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Pseudomonas Microbiome as a Potential Warehouse of Bio-Control Agents with Significant Antifungal Activity against Selected Fungal Pathogens of Rice Plant

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ABSTRACT

Feeding the world population amidst the challenge of climate change is a daunting task *ad the interim* optimizing dependability, source use, and environmental impacts of food production need due consideration. The only optional yet sustainable way to achieve these goals is to integrate Plant Growth Promoting Rhizobacteria (PGPR) for enhancing plant growth, nutrient use efficiency, abiotic stress tolerance, and disease resistance into agricultural production. This amalgamation, however, requires a large-scale, nevertheless, concerted effort among academicians, researchers, industry, and farmers to comprehend and accomplish plant-microbiome interactions into the open framework of modern agriculture. Several studies to date have explored PGPR microbiome structure and function under natural and agricultural environments in both model and crop plant species. The present work identifies a consortium of potential *Pseudomonas* sp. as rhizosphere microbiome against selected pathogenic fungal strains, *Pyricularia oryzae, Rhizoctonia solani, Fusarium oxysporum* that causes devastating loss in rice crop yield as a proto-typical from Madurai and Sivagangai Districts, TamilNadu, South India.

INTRODUCTION

It is well established that PGPRs have paramount potential to increase agricultural productivity. However, there is a great deal of variation in the performance of PGPR that might be due to the environmental factors, which may affect their growth and exert their effects on the plant. Environmental factors include climate change, soil characteristics, and the composite activity of the indigenous microbial flora of the soil (Gupta *et al.,* 2015). As of now, modern tools such as biosensors, nano-fertilizers in the fields of biotechnology, a nanotechnology that has been applied in agriculture to enhance productivity and yields of crop. It is important to develop a better insight into the complex environment of the rhizosphere and associated bacteria, their mechanisms of action, and exploitation of PGPR in the rhizosphere. Potential strain could be managed via high throughput genetic engineering to improve the colonization efficacy and their effectiveness. The use of multi-strain inoculums of PGPR with known functions is of current interest as these formulations may increase consistency in the field. They offer the potential to address multiple modes of action, multiple pathogens, and temporal or spatial variability.

Pseudomonas has been suggested as a potential biological control agent due to its ability to colonies rhizosphere and protect plants against a wide range of fungal diseases such as black root-rot of tobacco, damping-off of sugar beet, rice sheath rot (Saravanakumar *et al.,* 2009), and has a prospect for genetically manipulating the producer organisms to improve the efficacy of these biocontrol agents. In India, *R. solani* exists widely in rice-wheat cropping systems and has caused economic losses of billions of dollars (Singh *et al.,* 2016).

PGPRs improve plant growth by preventing the proliferation of fungal phytopathogens and thereby support plant growth. Some PGPR synthesize antifungal antibiotics e.g., Fluorescent pseudomonad mixtures mediate disease resistance in rice plants against sheath rot. PGPRs produce enzymes that can lyse fungal cells e.g., *Pseudomonas stutzeri* produces extracellular chitinase and laminarinase that lyses mycelia of *Fusarium solani.* Certain PGPR degrade fusaric acid produced by *Fusarium* sp. causative agent of wilt. *Pseudomonas fluorescens* produces 2,4-diacetyl phloroglucinol which inhibits the growth of phytopathogenic fungi (Saravanakumar *et al.,* 2009).

Pseudomonads exhibit strong antifungal activity against *P. oryzae* and *R. solani* mainly through the production of antifungal metabolites (Prasanna and Rao, 2009). Strains of *P. oryzihabitans* and *X. nematophila* produce secondary metabolites and suppress *Pythium* and *Rhizoctonia* sp. which also causes damping-off of cotton (Kapsalis *et al.,* 2008; Saharan and Nehra, 2011). Fluorescent pseudomonads also exhibit strong antifungal activity against *R. bataticola* and *F. oxysporum* found in rice rhizosphere, through the production of antifungal metabolites (Kumar *et al.,* 2002).

X. oryzae and *R. solani* the bacterial leaf blight (BB) and sheath blight (ShB) pathogens of rice are suppressed by indigenous *Pseudomonas* strains isolated from the rhizosphere of rice cultivated in coastal agro-ecosystem under both natural and saline soil conditions (Paul and Lade, 2014). Isolates of *P. fluorescens* from rice rhizosphere are also shown to exhibit strong antifungal activity against *P. oryzae* and *R. solani* mainly through the production of antifungal metabolites (Reddy *et al.,* 2008). Rokni-Zadeh et al. (2011) demonstrated the antagonistic potential of non-pathogenic rhizosphere isolates of fluorescent *Pseudomonas* in biocontrol of Olive knot disease.

Pseudomonas exhibited biocontrol potential against phytopathogenic fungi *in-vivo* and *in-vitro* conditions from chickpea rhizosphere (Saraf *et al.,* 2008). *P. putida* screened for biocontrol of root-rot disease complex of chickpea exhibited antifungal activity against *Macrophomina phaseolina*. *Pseudomonas* strains act as effective candidate suppressing *P. capsici* in all seasons and antagonizes reproductive phases of footrot fungus, *Phytophthora capsici*, (Paul and Sarma, 2006). Secondary metabolites produced by *P. aeruginosa* Sha8 reduce the growth of both *F. oxysporium* and *Helminthosporium* sp. (Hassanein *et al.,* 2009). Wild rhizobial cultural filtrates have significant antagonistic effects against soil-borne pathogenic fungi and therefore enhance plant resistance to diseases (Saharan and Nehra, 2011). Strains of Pseudomonads isolated from the rhizosphere soils of paddy in Malaysia screened for their plant growth-promoting activity were antagonistic to *Pyricularia oryzae* in dual culture assay (Noori and Saud, 2012). Recently, Yasmin et al. (2017) demonstrated the biocontrol of bacterial leaf blight of rice and profiling of secondary metabolites produced by rhizospheric *Pseudomonas aeruginosa* BRp3. Recently, a broad-spectrum antifungal compound Phenazine-1-carboxamide (PCN) was identified as a novel broad-spectrum anti-fungal strain of *Pseudomonas aeruginosa* (SU8) and the effect of its crude

metabolites against *Rhizoctonia solani* and *Pyrcularia oryzae* were evaluated (Zhang *et al.*, 2015). Results indicate that if develop PCN could serve as a novel biocontrol agent against *R. solani* (Xiang *et al.*, 2018).

MATERIALS AND METHODS

Isolation

Soil samples were collected from Vaigai basin located paddy fields in Madurai District. Isolation of pseudomonads from the rhizosphere was performed as described earlier (Nagajothi and Jayakumararaj, 2015). Briefly, soil suspension was obtained by shaking 10 g of soil sample having roots with tightly adhering soil in 90 ml of 0.1 M $MgSO_4.7H_2O$ buffer for 10 min at 180 rpm on a rotary shaker. The resulting suspensions were serially diluted and 0.1 ml aliquots of each dilution were spread onto King's medium B (KB) agar in triplicates. Purified single colonies were further streaked onto KB agar plates to obtain pure cultures. Stock cultures were made in Luria Bertani (LB) broth containing 50% (w/v) glycerol and stored at -80°C until further use.

Fungal strains

Three fungal plant pathogens *Pyricularia oryzae*, *Rhizoctonia solani,* and *Fusarium oxysporum* were collected from TamilNadu Agricultural University (TNAU), Coimbatore, and TNAU&RI, Madurai, TamilNadu, India. Further, purification was done by single spore isolation technique and cultures maintained at 27°C on Potato Dextrose Agar (PDA) slants until further use.

In-vitro fungal inhibition assay

Seven to ten days old cultures of *P. oryzae*, *R. solani,* and *F. oxysporum* species were used in the experiment. A fungal bioassay was performed using the paper disc method (Bahraminejad *et al.*, 2008). Fungus suspension was spread over the PDA plates and an overnight grown *Pseudomonas* strain was used as a source of an antifungal agent. A fresh colony of *Pseudomonas* isolated from rhizosphere soil was inoculated in LB broth and incubated at 27°C for 24 h. Paper discs were soaked in 5 ml of bacterial culture for 30 seconds and later placed on agar plates. The discs were dried between each application and were applied on agar plates within 15 minutes after fungus

inoculation and plates incubated at 27° C for 3 - 7 days. At the end of the incubation period, the plates were checked for clear zones of inhibition formed around the discs.

RESULTS

In-vitro antifungal activity

A total of 24 PGPR strains were isolated from paddy rhizosphere soil of Madurai and Sivagangai districts. These strains were analyzed for *in-vitro* antifungal activity against *Pyricularia oryzae*, *Rhizoctonia solani,* and *Fusarium oxysporum* (Table 1; Figure 1a, b, c) Out of ten selected strains of Madurai, the maximum zone inhibition was shown by SM4 (15mm) followed by SM2, SM11 and TM3 (10 mm) respectively against *P. oryzae*. The minimum zone of inhibition was shown by SM9 (9 mm), KM2 (5 mm), and KM1 (4 mm) strains. However, there was no activity shown byTM1, TM5, and KM3 strains against *P. oryzae*.

Likewise, the maximum zone of inhibition was shown by SM2 (14 mm) followed by SM4 (12 mm), SM11, KM2(11 mm), SM9, KM1, and KM3 (10 mm) strains respectively against *R. solani* whereas the minimum zone of inhibition was shown by TM1 (8 mm) and TM5 (6 mm). There was no activity observed by TM3 strain against *R. solani*. Similarly, for antifungal activity against *F. oxysporum*, the maximum zone of inhibition was shown by SM11 (19 mm), SM2 (12 mm), SM9, and TM3 (10 mm) respectively whereas the minimum zone of inhibition was shown by SM4 and KM3 (8 mm), followed by KM2 (7 mm) and KM1 (5 mm) strains. However, there was no significant activity shown by TM1 and TM5 against *F. oxysporum*.

Out of 14 selected isolates from Sivagangai, the maximum zone of inhibition was shown by MS8 (20 mm) followed by VS4, SS6 (12 mm) and VS8, SS2, SS5 (10 mm) respectively whereas the minimum zone of inhibition was shown by VSI (9 mm), followed by VS7, MS5 (8 mm) MS7 (5 mm), MS2 (4 mm) strains as against *P. oryzae* (Figure 1a). However, no activity was observed by VS9, SS8, and SS10 strains. Likewise, antifungal activity against *R. solani*, the maximum inhibition was observed by VS7 (28 mm) followed by MS8 (15 mm), SS6 (13 mm), VS1, VS8, MS2 (12 mm), VS4, VS9, SS2, MS5, MS7 (10 mm) respectively whereas minimum zone of inhibition exhibited only by SS10 (7 mm). However, there was no activity exhibited by SS5 and SS8 against *R. solani* (Figure 1b).

The maximum zone of inhibition was shown by VS4, MS8 (12 mm) followed by VS8, MS2 (11 mm) VS7, SS6, MS7 (10 mm) respectively whereas the minimum zone of inhibition was observed only by VS1 (8 mm). However, there was no activity exhibited by strains (VS9, SS2, SS5, SS8, SS10, and MS5) against *F. oxysporum* (Figure 1c). Based on results obtained, it could be stated that antifungal activity was in the order of ***R. solani* > *F. oxysporum* > *P. oryzae*.** Interestingly, antifungal activity exhibited by the isolates indicated a close relationship between the production of HCN and siderophores.

DISCUSSION

It has been well established that PGPR improves plant growth by preventing the proliferation of phytopathogens and thereby support plant growth. Some PGPR synthesize antifungal antibiotics, which inhibit the growth of phytopathogen fungi. *Pseudomonas* has been suggested as a potential biological control agent due to its ability to colonize the rhizosphere and protect plants against a wide range of important agronomic fungal diseases. Haas and Keel (2003) reviewed the regulation of antibiotic production in root-colonizing *Pseudomonas* spp. and relevance for biological control of plant disease.

Bio-antagonistic ability in *Pseudomonas* is mainly due to a synergistic combination of different metabolites and hydrolytic enzymes (Trivedi *et al.*, 2008; Noori and Saud, 2012). Furthermore, studies demonstrate that the most efficient antagonists in the soil belong to this genus (Haas and Defago, 2005; Bakker *et al.*, 2007). The strains were analyzed for *in-vitro* antifungal activity against *P. oryzae, R. solani,* and *F. oxysporum*. Based on results obtained, it could be stated that antifungal activity was in the order of *R. solani* > *F. oxysporum* > *P. oryzae*.

Identification of conserved traits in fluorescent pseudomonads with antifungal activity was carried out by Ellis and his coworkers (2000). Gaur et al. (2004) confirmed that fluorescent *Pseudomonas* exhibit strong antifungal activity against *P. oryzae* and *R. solani* mainly through the production of antifungal metabolites. Fluorescent *Pseudomonas* also exhibits strong antifungal activity against *Rhizoctonia bataticola* and *F. oxysporum* found in rice and sugarcane rhizosphere, mainly through the production of antifungal metabolites (Prasanna *et al.*, 2009). Besides, it has been demonstrated that *Pseudomonas* showed broad-spectrum antifungal activity

on Mueller Hinton medium against *Aspergillus*, one or more species of *Fusarium* and *Rhizoctonia* (Akgul and Mirik, 2008).

Interestingly, in the present study, antifungal activity exhibited by the isolates indicated a close relationship between the production of HCN and siderophores. In a study, Ningthoujam and coworkers screened for activity against some major rice fungal pathogens such as *Curvularia oryzae*, *P. oryzae*, and *F. oxysporum* showed potent antagonistic activities in dual culture assay. Considering the diversity of the factors proposed for priming/ synergy so far, plants seem to have developed diverse ways of positive crosstalk in the defense signaling to prime and enhance defense responses, so that they can protect themselves more effectively from the invasion of pathogens (Desaki *et al.*, 2012).

Reddy and Rao (2009) isolated PGPR strains belonging to fluorescent *Pseudomonas* from the rhizosphere of rice. Among 30 strains that were confirmed as *P. fluorescens*, this *P. fluorescens* strain was characterized by PCR-RAPD analysis and biochemical methods. Ten exhibited strong antifungal activity through the production of antifungal metabolites. Furthermore, in a study with wheat plant rhizosphere soil collected from different wheat-growing regions of Kashmir valley were evaluated for the presence of *P. fluorescens* using King's B medium. Based on colony morphology, siderophore production, and biochemical tests out of 136 rhizosphere soil samples, only 52 isolates were identified as *P. fluorescens*. Some species exhibited remarkable antifungal activity against *F. oxysporam* with different levels of inhibition pattern (Showkat *et al.*, 2012).

CONCLUSION

PGPR help in disease control in plants. Some PGPR, especially if they are inoculated on the seed before planting, can establish themselves on the crop roots. PGPR is a component in integrated management systems in which reduced rates of agrochemicals and cultural control practices are used as biocontrol agents.

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Table No.1 *In-vitro* antifungal activity of the isolates against selected pathogens

STRAINS	*Fugal Pathogens*		
	Pyricularia oryzae	***Rhizoctonia solani***	***Fusarium oxysporum***
SM2	++	+++	+++
SM4	+++	+++	++
SM9	++	++	++
SM11	++	++	++++
TM1	-	++	-
TM3	++	-	++
TM5	-	++	-
KM1	+	++	+
KM2	+	++	+
KM3	-	++	++
VS1	-	+++	++
VS4	+++	++	+++
VS7	++	+++++	++
VS8	++	+++	++
VS9	-	++	-
SS2	++	++	-
SS5	++	-	-
SS6	+++	+++	++
SS8	-	-	-
SS10	-	++	-
MS2	++	+++	++
MS5	+	++	-
MS7	+	++	++
MS8	++++	+++	+++

Note: - = no inhibition; + = weak inhibition; ++ = moderate inhibition; +++ = strong inhibition

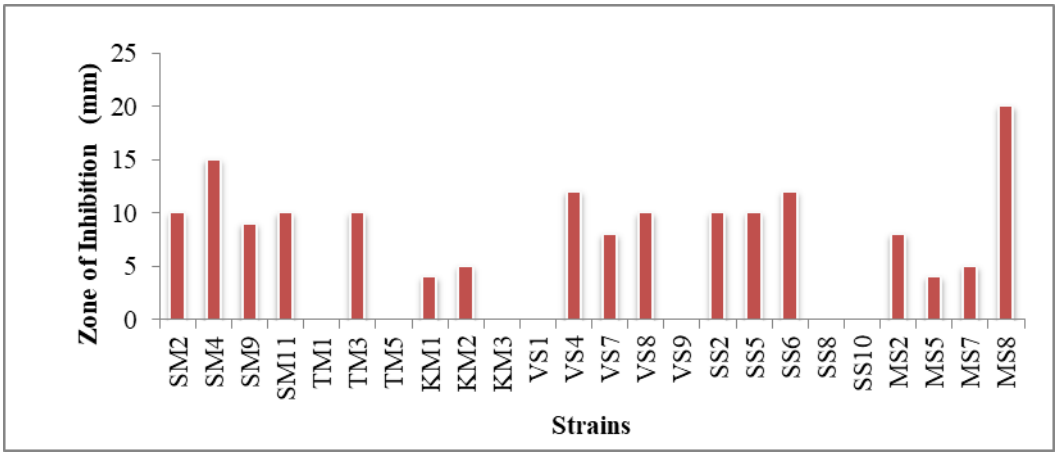

Figure No. 1a Antifungal activity of the isolates against *Pyricularia oryza*

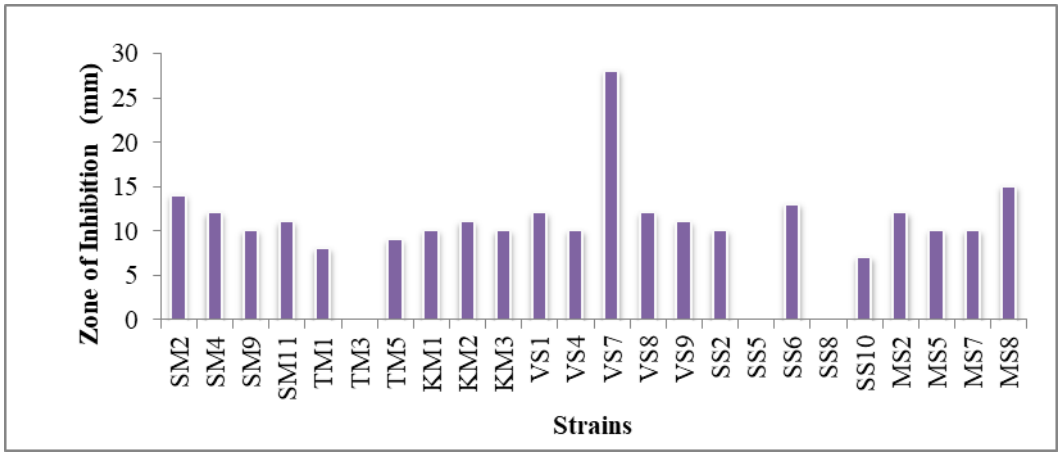

Figure No. 1b Antifungal activity of the isolates against *Rhizoctonia solani*

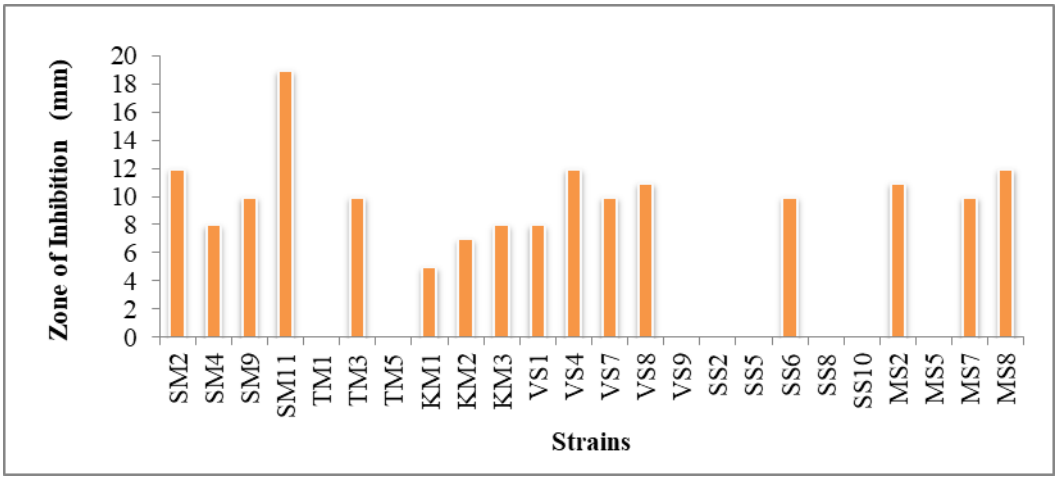

Figure No. 1c Antifungal activities of the isolates against *Fusarium oxysporum*