CSIRO PUBLISHING The South Pacific Journal of Natural and Applied Sciences, **29**,7-11 , 2011
www.publish.csiro.au/journals/spjns 10.1071/SP11002

Aspergilli and rhizobia are better co-inoculants as biofertilizers

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Abstract
The application of synthetic phosphorous fertilizer is a routine agricultural practice. However, in nature in the soil, several phosphate-solubilising microbes (PSM) have been identified in the rhizosphere, and are of significance in organic farming. Two major PSM are Rhizobium sp. and Aspergillus sp. that has been identified to have the potential to establish as biofilms in the soil root-zone environment. As the fungi are capable of solubilising both organic and rock phosphates, co-inoculation of these two microbes will enhance the availability of available phosphates to plants and in turn will reduce the requirement of synthetic fertilizers. The present study aims to evaluate the survivability of these two organisms in vitro conditions as co-inoculants. Aspergillus sp. seemed to be more synergistic or associative in growth with the rhizobial strains, and this organism is known well as a rigorous PSM. The effect of rhizobial toxins on the isolated fungal strains and the fungal toxins on the isolated rhizobial strains were tested in this study. Results show that Aspergillus flavus 2 and Rhizobium sp. K 1 strains were found to be highly antagonistic and will be eliminated for further studies. Nevertheless, synergism was found to be highly variable amongst not only within rhizobial strains, but also amongst the isolated fungal strains.

Keywords: *Aspergillus sp., Rhizobium sp., Phosphorous, Antagonism, Phosphate solubilising microbes.*

1 Introduction

Nitrogen (N), phosphorous (P), potassium (K) and sulphur (S) are the major nutrient elements of agro economic significance and are utilized by plants owing the credit to the synergistic and associative activity of the plant-microbe interactions. *Aspergillus* sp., a soil-borne fungi and *Rhizobium* sp., a well-established N_2-fixing microbe in the soil are also major phosphate solubilisers of the soil (Arcand and Schneider, 2006). These two species are one amongst the most powerful PO_4 solubilising macro- and micro-organisms (Abd-Alla *et al*., 2001) that has potential use as bio inoculants in the soil.

The interaction between *Rhizobium* sp. and soil-borne fungi has made major contributions in inhibiting many of the soil-borne plant pathogens, and participation in the N_2, P and S cycle. Whilst the symbiotic partner of leguminous plants, *Rhizobium* sp. only mineralizes soluble P of inorganic origin, *Aspergillus* sp. has also been known to play a role in the mineralization of insoluble P of both organic and inorganic (rock phosphates) origin (Barroso and Nahas, 2007). Although both these organisms are beneficial to the soil, they both are in turn dependant on the carbon sources released by the leguminous plants, and thus in turn compete for similar substrate. Hence, in nature there is a probability that rhizobial and aspergilli strains isolated from the soil samples either will demonstrate substrate competition or will live associatively depending on similar substrates. A similar study has earlier been reported at the field level for utilizing the innate rock phosphate by administering the fungal-rhizobial co-inoculants to the soil to form the fungal-rhizobial films, FRB (Seneviratne *et al.*, 2009) in the rhizospheric regions of the both the leguminous plants and non-leguminous plants (Seneviratne and Jayasinghearachchi, 2003; Seneviratne and Jayasinghearachchi, 2005; Jayasinghearachchi and Seneviratne, 2006).

Thus, this paper discusses observations of FRB relationships that are being evaluated in the laboratory conditions, before being taken to the pot culture and then to the field experiments. In this case study, the fungal and rhizobial strains were isolated from various soil samples collected from different regions of Chennai, Tamil Nadu, India, which were both salt laden and P deficient for cultivation.

2 Materials and Methods

2.1 Isolation of *Rhizobium sp. a*nd Fungi

Soil samples were randomly collected from various regions in and around Chennai, Tamil Nadu, India, including Mylapore, ECR, Alwarpet, Muglivakkam, Perungudi and Kodambakkam. Soil samples were diluted and *Rhizobium* sp. and fungi were isolated on Yeast Extract Mannitol Agar (YEMA) medium using spread plate technique (Cappuccino and Sherman, 1998).

2.1.1 Isolation of *Rhizobium sp.*

Rhizobial strains were isolated and subcultured on YEMA plates and finally to slants. Pure cultures thus obtained were characterized biochemically, and were differentiated as fast-growers and slow-growers using Bromothymol Blue (BTB) medium, YEMA medium supplemented with 0.5 % alcoholic solution of bromothymol blue.

2.1.2 Isolation of Fungi

Fungal cultures were obtained by transferring the single colony that grew on the YEMA plates, first on Sabouraud's Dextrose Agar plates. Then pure cultures were obtained using agar block technique, identified using macroscopic and microscopic (Lactophenol Cotton Blue) examination (Figure 1) and were maintained on SDA slants.

a. Extraction of extracellular fungal compounds

Fungal culture filtrates were prepared by allowing the formation of fungal matte on the surface of Sabouraud's

Dextrose broth in 250 ml Erlenmeyer's flask until sporulation, usually for one week under room temperature (~22ºC) in the dark. Fungal mat was removed by using sterile filter papers. Concentrated soluble extracellular compounds secreted into the fungal broth filtrates were used as such for well diffusion method. Sterile membrane filter papers were cut into discs and 0.1 ml of filtered filtrate of fungal cultures were placed using micropipette, and air-dried and was used for disc-diffusion method.

b. Bioassay for substrate competition between *Rhizobium* sp. and fungi

Bioassay for substrate competition between *Rhizobium* sp. and fungal cultures were performed using white YEMA plates devoid of Congo Red.

Well-diffusion method: YEMA plates were prepared, and lawn culture of the rhizobial strains was prepared using 48 hours broth cultures for inoculation at 22ºC. One ml of the fungal filtrate was placed inside the wells, and was incubated at room temperature for a week. The diameter of the fungal colony thus formed after incubation was measured and were grouped as in Table 1.

Disc Diffusion Method: YEMA plates were prepared, and lawn culture using 24 hours old broth culture of rhizobial strains was inoculated and allowed to stand for 10 s. Air-dried paper discs impregnated with filtered fungal filtrate were placed on these Petri plates and incubated at room temperature (~22ºC) for 48 hours in the dark. Zones of inhibition for growth of rhizobial strains around the discs were measured in mm (Table 2).

3 Results

3.1 Isolation of *Rhizobium sp. a*nd Fungal Strains

Eight strains of *Rhizobium* sp. were isolated and pure cultures were maintained. On characterization of these strains on BTB medium, 6 strains were observed as fast-growers and 2 strains identified to be slow-growers. 2 rhizobial strains were observed from Mylapore region (M-1 and M-2), 2 from ECR (E-1 and E-2), 1 from Alwarpet (A-1), 1 from Muglivakkam (Mu-1), 1 from Perungudi (P-1) and 1 from Kodambakkam (K-1). Rhizobial strains isolated from Mylapore (M-1 and M-2), ECR (E-1 and E-2), Alwarpet (A-1) and Muglivakkam (Mu-1) were fast-growers, and rhizobial strains isolated Perungudi (P-1) and Kodambakkam (K-1) were slow-growers.

Aspergillus species predominated in growing synergistically with *Rhizobium* sp. on YEMA medium. 5 strains of aspergilli were isolated; of which 3 species were identified, namely *A. flavus*, *A. niger* and *A. tamarii*. Among the 5 strains of aspergilli, each 2 strains of *A. niger* and *A. flavus* were isolated and identified (Figure 1). They were not characterized to the level of sub-species.

3.2 Bioassay for Substrate Competition Between *Rhizobium sp. a*nd Fungi

In well-diffusion method, fungal spores were dispensed in the wells cut on plates seeded with lawn culture of rhizobial strains. This technique allowed the measurement of the effect of any extracellular secretions by the rhizobial strains against the inoculated fungal strains (Table 1).

Amongst the isolated rhizobial strains, K-1 is the most antagonistic inhibiting the growth of all the isolated aspergilli strains except for *A. niger* 2. Amongst the isolated fungal strains, *A. flavus* 2 was observed to be the most sensitive strain in the presence of all the isolated rhizobial strains except for E-2 and A-1. E-2 and A-1 strains were the least antagonistic strains, thereby demonstrating a synergistic effect in the growth of the isolated fungal strains. Of the isolated fungal strains, *A. niger* 2 exhibited least sensitivity to the isolated rhizobial strains, followed by *A. flavus* 1 and *A. niger* 1, and then by *A. tamarii*. The two isolated *A. niger* strains were observed to be associative with Mu-1 rhizobial strain.

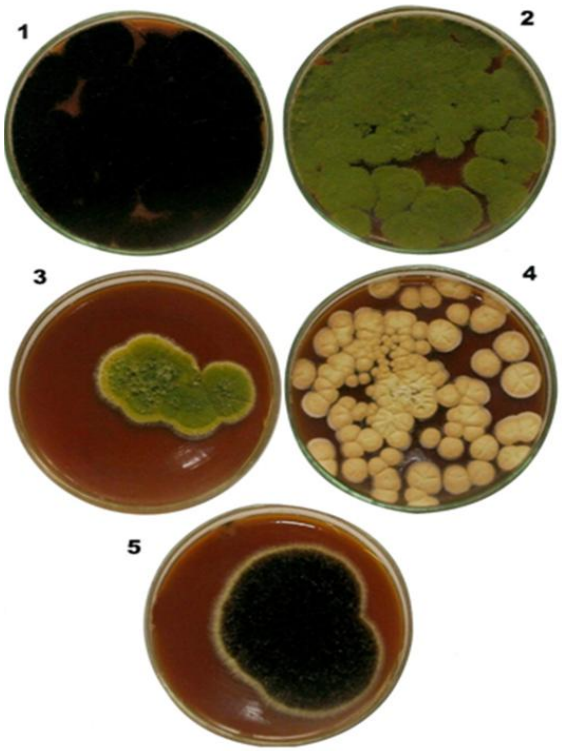

Figure 1. Pure culture of fungi isolated on SDA (fungal species were identified using the manual by Prakash, 2004) 1. *A. niger*1; 2. *A. flavus*1; 3. *A. flavus*2; 4. *A. tamarii*; 5. *A. niger*2

Table 1. Antagonistic effect of rhizobial strains against fungal strains.

Rhizobial strains	*A. flavus* 1	*A. niger* 1	*A. tamarii*	*A. flavus* 2	*A. niger* 2
M 1	MG	MG	MG	NG	MG
M 2	MG	MG	MG	NG	MG
E 1	MG	GG	NG	NG	GG
E 2	GG	GG	GG	MG	TG
A 1	GG	GG	TG	MG	GG
Mu 1	GG	TG	GG	NG	TG
P 1	GG	GG	MG	NG	GG
K 1	NG	NG	NG	NG	GG

n = 3 (triplicates) were processed in each sample category
TG: Tremendous Growth; GG: Good Growth; MG- Minimum Growth; NG- No Growth
Diameter of Fungal Colony in mm: TG- >200mm; GG- 199 mm to 100 mm; MG- < 99 mm; NG- 0 mm

3.3 Bioassay for the Effect Extracellular Secretions of Fungi on *Rhizobium sp.*

Fungal spores were removed from pre-filtered fungal filtrates by using membrane filter technique, and were impregnated on circular membrane discs and air-dried. Disc-diffusion technique was thus employed to observe the effect of various extracellular compounds secreted into the solution by the isolated fungal strains on lawn culture of 24 hours isolated rhizobial strains prepared on YEMA plates. The inhibition of isolated rhizobial strains to fungal extracellular secretions was strain dependant (Table 2).

Table 2. Antagonistic effect of fungal strains against rhizobial strains.

Rhizobial strains	*A. flavus* 1	*A. niger* 1	*A. tamarii*	*A. flavus* 2	*A. niger* 2
M 1	WA	WA	MA	NA	WA
M 2	WA	WA	MA	NA	WA
E 1	WA	WA	NA	NA	MA
E 2	WA	WA	MA	WA	MA
A 1	WA	WA	SA	WA	WA
Mu 1	SA	SA	WA	NA	SA
P 1	MA	WA	WA	NA	SA
K 1	NA	NA	NA	NA	WA

Source (Patel, 1974): NA- Non Antagonistic; WA – Weakly Antagonistic; MA- Moderately Antagonistic; SA – Strongly Antagonistic

Diameter of Zone of Inhibition in mm: SA- >200mm; MA- >150mm to <200mm; WA-<150mm; NA – 0 mm

A. flavus 2 is most associative and will grow readily with all the isolated rhizobial strains, though E-2 and A-1 rhizobial strains exhibit some kind of weak antagonism. *A. flavus* 1 and *A. niger* 1, though both being weakly antagonistic to most of the isolated rhizobial strains and shows no antagonism to K-1 rhizobial strain, they both are strongly antagonistic to MU-1 strain, and *A. flavus* 1 is moderately antagonistic to P-1 strain. *A. tamarii* and *A. flavus* 2 seemed to exhibit highly variable response to rhizobial strains. *A. tamarii* is weakly antagonistic to Mu-1 and P-1, moderately antagonistic to M-1, M-2 and E-2, strongly antagonistic to A-1 and there was no zones of inhibition to E-1 and K-1. *A. niger* 2 is mostly weakly antagonistic to M-1, M-2, A-1 and K-1, moderately antagonistic to E-1 and E-2 and strongly antagonistic to Mu-1 and P-1. K-1 strain of rhizobia hardly responds to any of the extracellular secretions of the isolated aspergilli species, except for *A. niger* 2, while A-1 strain is weakly antagonistic to all rhizobial strains except for *A. tamarii*. M-1 and M-2 strains responds in a similar fashion to the aspergilli species isolated, weakly antagonistic to both the strains of *A. niger* and *A. flavus* 1, no response to *A. flavus* 2 and moderately antagonistic to *A. tamarii*. E-2 is weakly antagonistic to *A. niger* 1 and both the strains of *A. flavus*, and moderately antagonistic to *A. niger* 2 and *A. tamarii*. E-1 is weakly antagonistic to *A. flavus* 1 and *A. niger* 1, no response to *A. tamarii* and *A. flavus* 2 and moderately antagonistic to A. *niger* 2. Mu-1 is the most sensitive of all the isolated rhizobial strains exhibiting strong antagonism to both the strains of *A. niger* and *A. flavus* 1, weak antagonism to *A. tamarii* and no response to *A. flavus* 2. P-1 shows no response to *A. flavus* 2, but moderately antagonistic to *A. flavus* 1, weakly antagonistic to *A. niger* 1, but strongly antagonistic to *A. niger* 2, and no zone of inhibiton was found to *A. tamarii*.

4 Discussion

Phosphorous, the second most important macronutrient for the plants, are usually precipitated in the soil as phosphates of calcium (Ca), magnesium (Mg), iron (Fe) and aluminium (Al), thereby making them unavailable for plants. In addition to the inorganic forms of P, organic matter also contributes to the total phosphorous content of the soil. Several rhizobacterial species have been well established as powerful P-solubilizers like *Bacillus* sp., *Pseudomonas* sp. and *Rhizobium* sp. (Rodriguez and Fraga, 1999); and also a few filamentous fungal species like *Aspergillus* and *Penicillium* have been known to be better solubilizers of rock phosphates by converting inorganic (rock) phosphates into available phosphate ions (Kucey, 1988; Narsian and Patel, 2000). These phosphate solubilising fungal species are also capable of solubilising organic phosphates (Barroso and Nahas, 2007), and thus can play a vital role in the phosphor cycle in organic-rich soil. In addition, the solubilisation of phosphates, mycorrhizal fungal species coexisting with the roots of plants also aids in the uptake of phosphate ions (Barea *et al.*, 2002; Bolan, 1991; Thingstrup *et al.*, 2000; Trolove *et al.*, 2003).

Of all the phosphate solubilising microorganisms, *Rhizobium*, which is of agronomic significance as they are the symbiotic N_2-fixer for the leguminous plants, and fungi like *Aspergillus* sp. and *Penicillium* sp., which are heterotrophic to the C sources secreted by the plants, are major contributors of the available PO_4^{2-} ions to the plants in the soil. Thus, both these organisms compete for survival in the soil, and may either collectively carry out the process of mineralization of P by forming FRB (Seneviratne *et al.*, 2009) or can show antibiosis by secreting microcins. For the current study, *Aspergillus* sp. always seemed to predominate while isolating *Rhizobium* sp. on YEMA plates, and hence was hypothesized to be capable of establishing Aspergilli-rhizobial (ARF) in the rhizosphere. In other words, both these microbes in question should be capable of becoming potential co-inoculants for being used as biofertiliser in organic farming.

Antagonism between fungi and bacteria, with special reference to rhizobium has been well established and is strain specific (Anusiya and Sullia, 1984) and is consistent in the present study as evident in Table 1 and Table 2. However, unlike in the previous study (Patel, 1974), the isolated fungal strains mostly showed weak antagonism against the isolated rhizobial strains. In particular, *A. flavus* 2 seemed to be more associative with all the rhizobial strains, and likewise K-1 rhizobial strain was observed to demonstrate weak or no antagonism to all of the fungal species tested. In a parallel study conducted to test the inhibitory effect of the rhizobial toxins on the fungal strains, K-1 rhizobial strain inhibited all the fungal species tested except *A. niger* 2; and *A. flavus* 2 demonstrated susceptibility to all the rhizobial strains tested except for E-2 and A-1 strains. Thus these two strains, K-1 rhizobial strain and the fungal strain, *A. flavus*

2, cannot be preferred for further analyses of phosphate solubilisation by co-inoculation. This is because, co – inoculation of these two strains will result either in the reduction of the establishment of fungal matte or will inhibit the establishment of the rhizobial affecting the formation and development of root nodules. This is in parallel with earlier studies clearly depicting the role of rhizobial strains and their toxins in the inhibition of fungal plant pathogens (Chan *et al.*, 2002; Sharif *et al*., 2003; Ozkoc and Deliveli, 2001) and the inhibitory role of fungal toxins in nodule formation and development on leguminous plants by the rhizobial strains (Habte and Barrion, 1984). Hence, all the rhizobial strains except K-1 and all the fungal strains except *A. flavus* 2 will be further analysed individually for the rate of phosphate solubilisation and the same when co-inoculated. Nevertheless, the chosen rhizobial strains and fungal species have the potential to form FRB to act effectively as bio-inoculants and will require to be investigated in this direction using pot culture technique for leguminous plants and non-leguminous plants separately, which could later be then tested in the field for their efficacy. In addition, this is also observed from the present study and other related studies (Suh *et al*., 1995) that rhizobial and aspergilli strains are better co-inoculants for use as biofertilizers than with other fungal strains like *Penicillium* sp.

Acknowledgements

I am grateful to Dr. N.K. Udaya Prakash, Marina Labs, Chennai (Omni Environment Inc., USA) and Veltech Hightech Engineering College, Chennai for helping us to identify fungal cultures up to species level, and Dr. Madhurama Gangawar, Senior Scientist, Punjab Agricultural University for reviewing this paper.

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