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ASSESSMENT OF PHOSPHORUS USE EFFICIENCY ON ACID TOLERANT ARBUSCULAR MYCORRHIZAL FUNGI USING RADIOTRACER TECHNIQUE AND THEIR PLANT GROWTH PROMOTION IN SORGHUM WITH COMBINED INOCULATION OF PHOSPHOBACTERIUM AND *AZOSPIRILLUM*

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ABSTRACT

This paper focuses on isolation of acid tolerant arbuscular mycorrhizal (AM) fungi and assessment of phosphorus use efficiency on mycorrhizal sorghum plants through radio tracer technique. The triple inoculation effect of AM, *Azospirillum* and phosphobacterium was also evaluated in growth enhancement of sorghum. Twelve different AM fungi were identified from level of acid tolerant in pH 5.3 based on percent root colonization and total spore count through reinfection study among all AM seven acidic soils. Two AM fungi namely *Acaulospora scrobiculata* and *Funneliformis geosporus* were found to be higher fungi isolated. Pot house experiment showed mycorrhizal plants have enhanced ^{32}P specific activity except for control with 100% NPK. Inoculation with two arbuscular mycorrhizal (AM) fungi namely *Acaulospora scrobiculata* and *Funneliformis geosporus* along with *Azospirillum* and phosphob acterium registered a 2-3 fold increase in P-use efficiency, total dry matter content, N uptake and P uptake with 4 fold increase and phosphatase activities in sorghum at 45 days of plant growth.

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INTRODUCTION

Acidic soils in India are widespread and occupy about 30% of the cultivated area around 48 mha (Panda, 1998). Among all states in India, Tamil Nadu is one of the affected states by soil acidity. There are mainly two districts Nilgiri and Pudukottai shows higher amount of acidic soil reaction. Phosphorus (P) deficiency is an important soil fertility problem in acidic soils because of its higher fixation by cations like aluminium (Al^{3+}), manganese (Mn^{2+}) and iron (Fe^{2+}) and very low recovery around 10-25% throughout the world from conventional high-soluble phosphatic fertilizer applied to the soil (Srivastava *et al.,* 2007). Hence, the concentration of bioavailable P for plants in the soil is very low reaching the level of 1.0 mg kg^{-1} soil including acidic soil (Goldstein, 1994). However, the use of conventional P fertilizers is highly limited in developing regions due to cost (Sanchez, 2002) and adverse environmental effects of P losses must be minimized (Tunney *et al.*, 1997). As rock phosphate (RP) has lower cost than others and show moderate recovery in acidic soil it is increasingly recognized as a potential fertilizer (Goenadi *et al.*, 2000). The use of phosphate solubilizing microorganisms, including arbuscular mycorrhizal (AM) fungi, has been proposed as a low-cost and low-energy mechanism to help increase the agronomic effectiveness of RP fertilizers and to enhance phosphorus use efficiency (PUE) in AM plants (Kumutha, 2001; Gyaneswar *et al.*, 2002). PUE is the efficiency with which P is used to produce dry matter when plant is in P deficient (Koide, 1991). Variation in PUE also occurs among plant species and as a consequence of variation in P availability and AM fungi colonization (Haynes *et al.*, 1991). Radio-tracer technique is one of the efficient methods and has been used for the assessment of PUE in AM plants by ^{32}P labelled phosphatic fertilizer (Dhinakaran and Savithri, 1997).

The AM symbiosis is recognized for its ability to increase the inflow of slowly mobile nutrients to plant roots, predominantly P (Tinker *et al.*, 1992). P taken up by AM fungi is considered to be from the same labile pool used by plant roots. However, there is increasing indication that mycorrhizal roots may be capable of using insoluble sources of inorganic P originated from phosphatic fertilizer application in soil that are not available to the roots (Duponnois *et al.*, 2005). Interactions between plant growth promoting rhizobacteria (PGPR) like phosphobacterium (P solubilizing bacteria) and AM fungi in the mobilization of P from RP have been consistently reported (Caravaca *et al.*, 2004; Cabello *et al.*, 2005). A potential nitrogen fixing bacteria *Azospirillum* and the phosphate solubilising bacteria have closer interactions in mycorrhizosphere and survive for a longer period in the rhizosphere of mycorrhizal roots (Karthikeyan and Prakash, 2008; Bharti and Pravesh, 2011). Hence, exploiting the potentials of these beneficial interactions will benefit the growth of plants in acid soil by enhancing the uptake of mineral nutrients. These interactive studies are in huge numbers in normal soil as maize (medium acid tolerant) host plant whereas in acid soils with sorghum plant (slight acid tolerant), the reports are very scanty. Further the impact of AM isolates from normal soil and inoculation with phosphobacterium and *Azospirillum* may not be

to the expected level in acid soils due to high P fixation.

Like symbiotic interaction studies, information about AM fungi and mycorrhizal (+AM) host plant responses at low pH (< 5.5) acidic soil is limited. Maximum enhancement of plant growth in acid soil varies with AM fungal isolates and soil pH, indicating adaptation of AM isolates to variable edaphic conditions (Clark, 1997; Clark *et al*., 1999). Hence, in acid soil, it is much worthful to isolate the efficient novel strains of arbuscular mycorrhizal fungi to increase plant phosphorous uptake and simultaneously to increase Al and Mn tolerance to plants is enormous because AM fungi accumulate these toxic ions in extramatrical fungal hyphae. It is hypothesized that the AM isolates obtained from acid soil must have the higher capacity to tolerate extended level of soil acidity, absorb enhanced level of P and promote plant growth. Hence, it is essential to identify an efficient isolate of AM fungus for enhancing P absorption as well as suitable for acid soils and are considered important to utilise these symbiotic interactions with other PGPR like *Azospirillum* and phosphobacterium.

With this background, the main objectives of the present study were to isolate the acid tolerant arbuscular mycorrhizal fungi, to assess the PUE in AM sorghum plant by ^{32}P radio-tracer technique and to investigate the impact of triple inoculation of AM fungi, phosphobacterium and *Azospirillum* to improve the sorghum plant growth as well as mineral nutrient acquisition.

MATERIALS AND METHODS

Soil sampling

Seven acidic soil samples with different pH from 4.4-6.6 were collected, of which first six samples from NPRC (National Pulses Research Centre) of Vamban (10º22' N, 78º49' 45.12'' E) in Pudukottai district and one sample from Western Ghats tropical rain forest (11º24' N, 76º43' E) in Nilgiri district of Tamil Nadu were collected (Table 1, Fig 1.). First six sampling sites were used as cultivable land of different crops with low input cropping system provided standard dose of fertilization and last one from undisturbed natural soil. From each site, about 2 Kg soil samples were collected from the top layer at 15-20 cm depth at four randomly selected locations during September, 2009. Samples collected from a site were pooled together and mixed well by quartering method, and representative subsamples were made. After removal of stubbles, pebbles and gravels manually, the subsamples were air-dried for 2 weeks and then packed into seven lots for storage at 4ºC for further use.

Determination of soil physio-chemical properties

The soil samples were used for assessment of physio-chemical properties (Table 1.) were further air-dried and analyzed. Soil pH was determined in 1:1, soil: water soon after the soil samples were brought to the laboratory. The total nitrogen (N) and available phosphorus (P) were determined according to Jackson (1973) and exchangeable potassium (K) was determined after extraction with ammonium acetate (Jackson, 1973).

Isolation AM fungal spores and identification of different AM isolates

AM fungal spores were isolated from seven different soils by standard wet-sieving and decantation method (Gerdemann and Nicolson,1963; Muthukumar *et al.,* 1996). AM isolates were identified by spore morpho-typing by intact and crushed spores through stained in polyvinylalcohol-lactophenol (PVLG) and Melzer's reagent (Schenck and Perez, 1990). Spore colour was examined under both phase contrast and binocular stereomicroscope on fresh specimens immersed in water. The identification of AM fungal spores was based on spore colour, size, surface ornamentation and wall structure with reference to the descriptions and images provided by the International Collection of Vesicular and Arbuscular Mycorrhizal Fungi (http://invam.caf.wvu.edu) and originally published species descriptions (http://schuessler.userweb.mwn.de/amphylo/).

Screening of acid tolerant AM fungi

These AM fungi were further grown and maintained as monospecific culture in sterilized sand: soil mixture (1;1) with pH 5.3 as sorghum host plant variety COFSH- 9 (Clark *et al.,* 1999). Twelve isolates grown as monospecific culture were tested for their reinfectivity to the host plant as well as for the potential of establishment of the spores (Karthikeyan and Prakash, 2008). The mother inoculum of all the twelve AM isolates containing spores, infected roots and mycelium were mixed @ of 15 g/pot containing 3 kg of vermiculite with pH 5.3 and sterilized sorghum seeds (variety same as previous) were sown. After 30 days, the plants were uprooted and roots were taken for assessing AM colonization by trypan blue staining (Koske and Gemma, 1989) and total spore counts (spore density) in soil were also done in per 100 g soil (Clark *et al.,* 1999). Based on percent AM colonization and total spore count two acid tolerant AM fungal taxa namely *Funneliformis geosporus* (AM 1), *A. scrobiculata* (AM 2) were selected for evaluation of phosphorus use efficiency (Dhinakaran and Savithri, 1997). AM 1, AM 2 and PB1 were given names for simplicity of literature.

Pot house experiment using Radio tracer (^{32}P) in sorghum

A pot house experiment was conducted at Department of Soil Science and Agricultural Chemistry of Tamil Nadu Agricultural University for testing the phosphorus use efficiency in AM sorghum plant with inoculation of two selected acid tolerant AM fungal isolates namely *Funneliformis geosporus* (AM 1), *A. scrobiculata* (AM 2) in comparison with *G. intraradices* as standard (mild acidic pH tolerant) and in combination with *Azospirillum lipoferum* (strain Az-204) and phosphobacteria *Bacillus megaterium* (PB 1) obtained from Department of Agricultural Microbiology, Tamil Nadu Agricultural University using ^{32}P labelled rock phosphate in acid soil (pH 5.3) with proper care of mutagenic radioactive materials (Dhinakaran and Savithri, 1997).

Seeds of sorghum plant, *Sorghum bicolor* (L.) Moench subsp. *bicolor* (variety same as mentioned before) were treated with fungicide carbendazim @ 2gm/kg of seeds for 2 min and washing with sterile distilled water to make the seeds free from fungicide, before sowing. Three plants were maintained in each pot. Plastic pots were filled with 10 kg sterilized autoclaved acidic soil in each pot collected from National Pulses Research Centre, Vamban in Pudukottai district of Tamil Nadu. The soil characteristics were pH 5.3, EC 0.02 dS/m, available N 190 kg/ha, available P_2O_5 6.9 kg/ha and available K_2O 108 kg/ha.

The treatments are as follows

T1- *F. geosporus* (AM 1), T2- *A. scrobiculata* (AM 2), T3- *G. intraradices* (AM 3), T4- *F. geosporus* + AZ 204 (*A. lipoferum*), T5- *A. scrobiculata* + AZ 204, T6- *G. intraradices* + AZ 204, T7- *F. geosporus* + AZ 204 + PB1 (*B. megaterium*), T8- *A. scrobiculata* + AZ 204 + PB1, T9- *G. intraradices* + AZ 204 + PB1, T10- Uninoculated control (75% N & P), T11 Uninoculated control (100% N & P).

The treatments were replicated three times in a completely randomized block design (RBD). Fertilizer application was done @ 120:60:60 kg/ha of N, P_2O_5 and K_2O in the form of urea, rock phosphate and muriate of potash for 100% N, P & K. Rock phosphate was labelled with ^{32}P (appro. Specific activity of 0.6 mci/g of P) obtained from Board of Radiation and Isotope Technology, Mumbai, before application. Treatment 1-3 & 10 received 100% N, K and 75% P. T4-T9 received 75% N, P and 100% K, T11 received 100% N, P & K. *A. lipoferum* (AZ 204) and *B. megaterium* (PB1) were applied @ 5ml/pot containing 10^6 cfu/ml during sowing. Three AM cultures *viz. F. geosporus, A. scrobiculata* and *G. intraradices* maintained in sorghum roots as monospecific culture were used. AM cultures were applied @ 50 g/pot containing 150 spores/ 100 g inoculum during sowing.

Labelling of radio-active phosphorous ^{32}P with fertilizer

For labelling of rock phosphate carrier-free P (obtained as orthophosphoric acid in diluted HCl medium from the Board of Radiation and Isotope Technology, Mumbai) and rock phosphate were mixed in proper proportion in a silica basin. After that it was dried under infrared lamp and later ground to powder using a mortar and pestle.

Assay of radioactive phosphorus in plant material

The assay of radioactive phosphorus in plant material was done by the method described by Dhinakaran and Savithri, 1997. The specific activity was estimated in the Geiger Muller counter (Type GCS 13 B of Electronics Corporation of India Limited, Hyderabad) for 60 sec and expressed as radioactive ^{32}P counts per minute or cpm. The counting was also taken for the labelled sample. Three counts were taken for each sample and the average count rate was obtained. Plant samples were taken at 45 days after sowing (DAS). From the radiation count obtained from the plant sample the specific activity, percent phosphorous derived from fertilizer and soil, uptake of P from fertilizer and soils as well as phosphorus utilization efficiency were also calculated (Dhinakaran and Savithri, 1997).

Per cent phosphorous derived from fertilizer (% Pdff)

$$\%\,\text{Pdff} = \frac{\text{Specific activity of plant sample}}{\text{specific acivity of the standard}} \text{ X } 100$$

Per cent phosphorous derived from soil (% Pdfs)

It was calculated as below

% Pdfs = (100 - % Pdff)

Phosphorous uptake from fertilizer

P uptake from

$$\text{fertilizer (mg pot}^{-1}) = \frac{\%\,\text{pdff}}{100} \text{ X Total P uptake (mg pot}^{-1})$$

Phosphorous uptake from soil

P uptake from

$$\text{soil (mg pot - 1)} = \frac{\%\,\text{Pdfs}}{100} \text{ X Total P Uptake (mg pot}^{-1})$$

Phosphorous use efficiency (PUE)

$$\%\text{PUE} = \frac{\text{P uptake from fertilizer}}{\text{Quantity of P added fertilizer}} \text{ X } 100$$

Plant and enzyme analysis

Plants were harvested, dried and weight of total dry matter taken and ground to fine powder after 45 DAS. After that the plant samples were subsequently analyzed for total nitrogen content by Kjeldhal method, phosphorus content by vanadomolybdate method (Jackson, 1973).

The assay of phosphatase (Orthophosphoric-monoester phosphor-hydrolase (EC.3.1.3.2) activity was determined at 45 DAS by measuring the p-nitrophenol (PNP) released by phosphatase activity when each treatment soil was incubated with buffered (pH 7.0) sodium p-nitrophenyl phosphate solution and toluene at 37°C for 24 h (Wang et al., 2006). The enzyme activity was expressed as μg PNP g^{-1} 24 h^{-1}.

Data analysis

The data were tested by one-way of analysis of variance (ANOVA) and means were compared by least significant difference (LSD). The statistical tests were applied using SPSS software package version 22.0 developed by SPSS Inc., Chicago, IL 60606, USA.

RESULTS AND DISCUSSION

Soil physiochemical properties

Physio-chemical properties of the seven different acidic soil samples showed significant variation in pH, EC, phosphorus and potassium content (Table 1.). The nitrogen content in six soil samples of Pudukottai district showed less variation but the Western Ghat forest soil showed the higher nitrogen content. The soil pH range categorizes the nature of soils from slightly acidic to extremely strongly acidic. Among all soils, the soil from pearl millet field (A4) showed comparatively lower nutrient level for phosphorus and potassium. All soils mainly were observed to be deficient in nitrogen and potassium with low to medium levels of phosphorus. Similar variation was observed in soil samples collected from different acidic regions of Tamil Nadu, India (Muthukumar and Udayan, 2000).

Occurrence of AM fungi in acidic soils

Totally twelve AM fungal isolates were isolated and identified from seven different acidic soils (Table 2.). These were *Acaulospora scrobiculata* (Fig 2c.), *A. laevis, A. foveata* (Fig 2a.), *Sclerocystis sinuosa, G. multisubstensum, Rhizophagus clarus, Funneliformis geosporus* (Fig 2b.), *Gigaspora gigantea, Claroideoglomus etunicatum, G. microcarpum, G. aggregatum, Scutellospora calospora*. The occurrence of AM fungal isolates in acidic soil showed less abundance in fungal diversity compared to normal soil (pH 6.8-7.3). The probable reason behind the low abundance of AM fungi in acidic soil is the soil pH, presence of toxic concentration of ions Al, Mn

Table 1: Physio-chemical parameters of acid soils

Properties	Soil samples						
	D1	D2	A4	A9	E10	E11	F1
Vegetation	Mango	Mango	Fallow	Fallow	Pigeonpea	Pigeonpea	Western Ghat forest
pH	4.4 ± 0.01	4.9 ± 0.01	5.3 ± 0.01	5.4 ± 0.01	5.5 ± 0.02	6.6 ± 0.02	4.5 ± 0.01
EC(dSm^{-1})	0.15 ± 0.01	0.07 ± 0.01	0.05 ± 0.01	0.13 ± 0.01	0.09 ± 0.01	0.18 ± 0.01	0.09 ± 0.01
Soil colour	red	red	black	black	black	black	black
Available N (Kg/ha)	221 ± 6.56	179 ± 5.89	190 ± 6.01	210 ± 6.21	196 ± 6.05	182 ± 5.93	624 ± 13.65
Available P_2O_5 (Kg/ha)	9.4 ± 0.31	13.5 ± 0.39	6.9 ± 0.03	34.5 ± 0.99	73.5 ± 2.07	63.7 ± 1.59	16.7 ± 0.48
Available K_2O (Kg/ha)	134 ± 3.68	179 ± 5.91	108 ± 3.02	147 ± 3.71	159 ± 3.84	238 ± 6.89	149 ± 3.73

Note- Sample name given according to collection site like sample from D1 block named D1; Standard errors in parentheses; Means were compared by LSD and SE calculated for three replications

Table 2: Screening of AM isolates based on reinfection potential using maize

AM Isolates	% AM fungal colonization at 40 DAS	No. of spores /100g of soil sample
Acaulospora foveata	64.4 c	200 ab
Acaulospora laevis	35.4 b	90 ca
Acaulospora scrobiculata	91.3 d	540 abc
Glomus aggregatum	42.1 cc	85 aa
Rhizophagus clarus	60.0 ab	175 abc
Claroideoglomus etunicatum	49.0 bc	180 bcd
Funneliformis geosporus	81.0 d	480 cd
Glomus microcarpum	21.6 a	60 ac
Glomus multisubstensum	11.2 a	240 b
Sclerocystis sinuosa	26.4 de	80 aa
Gigaspora gigantea	46.2 ab	110 cc
Scutellospora calospora	24.7 abc	75 cd
SE (d)	1.70	2.62
CD (p = 0.05)	3.68	5.63

Note-The data were tested by one way ANOVA and were significant at level $P < 0.05$; Mean values followed by the same letter are not significant by LSD at the 5% level.

Table 3: Effect of combined inoculation of AM fungi, *Azospirillum* and phosphate solubilizing bacteria on specific activity in sorghum at 45 DAS under pot house using ^{32}P radio tracer

Treatments	Specific activity cpm/mg ^{32}P
F. geosporus (AM1)	48.2
A. scrobiculata (AM2)	48.4
G. intraradices (AM3)	42.2
AM 1 + AZ 204	45.6
AM 2 + AZ 204	48.6
AM 3 + AZ 204	41.0
AM 1 + AZ 204 + PB1	43.8
AM 2 + AZ 204 + PB 1	45.2
AM 3 + AZ 204 + PB 1	38.6
Uninoculated control (75 % P)	43.3
Uninoculated control (100% P)	50.6
SE (d)	1.34
CD (p = 0.05)	2.72

Note-The data were tested by one way ANOVA and were significant at level $P < 0.05$; cpm- counts per minute

and Fe, different degrees of adaptability of AM in acidic soil (Clark *et al.,* 1999; Borie *et al.*, 2010).

Assessment of acid tolerance of AM fungal isolates

There was a significant variation observed in both mycorrhizal parameters namely percent AM fungal colonization and total AM fungal spore counts or spore density among all twelve AM fungal isolates screened for acid tolerance (Table 2.). It showed the AM fungal isolates have different degree of tolerance for survival in the particular pH of acidic soil. Among all isolates only two isolates *i.e. Funneliformis geosporus* (AM 1) and *A. scrobiculata* (AM 2) performed better and showed exceptionally high values in both percent colonization in roots (81.0%, 91.3%) and spore density (480, 540). Few AM fungal isolates *Acaulospora foveata* and *Rhizophagus clarus* also showed moderate level of root colonization (64.4, 60.0) but have low quantity of spore density (200, 175). Similar results were observed from previous studies (Borie *et al.,* 2010). Hence, only two AM fungal isolates were selected for further studies.

Combined triple inoculation effect on specific activity, P uptake and PUE of AM sorghum plant

There was a significant increase in specific activity of ^{32}P recorded in all AM plants than uninoculated controls. Treatments with single inoculation of AM isolates showed higher value of specific activity compared to dual and triple inoculation with *Azospirillum* and phosphobacterium (Table 3.). Overall there was much variation noticed in specific activity. Among combined inoculation with AM fungi *Acaulospora scrobiculata* (AM 2) and *Azospirillum*, recorded higher specific activity (48.6 cpm/mg P). Among single inoculation of AM isolates *Acaulospora scrobiculata* inoculation showed maximum specific activity (48.4 cpm/mg P). Whereas, the treatment *Glomus intraradices* + AZ 204 + PB 1 recorded minimum specific activity (38.6 cpm/mg P). Generally Specific activity of the control with 100% NPK is significantly higher than any other treatment. Similarly mycorrhizal plants showed significant increase in P uptake from fertilizer, % Pdfs and P uptake from soil than both uninoculated controls (Table 4.). But for % Pdff mycorrhizal plants showed significant increase than control with 75% P except the control with 100% NPK which showed maximum % Pdff value because of high ^{32}P specific activity. There was a significant increase in % PUE observed in all mycorrhizal treatments whether single, dual or triple inoculation with AM, *Azospirillum* and phospho bacterium than control (Table 5.). Maximum PUE 17.1% was noticed in T8 treatment with 256.25% increase than control with 75% P which showed minimum PUE of 4.8%.

Plants inoculated with AM, *Azospirillum* and phospho baccteria obtains their P from three different sources from fertilizer, from P mobilization through AM (source fertilizer and soil) and from P solubilization by phosphobacteria (source soil) (Zhang *et al.,* 2014). But uninoculated plants can access only P from fertilizer. As a result, uninoculated control

treatment with 100% NPK showed higher specific activity and %Pdff due to all P source from fertilizer and lower %Pdfs, uptake of P from fertilizer and soil. On the other hand, all mycorrhizal plant showed lower specific activity and %Pdff but higher %Pdfs, uptake of P from fertilizer and soil. Srivastava *et al.,* 2007 reported similar observation to evaluate agronomic effectiveness of rock phosphate. Increase in specific activity and % PUE by mycorrhizal plants also been reported in mulberry (Kumutha, 2001; Antunes *et al.*, 2007; Osorio and Habte, 2013) compared to uninoculated controls.

Table 4: Effect of combined inoculation of AM fungi, *Azospirillum* and phosphate solubilizing bacteria on % Pdff, %Pdfs, uptake of P from fertilizer and soil in sorghum at 45 DAS under pot house using ^{32}P radio tracer

Treatments	% P derived from fertilizer (%Pdff)	Uptake of P from fertilizer (g/pot)	% P derived from soil (% Pdfs)	Uptake of P from soil (g/pot)
F. geosporus (AM1)	16.0	0.007	84.0	0.036
A. scrobiculata (AM2)	16.1	0.009	83.9	0.049
G. intraradices (AM3)	14.1	0.005	85.9	0.031
AM 1+ AZ 204	15.2	0.008	84.8	0.046
AM 2+ AZ 204	16.1	0.011	83.9	0.059
AM 3+ AZ 204	13.7	0.006	86.3	0.039
AM 1+ AZ 204 + PB1	14.5	0.013	85.5	0.079
AM 2+ AZ 204 + PB 1	15.0	0.018	85.0	0.104
AM 3+ AZ 204 + PB 1	12.8	0.010	87.2	0.069
Uninoculated control	14.3	0.005	85.7	0.027
Uninoculated control	16.8	0.006	83.2	0.028
SE (d)	0.07	0.003	2.36	0.015
CD (p=0.05)	0.10	0.005	5.64	0.029

Table 5: Effect of combined inoculation of AM fungi, *Azospirillum* and phosphate solubilizing bacteria on phosphorus use efficiency (PUE) in sorghum at 45 DAS under pot house using ^{32}P radio tracer

Treatments	Phosphorus use efficiency (% PUE)
F. geosporus (AM1)	6.7
A. scrobiculata (AM2)	8.6
G. intraradices (AM3)	4.8
AM 1+ AZ 204	7.6
AM 2+ AZ 204	10.5
AM 3+ AZ 204	5.7
AM 1+ AZ 204 + PB1	12.4
AM 2+ AZ 204 + PB 1	17.1
AM 3+ AZ 204 + PB 1	9.5
Uninoculated control	4.8
Uninoculated control	5.7
SE (d)	0.08
CD (p=0.05)	0.29

Table 6: Effect of combined inoculation of AM fungi, *Azospirillum* and phosphate solubilizing bacteria on total dry matter yield of sorghum plant at 45 DAS

Treatments	Total dry matter yield (g per plant)	Percent increase over T11
Glomus geosporum (AM 1)	3.12	6.8
Acaulospora scrobiculata (AM 2)	3.58	22.6
Glomus intraradices (AM 3)	3.01	3.0
AM 1+ AZ 204	3.37	15.4
AM 2+ AZ204	4.06	39.0
AM 3+ AZ 204	3.24	10.9
AM 1 + AZ 204 + PB 1	4.04	38.3
AM 2+ AZ 204 + PB 1	5.06	73.3
AM 3+ AZ 204 + PB 1	3.51	20.2
Uninoculated control	2.85	-
Uninoculated control	2.92	-
SE(d)	0.04	
CD(p=0.05)	0.10	

Note- Values represents the mean of three replications

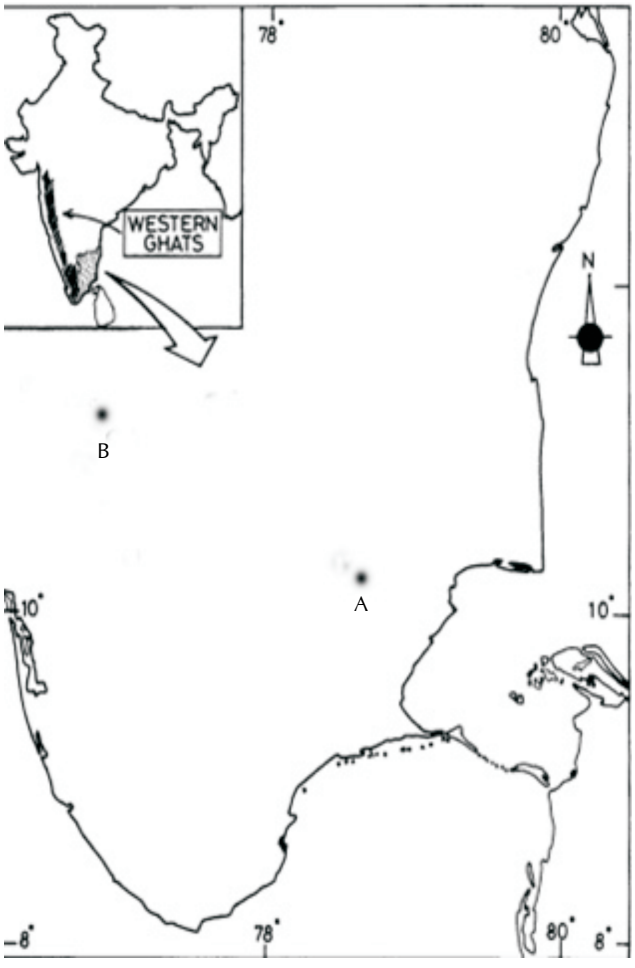

Figure 1: Map showing location of study sites in two districts of Tamil Nadu. A. Pudukottai; B. Nilgiri

Combined triple inoculation effect on total dry matter yield and nutrient uptake

There was a significant increase in total dry matter yield observed in all mycorrhizal treatments whether single, dual or triple inoculation with AM, *Azospirillum* and phosphobacterium than control (Table 6.). Treatment T8 showed maximum total dry matter yield of 5.06 g/plant and 73.3 % increase from control with 100% NPK. Uninoculated control with 75% P showed minimum total dry matter yield. There was also a positive correlation (r = 0.978) observed between % PUE and total dry matter yield. Karthikeyan and Prakash (2008) reported triple inoculation of AM, *Azospirillum* and phosphobacterium increases dry matter yield around two fold in *Eucalyptus* sp. The possible reason behind this plant acquired more nutrient N, P and can tolerate at extended level of stress condition than uninoculated control plant (Wang

Table 7: Effect of combined inoculation of AM fungi, *Azospirillum* and phosphate solubilizing bacteria on N and P uptake in sorghum plant at 45 DAS

Treatments N uptake	Percent increase (mg per plant)	P uptake over T11	Percent increase (mg per plant)	over T11
Glomus geosporum (AM 1)	123.5	32.6	4.3	26.5
Acaulospora scrobiculata (AM 2)	144.9	55.6	5.9	73.5
Glomus intraradices (AM 3)	98.4	5.7	3.6	6.0
AM 1+ AZ 204	154.3	65.7	5.5	61.7
AM 2+ AZ204	191.6	105.8	7.0	105.8
AM 3+ AZ 204	112.7	21.0	4.5	32.3
AM 1 + AZ204 + PB 1	188.6	102.6	9.2	170.6
AM 2+ AZ204 + PB 1	246.4	164.4	12.2	258.8
AM 3+ AZ 204 + PB 1	125.3	34.6	7.9	132.3
Uninoculated control	86.9	-	3.2	-
Uninoculated control	93.1	-	3.4	-
SE(d)	0.40		0.30	
CD (p = 0.05)	0.83		0.66	

Note- Values represents the mean of three replications

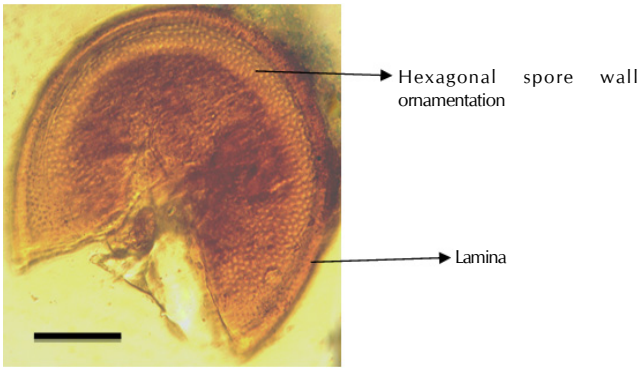

Figure 2a: Crushed spore of *Acaulospora foveata* in

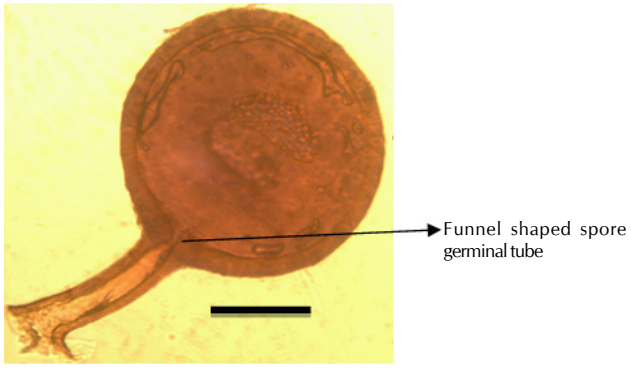

Figure 2b: Intact spore of *Funneliformis geosporus* in PVLG Melzer's reagent; bar 100 μm

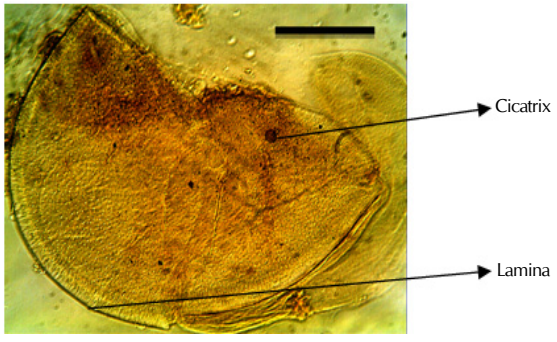

Figure 2c: Intact spore of *Acaulospora scrobiculata* in PVLG Melzer's reagent ; bar 100 μm; cicatrix- the point of attachment of sporiferous saccule

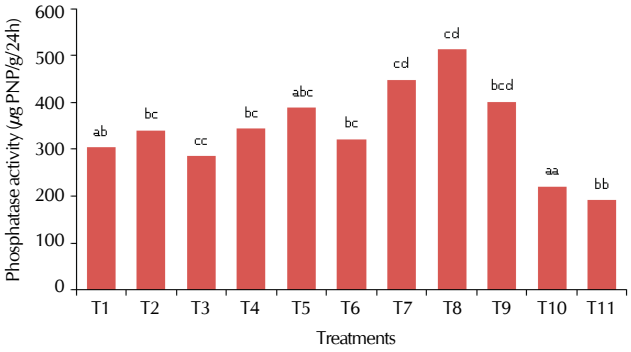

Figure 3: Phosphatase activities in the soil of Sorghum in response to different mycorrhizal inoculation treatments (T1-T11 same as mentioned before); PNP: p-nitrophenol; different letters above the bar indicate significant differences by LSD at $p < 0.05$

et al., 2006). Similarly there was a significant increase recorded in N and P uptake in all mycorrhizal plants whether single, dual or triple inoculation with AM, *Azospirillum* and phosphobacterium than control (Table 7.). It was observed that dual inoculation with AM and *Azospirillum* enhanced more N uptake than single inoculation with AM but simultaneously triple inoculation did not increases much from dual inoculation. In this regard, treatment T5 with dual inoculation performed much better than treatments T7 and T9 with triple inoculation. It denotes *Azospirillum* singly makes good compatibility with AM fungi and enhanced the level of N uptake. But overall treatment T8 recorded maximum N uptake of 246.4 mg/plant with 164.4% increase than control T11. For P uptake all triple inoculation treatments showed higher P uptake. Treatment T8 showed maximum P uptake of 21.2 mg/plant than control T11. It denotes phosphosbacteria share separate contribution for P uptake apart from AM in plants overall P uptake. There was also a positive correlation (r = 0.983) observed between % PUE and P uptake. Similar correlation was also observed in maize plant as previously reported (Wang *et al.*,2006). Being a PGPR phosphobacterium solubilizes more P trough mechanism of gluconic acid production, hydrolytic enzyme production it boosts up plant with enhanced level of P (Kachhap *et al.,* 2015). *Azospirillum* prefers to colonize on root surface and fix nitrogen. As nitrogen fixation is a high energy requiring process it needs more ATP. This P comes from either soil solution or AM fungal hyphae or P solubilized from phosphobacteria in triple inoculated plants. As a result, inoculation with AM and phosphobacteria enhanced N fixation and uptake to plants. It proves there is P turnover from AM and phosphobacteria to *Azospirillum*. On the other hand, inoculation of *Azospirillum* couldn't increase P uptake at satisfactory level. It proves there is little or no N turnover from *Azospirillum* to AM and phosphobacteria. In this regard sufficient literature was not found.

Combined triple inoculation effect on phosphatase activities in soil

There was a significant increase in soil phosphatase activity observed in all mycorrhizal plants whether single, dual or triple inoculation with AM, *Azospirillum* and phospho bacterium than control (Fig. 3.). Triple inoculation with AM, *Azospirillum* and phosphobacterium significantly enhanced the phosphatase activity but dual inoculations with AM and *Azospirillum* failed to enhance phosphatase activity at satisfactory level than single inoculation. It depicts phosphobacteria facilitate the phosphatase activity along with AM but *Azospirillum* can't do. There was also a positive correlation (r = 0.951) observed between phosphatase activity and P uptake.

In our results all the mycorrhiza treated plants increased phosphatase activities even triple inoculation with AM, *Azospirillum* and phosphobacterium showed around 2.67 times of enhancement than control T11. Similar observation was recorded as previous report based on maize as host plant (Wang *et al.,* 2006). Though there are profound literatures can be acquired in this regard the exact reason behind this is still quite puzzling. According to previous studies, the probable mechanisms on the enhancement of soil enzymatic activities may involve direct and indirect roles of AM fungi: First AM propagules themselves synthesize some hydrolytic enzymes in soil (Varma, 1998; Wang *et al.,* 2006). Secondly mycorrhizal plants may release more root exudates containing soil enzymes because of the larger root system and/or improved nutrition and/or resistances to stress of mycorrhizal plants. Thirdly, AM fungi alter soil microbial communities including nitrogen fixer *Azospirillum* and phosphobacterium in the rhizosphere directly or indirectly through changes in root exudation patterns, or through fungal exudates, the so called ''mycorrhizosphere effect'' (Wang *et al.,* 2006). Besides these, how inoculation with phosphobacteria and AM give much boos up for phophatse activities it can be explained from recent studies (Zhang *et al.,* 2014). Root exudates released from the AM hyphae may also have primed the bacteria and hyphal exudates containing formate, acetate, glucose and oligosaccharides (Alkan *et al.*, 2006; Toljander *et al.,* 2007). that can be readily assimilated by bacteria as C sources, leading to an alteration of the bacterial composition and activity of the rhizosphere (Zhang *et al.,* 2014). As a result, both AM fungi and bacteria enhanced the phosphatase activity and mineralize more P from plant unavailable sources.

In our study we have tried triple inoculation of acid tolerant AM, *Azospirillum* and phosphobacterium on enhancement of vigour of sorghum plant. Hence, it can be concluded from this study that this work might not be a novel work but triple inoculation gives three times more PUE, around 73.3% total dry matter yield, 164.4% and 258.8% increase in N and P uptake than control. So this combination has tremendous potential as a biofertilizer in acidic soil in near future after further research in field especially in acidic soil.

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