Fungal hyphae improve soil structure by helping to form water-stable soil aggregates (Miller and Jastrow, 1990; Tisdall *et al.*, 1997; Rillig and Steinberg, 2002). Mycorrhizal fungi also improve rhizosphere health by stimulating root exudation which promotes the growth of other beneficial soil microbes (Borowicz, 2001; Paul and Clark, 1996).

Glomalin is a glycoproteinaceous molecule produced by the hyphae of AM fungi (Wright and Upadhyaya, 1999; Rillig *et al.*, 2001). Glomalin is dark reddish-brown in colour; however, after extraction it loses the brown colour associated with organic matter. The brown colour of glomalin is attributed to the incorporation of iron as a structural component that may play a role in accumulation and/or function (Wright and Upadhyaya, 1998). The identification of glomalin has led to a re-evaluation of fungal contributions to Soil Organic Matter (SOM) and aggregate stability. Glomalin was identified at the USDA in the early 1990s in course of the work to produce monoclonal antibodies reactive with AM fungi. One of these antibodies reacted with a substance on the hyphae of a number of AM species (Wright *et al.*, 1996). This substance was named as glomalin after *Glomales*, the order to which all AM fungi belong. The glomalin fraction is operationally defined by its extraction procedure, but it is further characterized by total and immunoreactive protein assays (Wright *et al.*, 1996). The evidence that glomalin is produced by AM fungi, not plant roots, was obtained in the investigation of the reaction of the monoclonal antibodies against glomalin. In a blind experiment, immunofluorescence correctly identified glomalin only on roots that were later described as having AM colonization (Morton 1990).

Glomalin is reported to be present on the extramatrical hyphae of all AMF (except *Sclerocystis*) tested to date (Wright *et al.*, 1996). As hyphae degrade, this hydrophobic, highly stable glycoprotein sloughs off to coat organic matter and other soil particles. Wright *et al.* (1996) hypothesized that glomalin forms a conglomeration with root fragments and organic matter, thus, protecting it from degradation by microorganisms. Wright and Upadhyaya (1998) found a strong correlation between glomalin concentration and soil aggregation.

Several 'pools' of glomalin have been identified based on solubility characteristics: (*i*) easily extractable glomalin (EEG); (*ii*) total glomalin (TG);

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and (*iii*) a 'scum' at the air-water interface that occurs during the harvesting of hyphae from pot-cultured AM fungi. Hydrophobic and/or cationic interactions may be the mechanisms by which glomalin becomes deposited on soil or organic particles, and mesh or glass beads (Wright and Upadhyaya, 1996). Glomalin may move in and out of these operationally defined pools (i.e., EEG becomes scum and scum becomes TG). Steinberg and Rillig (2003) found that following soil incubation to measure decomposition, EEG increased while TG decreased. They speculated that partial degradation decreases sorption of glomalin to soil particles, which may increase solubility and the amount in the EEG pool.

The formation of humic substances or, more likely, glomalin, would provide the organic environment that plants need for productive growth. Glomalin would have evolved this function, because supposedly it was first formed when there was no other organic matter in the soil. In the present investigations, an attempt was made to assess the occurrence and amount of EEG and TG with an objective to find the distribution of AM fungi and to find the relationship between spore number and the amount of glomalin associated with the rhizosphere soils of some trees in Warangal district, Andhra Pradesh, India.

Material and methods

In the present investigations, 22 rhizosphere soils of different tree species (Table 1) were collected from different agro-edaphic regions of Warangal district (Mylaram (My), Chelpur (CH), and Bhupalapally (BPL)), brought to a laboratory, and stored at 4°C until experimentation. The investigations were divided into two parts. In the first part, resting spores of AM fungi were extracted from rhizosphere soil and enumerated. In the second part, a quantitative estimation of easily extractable and total glomalin (Wright and Upadhyaya, 1996) was done.

Extraction and enumeration of AM resting spores

The extraction and enumeration of AM resting spores was done by the method suggested by Gerdemann and Nicolson (1963). Ten grams of soil collected from rhizosphere was poured into 100 ml of water, subjected to shaking on a horizontal shaker for 30 minutes, and allowed to settle. The supernatant liquid was passed through a coarse sieve (500 to 800 mm) to remove large pieces of organic matter. The lower collected liquid was passed through sieves of decreasing pore size (250, 106 and 45 µm). This process was continued till all the colloidal materials passed through the sieves. All the debris collected on the sieves was spread on a petridish and observed under a stereo-binocular microscope for the presence of resting spores; thereafter, the total number of spores present was enumerated.

Extraction of EEG

One gram of soil taken in an autoclavable centrifuge tube with 8 ml of 20 mM sodium citrate (pH 7.0) was autoclaved for 30 minutes at 121°C. Later it was centrifuged for 15 min at 8000 xg. The supernatant that contained the EEG was collected, quantitatively measured, and then stored at 4°C until further use.

Extraction of TG

One gram of soil was placed in an autoclavable centrifuge tube with 8 ml of 50 mM sodium citrate (pH 8.0) and autoclaved for 60 min at 121°C and then centrifuged for 15 min at 8000 x g. The supernatant containing the protein (TG) was collected and stored at 4°C. This process was repeated until all the TG was extracted. The supernatant contains the protein (TG). The extracts obtained in each round were pooled together and the final total volume was measured with a graduated cylinder.

Quantitative estimation of glomalin

The quantitative estimation of glomalin was done using the method suggested by Lowry *et al.*, (1951). Five millilitres of alkaline copper sulphate reagent was added to 1 ml of extract and incubated at room temperature for 10 minutes. To this 1 ml of 1 N NaOH and later 0.5 ml of FC reagent were added and the optical density of the resultant blue colour was read at 660 nm. The concentration of glomalin was calculated from the standard graph plotted for bovine serum albumin (BSA).

The results obtained were subjected to statistical analysis using Smith's Statistical Package (SSP version 2) at alpha levels of 0.05. Analysis of variance (ANOVA) was calculated for different parameters.

Results and discussion

The results obtained with regard to total number of spores and the quantity of EEG and TG are presented in Table 1.

A critical perusal of Table 1 reveals that the AM fungal spore population varied with the host tree species and the type of soil. The highest number of spores was recorded from rhizosphere of *Sesbania grandiflora* (BPL-19; 512) and the lowest from

Table 1 Quantitative estimation of Easily Extractable Glomalin (EEG) and Total Glomalin (TG) isolated from the rhizosphere soils

S. no.	Sample	Host tree	Total no. of spores/10 gm soil	EEG ug/ml	EEG* mg/1 gm soil	TG ug/ml	TG* **mg/1 gm soil**
1	My-01	*Acacia nilotica*	273	140	1.15	80	0.66
2	My-02	*Albizzia lebbeck*	269	150	1.22	190	1.47
3	My-03	*Dalbergia sissoo*	289	170	1.31	170	1.34
4	My-04	*Eucalyptus tereticornis*	320	240	1.89	180	1.43
5	CH-01	*Polyalthia longifolia*	319	260	2. 0	240	1.91
6	CH-02	*Pongamia pinnata*	338	300	2.39	330	2.69
7	CH-03	*Acacia nilotica*	325	260	1.98	420	3.33
8	BPL-01	*Punica granatum*	482	420	3.31	280	2.25
9	BPL-03	*Tectona grandis*	283	165	1.32	180	1.13
10	BPL-04	*Acacia nilotica*	293	180	1.51	310	2.46
11	BPL-05	*Albizzia lebbeck*	362	390	3.17	320	2.49
12	BPL-07	*Dalbergia sissoo*	173	100	0.8	180	1.15
13	BPL-09	*Eucalyptus tereticornis*	295	200	1.65	190	1.44
14	BPL-11	*Polyalthia longifolia*	309	250	1.97	150	1.25
15	BPL-12	*Pongamia pinnata*	285	165	1.31	190	1.53
16	BPL-13	*Psidium guava*	302	230	1.8	130	1.09
17	BPL-14	*Azadirachta indica*	274	140	1.21	90	0.73
18	BPL-15	*Mangifera indica*	298	210	1.61	130	1.03
19	BPL-17	*Sapindus emerginatus*	270	150	1.29	100	0.96
20	BPL-18	*Saraka indica*	297	220	1.74	220	1.74
21	BPL-19	*Sesbania grandiflora*	512	450	3.6	260	2.08
22	BPL-22	*Terminalia catappa*	353	340	2.7	175	1.35

Note: *Means of triplicate values

Note to Table 1: Fisher's Two-Factor ANOVA for locations and parameters of glomalin estimation

Source of Variation	SS	df	MS	F	P-value
Between samples	16.2241486	20	0.811207	3.456707	0.003917
Between parameters	0.60384038	1	0.603840	2.573077	0.124372
Error	4.69352762	20	0.234676		
Total	21.5215166	41			

rhizosphere of *Dalbergia sissoo* (BPL-7; 173). The spore number appears to be significantly determined by the host plant rather than the type of soil. The quantities of EEG and TG also varied with the spore number that in turn is determined by the host plant. The levels of EEG were found to be high in rhizosphere of S*esbania grandiflora* (BPL-19 3.6 mg/1 gm soil) and low in *Dalbergia sissoo* (BPL-7; 0.8 mg/1 gm soil). On the other hand, the highest TG levels were noticed from the rhizosphere of *Pongamia pinnata* (CH-02; 2.69 mg/1 gm soil), whereas the rhizosphere of *Acacia nilotica* (MY-01; 0.66 mg/1 gm soil) was found to be lowest. From the above results, a positive correlation could be observed between spore number and EEG, whereas no such correlation could be observed between spore number and TG, and even within EEG and TG (Figure 1). There is a significant difference between the samples (between spore number/between EEG/between TG) $P< 0.01$ (0.003917), whereas no significant difference was

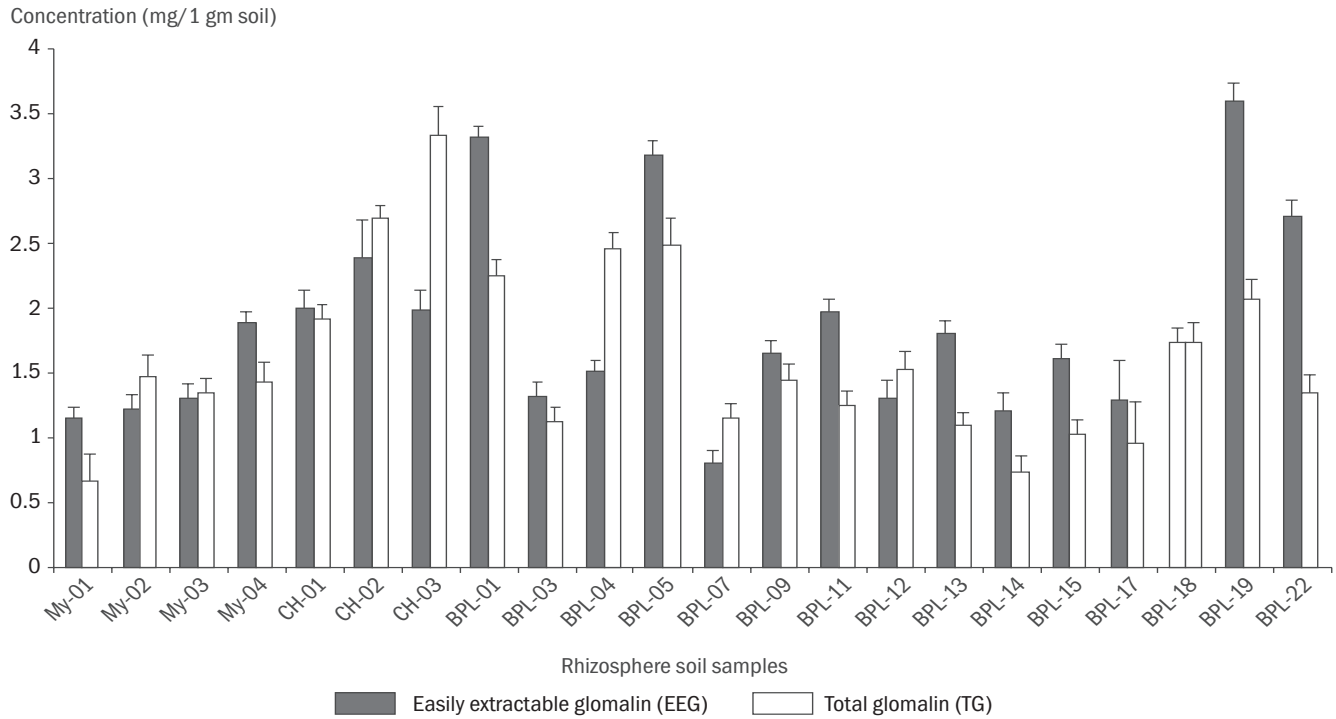

Figure 1 Concentration of Easily Extractable Glomalin (EEG) and Total Glomalin (TG) isolated from the rhizosphere soils
Note: The bars represent the means of the concentrations of EEG and TG (mg/ 1 gm soil) and the Y-error bars represent the standard error of the means.

observed between parameters (spore number, EEG, and TG) P>0.01 (0.124372). Thus, it is evident that the amount of glomalin is determined by the spore number and host species rather than the soil.

The results presented through Figure 1 clearly show the means and standard errors (SE) of the values of EEG and TG. The ANOVA table shows the values of 'P' and 'F' between locations and between parameters at 0.05 alpha values.

Glomalin has been found in abundance (typically 2 to 15 mg g-1 and up to >60 mg g-1) in a wide range of soil environments (acidic, calcareous, grassland, and cropland) (Wright and Upadhyaya, 1998; Wright *et al.*, 1999) and appears to be as ubiquitous as AM fungi themselves (Carlile and Watkinson, 1996; Olsson *et al.*, 1999; Wright and Upadhyaya, 1998). Driver *et al.* (2005) reported that glomalin is a component of the hyphal wall. However, Treseder and Turner (2007) found that hyphal length does not correlate with glomalin production. Lovelock *et al.*, (2004) noted that diameter is an important determinant of glomalin production. They further felt that glomalin concentrations might be less in fine hyphae compared to coarse hyphae, probably due to greater proportion of cytoplasm to hyphal wall volume in fine hyphae. Therefore, the higher production of glomalin by *G. mosseae* may be a result of hyphal characteristics, the inherent ability of the fungus to produce glomalin. Nonetheless, Nichols and Wright (2004) stated that overall variations observed among isolates, species, and hosts of AMF do not have a similar trend; thus, factors other than fungal activity (Bedini *et al.*, 2007) and host productivity (Violi *et al.*, 2007) must be considered.

It has been argued that plant species respond differently to AMF colonization because of the variation in plant dependence on AMF (Van der Heijden *et al.*, 1998; Treseder and Allen, 2000; Burleigh *et al.*, 2002). For example, nutrients gained from individual AMF species vary among plant species (Burleigh *et al.*, 2002; Smith *et al.*, 2004). In addition, a positive and significant enhancement of plant growth through glomalin production has been observed (Violi *et al.*, 2007; Bedini *et al.*, 2009). Although Bedini *et al.* (2009) reported that plant influence on glomalin-related soil protein (GRSP) levels was not significant when plant size was separated from mycorrhizal effect; they noted that host plant biomass might be related to GRSP production by AMF. Thus, given that the available information indicates that glomalin is located in AMF spore and hyphal walls (Driver *et al.*, 2005; Purin and Rillig, 2007), the evidence supports the hypothesis that greater spore production by AMF induces higher glomalin production.

Thus, the present study indicates that glomalin is widely distributed in soils under investigation, which also indirectly indicates the occurrence and distribution of AM fungi with different host tree species. The study

further reveals the fact that glomalin plays a significant role in the formation of soil aggregates, and the stability and productivity of the plant.

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Impact of long-term wastewater irrigation on the abundance of arbuscular mycorrhizal spores in the peri-urban soil of Varanasi

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Introduction

The growing competition for scarce water resources, coupled with laws limiting groundwater pumping, has led to utilization of low quality water in irrigated agriculture in the Indo-Gangetic plains(Gupta and Seth, 2007). However, applying wastewater to arable lands also involves certain environmental and agricultural risks (Sharma *et al.*, 2008). Wastewater differs from fresh water in terms of higher contents of electrolytes, dissolved organic matter, suspended solids, and biochemical and chemical oxygen demand. These varied constituents in the applied water can affect soil physico-chemical and biological properties. Increased amount of heavy metals could prove to be toxic for soil microorganisms. Although the concentration of heavy metals in waste water are low, long-term use of such waste water on agricultural lands often results in the build-up of elevated levels of these metals in soils. Among soil microorganisms like Arbuscular mycorrhizal fungi (AMF),an important biotic component of agricultural soils,are known to play a key role in the mobilization and immobilization of metal cations (Rakshit and Ghosh, 2009; Pal, 2011), thereby changing their availability for plants by effectively enlarging the rhizosphere. However, only a few studies have been carried out involving interactions between AMF and metals as a source of soil disturbance. To our knowledge, no studies have been reported on the long-term effects of increasing concentrations of waste water on the diversity of mycorrhizal propagules or on the influence of the host plant on AM fungal diversity in soils polluted by heavy metals. Our aim in this study was to determine the manner in which AM fungal diversity is affected by the addition of waste water for a long period of time.

Materials and methods

Study area

The experiment was conducted at an urban fringe of the subtropical area of Varanasi city, situated in the Eastern Gangetic plain (25°18´ N latitude and 83° 01´ E longitude and 76 m above the sea level) of northern India, with an average annual rainfall of 1100 mm and mean annual temperature ranges between 20–42°C and 9–28°C, respectively. This field site has been contaminated by surface application of sewage sludge and surface irrigation with waste water generated from domestic sewage, effluents discharged from small-scale fabric, plastic, battery industries, dyeing, metal plating, bicycle tyres, and heavy agricultural equipment located in the urban areas of Varanasi since the 1990s.

Soil sample collection

Soil samples were collected in triplicate from rhizosphere of 15 crop species at a depth of 0–30 cm and combined to form approximately 500 g of soil. Roots were separated and 100 g of air-dried soil was employed for extraction of AM fungal spores. Rest were air dried, crushed, and passed through a 2mm mesh-sieve and stored at ambient temperature before analysis of soil properties and concentrations of heavy metals by the standard soil analysis technique. The available Cd and Ni were extracted by DTPA solution and analysed in atomic absorption spectrophotometer. The pH of the soil was determined by using a combined electrode (Jackson,1967) and EC by conductivity metre (1:2.5 soil water suspension); organic matter by chromic acid wet digestion method (Walkley and Black,1939); plant available nitrogen by alkaline permanganate method (Subbiah and Asija,1956); phosphorus by $NaHCO_3$ (Olsenetal.,1954); and potassium by flame photometry (Jackson,1958). Metal concentrations and the selected soil properties are showed in Table 1. According to the Indian National Standards Institution (Awasthi, 2000), this soil is seriously contaminated with Cd as described in previous studies (Sharma *et al.*, 2008).

Arbuscular mycorrhiza spore extraction and spore count

AMF spores were isolated from 100 g of soil by wet sieving using two sieves with aperture sizes of 425

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Table 1 Chemical properties of the contaminated soil

Parameter		Value (Range)
pH (1:2.5 H2O *m/V*)		8.0–9.0
EC(1:2.5 H2O *m/V*) dSm^{-1}		0.072–0.316
OM (organic matter, gkg^{-1})		0.77–3.52
CEC (cation exchange capacity, cmol/kg)		16.5–18.3
Total N (%)		0.11–0.15%
Av P ($mg\ kg^{-1}$)		8.1–8.9
Available K ($mg\ kg^{-1}$)		62.5–82.3
DTPA(diethylenetriaminepentaacetic acid) -extractable Cd ($mg\ kg^{-1}$)		5.7–6.75(0.8)
DTPA(diethylenetriaminepentaacetic acid) -extractable Ni ($mg\ kg^{-1}$)		16.50–20.85(50)
Dehydrogenase activity (µg TPF/g/day)		552–592
Phosphatase activity (µg p- nitrophenol/g soil/ hour)	Acidic	10.5–12.7
	Alkaline	251–290
Urease activity (µg urea hydrolysed/g soil/hour)		361–384.7

Note: The values in brackets are the standard values of heavy metals for soils intended for agricultural uses with pH ranging from 6.5 to 7.5 based on Indian National Standard.

and 63 µm and decanting method (Gerdemann and Nicolson, 1963), followed by sucrose centrifugation using a 1.17 M sucrose solution at a speed of 2000 rpm for 5 minutes. After centrifugation, the supernatant was poured through 50 mm pore-size mesh and quickly rinsed with tap water. Spores were counted with a Don caster dish under the dissecting microscope and grouped according to morphological characteristics.

Enzymatic activity in the rhizosphere

In the laboratory, subsamples were taken from bulk samples and were further homogenized. Large roots or shells were removed and wet soil samples were added to polypropylene centrifuge tubes for analysis of enzymatic activities. For alkaline and acid phosphatase enzymatic activity, the base substrate used was p-nitrophenol bound with phosphate (Tabatabai and Brenmer, 1969). The artificial substrate (1 mL, 0.05 M), toluene to inhibit microbial growth during incubation, a pH buffer (pH 11 for alkaline phosphatase and pH 4.5 for acid phosphatase) were incubated in closed polypropylene centrifuge tubes at 37°C for 1 hour. At the end of incubation, enzyme activity was stopped by addition of 4mL of 0.5M NaOH; the mixture was filtered and the extract was analysed using a UV-VIS spectrophotometer at 420 nm. Absorbance of filtrates was compared with p-nitrophenol standards. To account for non-enzymatic substrate hydrolysis, values for controls were subtracted from sample replicates. For DHA activity 2, 3, 5-triphenyltetrazolium chloride (TTC) was added as substrate to the fresh moist soil samples and incubated at 37°C for 24 hours. After incubation, the triphenyltetrazoliumformazan (TTF) formed was extracted, centrifuged, and estimated spectrophotometrically at 485 nm. Dehydrogenase activity is expressed as microgram of TTF released per gram of dry soil per 24 hours (Casida *et al.*, 1964). The urease activity was determined by the method proposed by Kandeler and Gerber (1988) and Wang *et al.* (2007).

Results and discussion

Soils of the experimental sites were neutral to alkaline with a pH value higher than 7.5 (Table 1). Experimental sites recorded OM 0.77 to 3.52 g kg^{-1} and total N of 0.11%–0.15%, respectively (Table 1). The soil is low in organic carbon, medium to high in phosphorus, and medium to low in potassium content. The 2:1 extract of soil exhibited low EC. Continuous application of waste water to the soil led to higher concentrations of heavy metals ($mg\ kg^{-1}$) in the soil with DTPA (diethylenetriaminepentaacetic acid), extractable Ni and Cd ranged between 16.50–20.85 and 5.7–6.75, respectively. Among the two heavy metals studied, Cd in the soil was above the permissible limits of Indian and EU standards (Table 1).

Soil biochemical activity was higher compared to other soils of this dry land region.

AM fungal spore density varied greatly between plant species. AM fungal species belonging to the genus *Glomus* were found in rhizosphere samples of different crop species. The ability of *Glomus* to dominate soil rhizosphere indicated that *Glomus* has a broad host range. The host plant also had a significant effect on the total AMF spores produced in the rhizosphere, *Sorghum bicolour* being the trap plant that produced AMF spores most effectively possibly because of the higher root growth rate of this plant species, which can facilitate further contact with most AM fungi present in the soil. Host plants also exerted a varied effect on AMF diversity, with *T.erecta* and *S. bicolour* promoting significantly higher number as well as levels of diversity in their rhizosphere(s) than those produced by cauliflower and radish. Therefore, the impact of crop rotation on AMF is significant and should be considered in field management. Variation in spore density and arbuscular mycorrhizal fungi colonization in relation to crop plants can be linked

to factors, such as plant phenology, dependency on mycorrhiza, changes in the soil microenvironment, or unknown host characteristics. Total AMF spore number decreased significantly with increasing amounts of heavy metals in soil, from 330 spores (per 100 g of dry soil) in marigold with a heavy metal content mg kg^{-1} to 56 spores in cauliflower with a heavy metal content mg kg^{-1}(Table 2). The total number of AMF spores strongly decreased with increasing amounts of heavy metals, but the AMF propagules never disappeared completely in soils irrigated with waste water for the last twenty five years, suggesting a certain adaptation of these indigenous AMF to such environmental stress.

A negative correlation was shown between the total number of AMF spores and soil heavy metal content (Ni:r = -0.52, P < 0.05 and Cd: r = -0.56 , P < 0.05) corroborating its sensitivity to the presence of heavy metals (Table 3). Correlation coefficient was higher for the concentration of free cations (Cd^{2+}) in the soil solution compared to Ni as it is toxic to soil microbes. This fungi toxic effect of metal can cause inability of certain AMF species' to colonize the root system and/or to multiply in the rhizosphere. Only AMF species better adapted to the disturbed environment by the addition of metals many overcome the stress situation and complete their life cycles.

Spore count was negatively correlated with P (r = -0.48, P < 0.05) and N(r = -0.80, P < 0.01) in the experimental soil. This is due to the fact that higher nutrient content,especially P content, resulted in suppression of external hyphal growth or in an increase of P concentration in plant tissue. Suppression of growth and colonization rate of AM fungi due to high P content was reported earlier by Ahmed *et al.* (2000). A positive response between AM and soil K (r =0.70, P < 0.01) in the present experiment suggests that a minimum soil K was necessary for AM growth in certain plant species. No significant correlation between AM spore count and pH, EC, and organic matter content was observed. Spore count was positively correlated with enzyme activities for most of the soils.DHA was significantly correlated with mycorrhizal spore count. DHA was correlated positively (r =0.49, P < 0.01) with changes in the microbial biomass, including AMF propagules due to long-term waste water application while the urease activity was negatively affected by mycorrhizal fungal population (r = -0.71, P < 0.01). Mycorrhizal colonization clearly responded to distinctive increases in the activities of enzymes involved in P dynamics. ACP (r =0.72, P < 0.01) and ALP (r =0.68, P <

Table 2 Total number of AMF spores (per 100 g of dry soil) in rhizosphere of different crops

Type of host Plant	Plants	AMF spores
Cereals	Rice	94
	Sorghum	163
	Jowar	160
	Bajra	151
Vegetables	Palak	85
	Papaya	166
	Turmeric	84
	Radish	71
	Okra	154
	Cauliflower	56
Flowers	Marigold	330
	Hibiscus	82
	Jasmine	105
	Rose	73
	Bela	145
Range	56–330	
Mean	126.6	
SD	48.79	

Table 3 Pearson's correlation coefficient (r) between arbuscular mycorrhizal spore density and edaphic and biochemical factors

Parameters		Spore density(number of AMF spores / 100 g of dry soil)
pH (1:2.5 H2O m/V)		-0.36ns
EC(1:2.5 H2O m/V) dSm-1		-0.26 ns
OM (organic matter, g kg-1)		-0.15 ns
Total N (%)		-0.80**
Av P (mg kg-1)		-0.49*
Available K (g/kg)		0.70**
DTPA(diethylenetriaminepentaacetic acid) -extractable Cd (mg kg-1)		-0.56*
DTPA(diethylenetriaminepentaacetic acid) -extractable Ni (mg kg-1)		-0.52*
Dehydrogenase activity(µg TPF/g/day)		0.49*
Phosphatase activity (µg p- nitrophenol/g soil/ hour)	Alkaline	0.68**
	Acid	0.73**
Urease activity (µg urea hydrolysed/g soil/hour)		-0.71**

Notes: * p < 0.05, ** p < 0.01

0.01) was positively correlated with the development of mycorrhizal spores. As heavy metals cannot be chemically degraded, bio-remediation of metal-polluted soils is limited mainly to immobilization through phytostabilization by promoting plant growth to reduce or eliminate the bioavailability of metals. In this context, role of indigenous AMF constitutes an important functional component of the soil-plant ecosystem that is critical for sustainable productivity in wastewater treated soils.

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CENTRE FOR MYCORRHIZAL CULTURE COLLECTION

Mitochondrial genome sequences are more preferred reliable molecular markers in Arbuscular Mycorrhizal Fungi over nuclear genome sequences

Burla Sashidhar and Alok Adholeya

Introduction

Arbuscular Mycorrhiza Fungi (AMF) are vital components of the microbial soil community, forming the most commonly occurring underground network, probably the oldest and largest symbiotic association between the roots of more than 80% of all terrestrial plant species and members of the phylum Glomeromycota. AMF help plants in several ways such as by improving plant fitness by increasing soil health, seedling establishment, plant fecundity, and tolerance to some root pathogens. It also aids in improving soil nutrients uptake, especially of poor mobility nutrients like phosphorus which play an important role in carbon and nitrogen recycling, sequestration of toxic heavy metals, water relation and formation, etc., and the fungi itself is benefited from the plants for carbon nutrition. It is universally accepted that the more AMF species are present in one ecosystem, the more diverse the ecosystem will be. But, some AMF individuals perform better than others in improving the plants fitness and directly affect crop yields as a whole. Despite many positive effects on plant nutrition, knowledge of AMF genetics is limited because of its obligate biotrophic nature; single spore contains many nuclei with genetic differences. Until now, attempts to generate strain-specific genetic markers have proved unreliable. The current review is based on a newly published research article by Formey *et al.* (2012) on the method to generate reliable strain-specific genetic markers in different AMFs by designing specific primers for Polymerase Chain Reaction (PCR) that are able to differentiate five laboratory strains of *Rhizophagus irregularis*, all of which have been previously problematic to differentiate. Formey *et al.* (2012) found that intra- and interstrain variability in different isolates of AMF based on mitochondrial genome sequences that are invaded by mobile selfish DNA elements.

Nuclear genome polymorphism is high in arbuscular mycorrhizal fungi

AMF play an important role in altering the plant community structure and consequently increase plant diversity and productivity. In recent years, most of the research work on AMF was focused mainly on taxonomy, phylogeny, ecology, genetics, and functional symbiosis with application of different traditional and molecular methods but associated with a number of difficulties in studying AMF. Recently, Ehinger *et al.* (2012) found the relative abundance of four alleles of a single locus Bg112 after three successive generations. They have found that 'families' of single spore isolates derived from a single spore culture in the previous generation were more similar to one another than to families from other single spore isolates. The genetic diversity contained in one initial spore repeatedly gave rise to genetically different variants of the fungus with novel phenotypes. None of the alleles became extinct, and no new alleles were observed in this study.

Traditionally, AMF identification was based on the morphology of the spores (Gerdemann and Trappe, 1974; Morton, 1988; Walker, 1992). Other biochemical methods of identification and characterization of AMF were based on fatty acids profiling (Graham *et al.*, 1995), spore protein profiling through SDS-PAGE (Avio and Giovannetti, 1998), and isozymes analysis of malate dehydrogenase and esterase loci (Dodd *et al.*, 1996). These methods are not widely used but can complement the other methods of characterization and do not solely identify the AMF diversity. Intraspore rDNA variations were well reported in the genome of a single nucleus as well as in other organisms and/or among nuclei residing in the same cell (Buckler *et al.*, 1997). The genetic studies on multiple nuclei in the glomeromycotan mycelia raised many contradictory statements; some reported that nuclei residing in single spore are heterokaryotic (Kuhn *et al.*, 2001; Hijri and Sanders, 2005) while others data indicated that they are homokaryotic (Pawlowska and Taylor, 2004). A heterokaryotic genetic system implies absence of a fixed nuclear genotype for a fungal isolate, with populations of nuclei changing within a species. To address this issue, monoaxenic cultures of *G.*

intraradices was grown *in vitro* using transformed carrot roots and amplified fragment length polymorphism (AFLP) analysis on *G. intraradices* showed a high degree of genetic and phenotypic diversity among individual isolates (Koch *et al.*, 2004).

rRNA genes of the genomic DNA are available in high copy number with highly conserved as well as variable sections and hence they were explored as molecular marker for identification and characterization of AMF. These sequences allow in distinguishing taxa at many different levels (Saiki *et al.*, 1988). The first studies on the subgroup of order Glomales on molecular phylogeny using 18S rDNA subunit sequence as a basis for detection of AMF was conducted by designing the specific primer VANS1 (Simon *et al.,* 1992) and an identification system using SSCP later (Simon *et al.*, 1993) was proposed. From then, AMF are molecularly characterized using different sets of primers at different regions of the nuclear encoded rDNA genes containing internal transcribed spacers ITS1, ITS2, and the 5.8S gene because of its high polymorphic nature. The regions of small-subunit (SSU) rRNA gene (Schussler *et al.*, 2001), the regions of large-subunit (LSU) rRNA gene (Alves *et al.*, 2006; Clapp *et al.*, 2001; Raab *et al.*, 2005), and a combination of all rRNA gene sequences that constitute complete 1500 bp fragment of rDNA locus was used as identification tool in AMF (Stockinger *et al.*, 2010). Sometimes this has been proved with respect to single spore rDNA gene sequence analysis of the intra-species variations (Hijri *et al.*, 1999; Jansa *et al.*, 2002; Nilsson *et al.*, 2008; Sokolski *et al.*, 2010) are more than the inter-species variations (Borstler *et al.*, 2008; Rosendahl, 2008), and because of this, rDNA single genetic locus does not give a clear cut-off between intra-species and inter-species variation. Hence, other highly conserved house-keeping protein-encoding genes were used as targets for the marker development for AMFs, i.e., actin gene sequence analysis (Helgason *et al.*, 2003), -tubulin gene sequence analyses (Corradi *et al.*, 2004a; Corradi *et al.*, 2004b; Msiska and Morton 2009), and P-type H^+-ATPase (Corradi *et al.*, 2004a; Ferrol *et al.*, 2000) have been investigated, but success rate was very low. While genes for elongation factor (ef1-alpha), V-H^+-ATPase, and F-ATPase subunit sequences were analyses (Sokolski *et al.*, 2010) and found efficacious only for discriminating between unrelated *Glomus* species.

A number of other molecular methods have been investigated for studying the genetic diversity analysis and species identification. Which includes terminal restriction fragment length polymorphism (T-RFLP) with fluorescently labelled primers combined with restriction digestion to analyse the sequence variation either from single or mixed-species DNA samples (Dickie and Fitzjohn, 2007) and increasingly used as high an throughput fingerprinting technique in AMF ecology. But, the peak-profile of T-RFLP and database T-RFLP also raised a number of errors. Croll *et al.* (2008) used 10 simple sequence repeat (SSR), introns of a nuclear gene as molecular markers while Mathimaran *et al.* (2008) used a set of 18 SSR markers to analyse genetic variation among eight different isolates of *G. intraradices.* Only two isolates from these sets appeared to be identical clones. Genetic diversity among fungal isolates was high, but isolates from far distance locations like from two different continents were not substantially different (Borstler *et al.*, 2008). These results complemented those of Koch *et al.* (2004) and indicated that much of the global genetic diversity of *G. intraradices* could be represented just within one field site. Multilocus genotypes also have been identified from individual field collected spores of *G. mosseae* using markers from single copy nuclear genes GmFOX2, GmTOR2, and GmGIN1 (Stukenbrock and Rosendahl, 2005). An inorganic phosphate transporter gene sequence (852-bp section) has been used to discriminate between 10 *Glomus* fungal species represented by 25 strains. This gene was particularly valuable in differentiating between different similar morphotypes with nucleotide and amino acid sequence differences greater than 3%. This gene was also proposed as a reliable barcode for the *Glomeromycetes* (Sokolski *et al.*, 2011). Neither the SSR or RFLP markers listed earlier, nor the 'Single Nucleotide Polymorphisms' of Stukenbrock and Rosendahl (2005) and inorganic phosphate transporter gene have been successfully applied to the mycorrhizal roots from the field so far.

Nuclear genome verses mitochondrial genome

There has been a long debate over genome size and nuclear organization in AMF. Different studies conducted have showed different results varying in genome size and ploidy level. A large amount of sequencing data was generated (Martin *et al.*, 2008), but the sequence assembly and annotation seems not efficient and still not possible because of its high genetic polymorphism among nuclei of a single spore. A few reports estimated the nuclear DNA content of AMF isolates but none of these studies have shown similar results on genome sizes. Hijri and Sanders (2004) showed size of the nuclear genome was high and the genome size and ploidy level of DAOM197198 isolate has already been determined in *G. intraradices* DAOM197198 which was also chosen for the first complete genome sequencing project on AMF (Martin *et al.*, 2008) but it was a

long hard road to get the large genome sequence data assemble and annotate. The average genome size of AMF seems to be highly variable where Hijri and Sanders (2004), reported 15.7 Mb in *G. intraradices* DAOM197198 and 37 Mb in *Glomus etunicatum (*Hijri and Sanders, 2005), while it was reported 250 Mb in *Glomus versiforme* (Bianciotto and Bonfante, 1992) and 740 Mb in *Gigaspora margarita* (Hosny *et al.*, 1997). Recently, Sedzielewska *et al.*, (2011) showed that the genome size of *G. intraradices* AMykor isolate was 150 Mb which was in comparison with the nuclear DNA content of *G. intraradices* DAOM197198 and an *Arabidopsis thaliana* as reference genome sizes, measured using FC and Feulgen DNA image densitometry. These values are 10 times bigger than previously reported (Hijri and Sanders, 2004) but complement the results of FC data, indicate the true picture on the genome size of *G. intraradices* (Sedzielewska *et al.*, 2011).

In contrast, mitochondrial genome size is comparatively small (usually < 100 kb) which ranges widely from 70 783 bp for DAOM197198 to 87 754 bp for MUCL_43204 (Formey *et al.*, 2012). It has been shown that mitochondrial DNA in AMF was homogeneous within the isolates of the same species, which made it possible to sequence and assemble complete mtDNA sequence of an isolate *G. intraradices* by Lee and Young (2009). The sequencing showed that the genes are homogenous as there was no evidence of substantial sequence variations and hence mtDNA can act as good marker for identification which was true in terms of a long history as molecular markers in other organisms. In ectomycorrhizal fungal molecular identification and species characterization was carried out successfully using mtLSU rRNA analysis on roots colonized with EMF and the large sets of data generated were available for comparative study (Bruns *et al.*, 1998). The first sequences from the mitochondrial genome of the AMF have been documented the presence of unique sequences without substantial variations in an isolates of *G. intraradices* and *G. proliferum* mtLSU rRNA region (Raab *et al.*, 2005). Borstler *et al.* (2008) showed by using mtLSU rRNA gene, combined with RFLP approach that the genetic diversity within *G. intraradice* is substantially higher than previously expected. These results suggested that mtLSU rDNA gene sequences provide useful information on intra-specific variation that occurs in closely related *Glomus* species (Thiery *et al.*, 2010) and these variations are mostly because of the presence/absence nature of introns. Based on the advancement in the mitochondrial genome sequencing projects more AMFs mtDNA genomes were sequenced and assembled. Till date, there are at least eight different AMF mtDNA genome that have been sequenced, two of them were strains of *G. irregularis* – FACE#494 (Lee and Young, 2009) and DAOM197198 (HQ189519.1). Two other species of AM fungi – *Gigaspora margarita* (Pelin *et al.*, 2012) and *Gigaspora rosea* (Nadimi *et al.*, 2012) were sequence and have been published. Four different strains of *G. irregularis* AMF mtDNA genomes, MUCL_43204, MUCL_46239 and MUCL_46240/MUCL_46241 were sequenced and were submitted to Genbank with the accession numbers JQ514224, JQ514223 and JQ514225, respectively (Formey *et al.*, 2012).

Mobile genetic elements invade randomly in *Rhizophagus irregularies* mitochondrial genome and showed no variation within strain

This wealth of mtDNA genome sequence information was then studied further for comparative analysis of mtDNA genomes to intra- and interstrain variability. All the mt DNA genomes are circular with an average GC content of 37% (Formey *et al.*, 2012). The strains exhibit a different number of predicted open reading frames (ORFs), compared to FACE#494 having the lowest ORF content (19), while MUCL_43204 having the highest (34). One important feature was that all strains have the same classic set of proteins that was already annotated in the FACE#494 strain (Formey *et al.*, 2012). These variations in the number of predicted ORFs could partly explain the variation in genome size. Intraspecific polymorphic regions would be helpful to design markers for fundamental genetic studies *in vitro* or in the field, or for quality control of AM fungal inoculum. These variations have prompted to explore these mitochondrial genes as a potential alternative to nuclear markers because of their intra-strain sequence homogeneity and high copy number per spore (i.e., each AMF spore typically contains hundreds of mitochondria).

The mt genomes are unique and have been shown homogeneous within isolates of the same species of each strain. Further, some mitochondrial intergenic regions vary substantially among isolates of *G. irregularis* and closely related species, with the presence of numerous insertions/deletions and eroded mobile elements. The presence of this polymorphism is mainly because of three types of variability generating element (VGE) viz., homing endonucleases (HEOs), DNA polymerase domain-containing open reading frames (DPDCO) and small inverted repeats (SIR) (Formey *et al.*, 2012). The discovery these VGEs highlights the great intraspecific plasticity of the *G. irregularis* mt genome. These mobile genetic elements have eroded the mitochondrial genomes of *G. irregularis* strains, which range from just over 70 to 88 kb. Such interstrain variation had already been

reported in other fungi (e.g., *Schizosaccharomyces pombe*; Schafer, 2003), but it is important and yet unknown from where these mobile genetic elements were originated and how stable these variations exist within the isolate.

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Recent references

The latest additions to the network's database on mycorrhiza are published here for the members' information. The list consists of papers from the following journals.

- *Agriculture, Ecosystems & Environment*
- *Applied Clay Science*
- *Applied Soil Ecology*
- *Basic and Applied Ecology*
- *Ecological Modelling*
- *Environmental and Experimental Botany*
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- *Scientia Horticulturae*
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- *Soil Biology and Biochemistry*

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