Plant Archives Vol. 15 No. 2, 2015 pp. 763-768 *ISSN 0972-5210*

ANTAGONISTIC POTENTIAL OF MYCORRHIZA ASSOCIATED *PSEUDOMONAS PUTIDA* AGAINST SOIL BORNE FUNGAL PATHOGENS

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Abstract

Mycorrhizal spores were found to be closely associated with bacteria on their spore wall surface known as mycorrhiza associated bacteria (MAB) and its play a major role in the plant growth promotion by production of many known and unknown metabolites, nutrient solubilization and suppression of many soil borne pathogens in rhizosphere of crop plants. The present study was carried out to understand the antagonistic potential of mycorrhiza associated bacterium *i.e. Pseudomonas putida* (Genbank Accession number HM590707) against selected soil borne fungal pathogens *viz.*, *Fusarium oxysporum*, *Ceratocystis fimbriata* and *Sclerotium rolfsii*. The bacterium *P. putida* used in this experiment was isolated from *Glomus mosseae* spore. The antifungal properties of *P. putida* indicated that this bacterium could produce siderophores, ammonia, hydrogen cyanide (HCN), protease, chitinase, urease and ACC deaminase. The dual culture assay revealed that the *P. putida* found to inhibit 65.5, 68.8 and 62.2 per cent mycelial growth of *F. oxysporum*, *C. fimbriata* and *S. rolfsii* respectively under *in vitro* condition, which indicates the broad spectrum antagonistic activity of this bacterium. Similarly, it was observed that *P. putida* could suppress 58.8-61.1 per cent fungal biomass in liquid broth culture. This finding reveals the broad spectrum anti-fungal activity coupled with the production of anti-fungal metabolites of mycorrhiza associated *P. putida* can be explored as biocontrol agent for managing soil borne fungal pathogens under field condition.

Key words : Mycorrhiza associated bacteria, *Pseudomonas putida*, antagonistic activity, anti-fungal metabolites, soil borne fungal pathogens.

Introduction

Arbuscular Mycorrhizal (AM) fungi are ubiquitous in nature and constitute an integral component of terrestrial ecosystems, forming symbiotic associations with plant root systems of over 80% of all terrestrial plant species, including many agronomically important species. During AM fungal colonization of plants, the AM fungal spores provide shelter for some beneficial bacteria, which are known as mycorrhiza associated bacteria (MAB) (Garbaye, 1994). These mycorrhiza associated bacteria were reported to play a major role in plant growth promotion in fruit crop seedlings (Panneerselvam *et al*., 2012; Panneerselvam *et al*., 2013; Sukhada *et al*., 2013). AM fungi is one of the important bio-control agents for managing the wide range of soil borne fungal pathogens in horticultural production (Singh *et al*., 2000). The role of AM fungi and a concurrent decrease of plant disease symptoms by inducing the local and systemic resistance to disease of crop plants have been well demonstrated by several authors (Cordier *et al*., 1996; Lioussanne *et al*., 2008 and Pozo *et al*., 2002). However, the role of mycorrhiza associated bacteria on disease suppression was not explored much. Globally, pathogenic fungi are responsible for more than 70% of all major crop diseases (Agrios, 2005) and have caused significant economic losses typically reducing plant yields annually. Among the most common plant pathogenic fungi, the following three fungi *viz*., *Fusarium* sp, *Ceratocystis* sp. and *Sclerotium* sp. are reported to cause dreadful diseases in many horticultural crops.

Fusarium oxysporum causes the most destructive wilt or root rot disease in many fruit crops (Stover, 1962). *Ceratocystis fimbriata* causes the dreadful disease of pomegranate wilt (Somashekhara, 1999 and Huang *et al*., 2003) and fruit rots (Firmino *et al*., 2013). *Sclerotium rolfsii* causes southern blight disease known to be very destructive in numerous vegetable and fruit crops (Farr *et al*., 1989 and Mullen, 2001). These soil borne fungal pathogens are not being able to managed effectively by using fungicides alone. Many reports have suggested

adopting integrated disease management approaches including chemicals, biocontrol agents and growing resistant varieties as alternatives for sustainable crop production. In general, mycorrhizal colonization is one of the closest associations in most of the crop plants and also recent report (Bagyaraj *et al*., 2015) suggests that studying the mycorrhizal responses is essential for breeding programme, hence understanding the beneficial effect of bacteria being associated in AM fungal spores may be much useful for crop production. Many reports are available on bacterial biocontrol agents *viz*., *Pseudomonas*, *Burkholderia* and *Bacillus* spp. (Weller, 1988; Weller and Cook, 1983) and their role on plant disease suppression. Though, scientific findings have addressed the growth promoting activities of mycorrhiza associated bacteria, information on its antagonistic potential is still scarce.

In our previous works, the following mycorrhiza associated bacteria *viz*., *Pseudomonas putida*, *B. choshinensis*, *B. parabrevis*, *Pr. Rettgeri*, *P. aeruginosa*, *Brevibacillus* sp and *Bacillus subtilis* and their growth promoting potential has been proved in guava and sapota seedlings (Panneerselvam *et al*., 2012 and 2013), among the MAB isolates, *P. putida* was found to be superior in improving plant growth promotion in terms of plant height, stem girth, shoot and root weight, total biomass, plant nutrients uptake and mycorrhizal colonization. However, the antagonistic potential of this bacterium has not been studied so far, hence in the present investigation; we have studied the antagonistic activity of the *P. putida* against the fungal pathogens *Fusarium oxysporum*, *Ceratocystis fimbriata* and *Sclerotium rolfsii*.

Materials and Methods

Isolation and identification of mycorrhiza associated bacteria (MAB)

Glomus mosseae spores isolated from sapota rhizosphere were surface sterilized with 5% Chloramine-T (BDH Inc., Toronto, Canada) for 30 min (Walley and Germida, 1996) followed by six times rinsing with sterile distilled water. The surface-sterilized spores were crushed with sterile water and then plated out in nutrient agar medium and incubated at 30^0C for 2 days. The isolated bacteria were identified based on 16S rRNA gene sequencing. In total, four plant growth promoting bacteria were screened from *G. mosseae* spore, of which *P.putida* was found to be superior in enhancing plant growth promotion (Panneerselvam *et al*., 2013).

Cultures of pathogenic fungi

Culture of fungal pathogen *Fusarium oxysporum* was received from IMTECH, Chandigargh, India and *Ceratocystis fimbriata* and *Sclerotium rolfsii* were received from Soil Microbiology Lab, Division of Soil Science and Agrl. Chemistry, ICAR-IIHR, Bengaluru, India.

Screening of anti-fungal activity in both solid and liquid medium

In agar plate assay, a loopful of *P. putida* culture (24 hr grown) was streaked on four sides of the potato dextrose agar (PDA) plates. Mycelial discs (2 mm in diameter) of pathogenic fungi (*F. oxysporum*, *C. fimbriata* and *S. rolfsii*) from an actively growing culture were placed at the centre of the PDA plate. Control plates were maintained by inoculating only fungal culture. The plates were incubated at 30°C for 7 days. The zone of inhibition of radial mycelial growth of the fungi was measured. The percentage inhibition of radial mycelial growth was recorded as described by Noori and Saud (2012).

For liquid medium, 2 mm agar discs of pathogenic fungi from PDA plates were aseptically transferred into 250 ml flasks containing 100 ml of potato dextrose broth (PDB), which contain 1.0% *P. putida* culture broth (2.8×10^{10} cfu ml^{-1}) and incubated at room temperature for 7 days. Control was maintained by inoculating only fungal culture. The fungal mycelium was harvested after 7 days of inoculation, separated from the liquid culture by filtration through a Whatman No. 1 filter paper. The mycelial mat was dried at 70°C till it reaches constant weight. Then the per cent inhibition of fungal growth was calculated based on the data of dry biomass recorded from both in treatment and control.

Anti-fungal metabolite production

Siderophore production

Siderophore production efficiency of *P. putida* was determined by the method described by Schwyn and Neilands (1987). For this, 10µl of 24 h old pure bacterial culture grown in nutrient broth was inoculated onto Chrome Azurol S agar plates in triplicates and incubated at 30°C for 4 days and observed for orange halo formation around each colony.

Hydrogen cyanide production (HCN)

HCN production potential of *P. putida* was inferred by the qualitative method of Bakker and Schippers (1986). Change of colour from yellow to dark brown of filter paper previously dipped in 2% sodium carbonate prepared in 0.05% picric acid was rated positive.

Ammonia production

Method suggested by Cappuccino and Sherman

(1992) was used to test ammonia production by the MAB isolate. *P. putida* culture was inoculated in 10 ml peptone broth and incubated at 30°C for 48-72h. After incubation 0.5 ml of Nessler's reagent was added to the tube. Development of brown to yellow colour indicates the production of ammonia.

Volatile compounds

Production of volatile compound was assayed by inverted plate technique (Dennis and Webster, 1971). Two separate Peptone glucose agar (PGA) plates were taken, one plate was inoculated with the fungal culture and in the other plate, 48h old *P. putida* culture was streaked and the two plates were sealed mouth to mouth with parafilm. Controls were maintained with only fungal culture inverted over uninoculated PGA plate and incubated at 30°C for 5 days in triplicate. After incubation period the colony diameter of fungus was measured and compared to control.

Antifungal enzymatic activity

Protease activity

The protease activity of *P. putida* was determined according to Abo-Aba *et al*. (2006) in triplicate. Plates were inoculated with 10ml of 24-h-old pure bacterial culture in Petri-dishes containing 3% agar milk. Plates were incubated at 28±2°C and observed daily for formation of transparent halos around each colony up to 4 days.

Chitinase activity

P. putida was streaked onto colloidal chitin agar, which was prepared according to Hsu and Lockwood (1975) in triplicate. After the incubation at 30°C for 10 days, plates were checked for bacterial growth and the chitin cleared from agar surrounding the bacterial colonies was monitored.

Cellulase activity

The *P. putida* culture was streaked onto carboxymethyl cellulose agar in triplicate and plates were incubated at 30°C for 2 weeks. After that the growth of visible colonies in the plates was recorded. Hexadecyl trimethyl ammonium bromide (5ml, 1%) was used in each plate to distinguish the cellulolytic colonies from the others. After 20min, a clear zone around the colonies was considered as positive for cellulolytic activity.

Urease activity

The urease test was performed by growing the isolate of *P. putida* in urea broth containing the pH indicator phenol red (pH 6.8) and incubated at 37°C for 4-5 days. The positive reaction for the presence of urease activity was observed with change in broth coloration from yellow to deep pink.

ACC deaminase activity

Screening for ACC deaminase activity of *P. putida* was done based on its ability to use ACC as a sole nitrogen source. The *Pseudomonas* isolate was grown in 5 ml of TSB medium incubated at 28°C at120 rpm for 24h. The cells were harvested by centrifugation at 3000g for 5 min and washed twice with sterile 0.1 M Tris-HCl (pH 7.5) and resuspended in 1 ml of 0.1 M Tris-HCl (pH 7.5) and spot inoculated on petriplates containing modified DF (Dworkin and Foster) salts minimal medium (Dworkin and Foster, 1958), glucose,2.0g; gluconic acid, 2.0g; citric acid, 2.0g; KH_2PO_4, 4.0g; Na_2HPO_4, 6.0g; $MgSO_4.7H_2O$, 0.2g; micronutrient solution ($CaCl_2$, 200mg; $FeSO_4.7H_2O$, 200mg; H_3BO_3, 15mg; $ZnSO_4.7H_2O$,20mg; Na_2MoO_4, 10mg; KI, 10mg;NaBr, 10mg; $MnCl_2$, 10mg; $COCl_2$, 5mg; $CuCl_2$, 5mg; $AlCl_3$, 2mg; $NiSO_4$, 2mg;distilled water, 1000ml) 10ml and distilled water, 990ml; supplemented with 3mM ACC as sole nitrogen source. Plates containing only DF salts minimal medium without ACC as negative control and with $(NH4)_2SO_4$ (0.2% w/v) as positive control. The plates were incubated at 28°C for 72h. Growth of the isolate on ACC supplemented plates was compared to negative and positive controls.

Statistical analysis

The data were analyzed using Web Agri Stat Package version WASP2.0, Graphpad Prism 5 software and subjected to one way analysis of variance (ANOVA). Treatment difference was evaluated using least significant difference (LSD) at $p = 0.05$.

Results and Discussion

Isolation and identification of mycorrhiza associated bacteria

A total of four bacteria were isolated from *G. mosseae* spores and these isolates showed 100% sequence homology with *P. putida*, *B. choshinensis*, *B. parabrevis* and *Pr. rettgeri*. All the isolates were tested for their growth promoting attributes in sapota seedlings, of which *P. putida* was found to be superior (Panneerselvam *et al*., 2013), hence for the further study *i.e*. antagonistic potential against soil borne fungal pathogens such as *F. oxysporum*, *C. fimbriata* and *S. rolfsii* only *P. putida* was evaluated.

Evaluation of anti-fungal activity of *P. putida*

P. putida found to significantly suppress *F.oxysporum*, *C. fimbriata* and *S. rolfsii* in the potato dextrose agar (PDA) as compared to control (table 2).

Table 1 : Production of anti-fungal metabolites by *P. putida*.

Different metabolites	Rate of production
Siderophore production	+++
HCN production	+
Protease activity	+
Cellulase activity	-
Chitinase activity	+
Urease activity	+
ACC deaminase activity	+
Volatile substances	-

"+++" Stronger production, "++" Moderate production, "+" Low production, "-"no production

(Whipps, 2001; Bloemberg and Lugtenberg, 2001). Many earlier researchers have documented the antagonistic potential of *Pseudomonas flourescens*, *P. putida*, *P. graminis*, *P. chlororaphis*, *P. veronii* and *P. tolaasii* (Adhikari *et al*., 2001 and Chen *et al*., 1995). Pannerselvam *et al*. (2006) reported that the applications of *P. flourescens* and AM fungi in coffee plants under field conditions recorded higher phenylalanine ammonia lyase, peroxidase, polyphenol oxidase activities and total phenols in response to the coffee leaf rust compared to uninoculated control. These examples illustrated the high potential of fluorescent Pseudomonads on suppression of phytopathogenic fungi.

Table 2 : Antagonistic activity of *P. putida* against *F. oxysporum*, *C. fimbriata* and *S. rolfsii* under *in vitro* condition.

Treatment	*F. oxysporum*		*C. fimbriata*		*S. rolfsii*	
	Mycelial growth diameter (cm)	Per cent inhibition over control	Mycelial growth diameter (cm)	Per cent inhibition over control	Mycelial growth diameter (cm)	Per cent inhibition over control
P. putida	3.1b*	65.5	2.8b*	68.8	3.4b*	62.2
Control	9.0a	-	9.0a	-	9.0a	-
CD (0.01)	0.67	-	0.66	-	0.67	-
CD (0.05)	0.49	-	0.48	-	0.49	-

*Data are average of ten replications.

Table 3 : Antagonistic effect of *P. putida* on *F.oxysporum, C. fimbriata* and *S. rolfsii* in liquid broth assay.

Treatment	*F. oxysporum*		*C. fimbriata*		*S. rolfsii*	
	Mycelial dry weight (mg)	Per cent inhibition over control	Mycelial dry weight (mg)	Per cent inhibition over control	Mycelial dry weight (mg)	Per cent inhibition over control
P. putida	0.21b*	61.1	0.19b*	60.4	0.21b*	58.8
Control	0.54a	-	0.48a	-	0.51a	-
CD(0.01)	0.045	-	0.036	-	0.039	-
CD(0.05)	0.033	-	0.027	-	0.028	-

*Data are average of ten replications.

P. putida found to inhibit the mycelial growth of *C. fimbriata*, *F. oxysporum* and *S. rolfsii* in the range of 62.2 – 68.8% after seven days of incubation. Similar trend was noticed in potato dextrose broth (PDB) (table 3). In the broth assay, *P. putida* showed 61.1, 60.4 and 58.8 per cent suppression of mycelial growth with respect to *F. oxysporum*, *C. fimbriata* and *S. rolfsii* compared to control. These observations are corroborated with the earlier finding of Cook *et al*. (1995). Majority of antagonistic bacteria belong to the group of fluorescent Pseudomonads also known as effective biocontrol agents

Anti-fungal metabolite production

The *P. putida* tested for anti-fungal metabolite production showed positive to siderophore production, ammonia production and hydrogen cyanide production (table 1) and negative for the production of volatile substances. The results of antifungal enzymatic activity revealed that *P. putida* showed positive for protease, chitinase, urease and ACC deaminase activities whereas it was negative to cellulase activity (table 1). The microbial metabolites such as siderophore, HCN are reported to play a major role in disease suppression (Cao *et al*., 2005;

Thomashow and Weller, 1996). Siderophore producing bacteria have competitive advantage over the pathogens for the absorption of available iron. *P. fluorescens* and *P. putida* have been shown to be successful seed and soil inoculants for siderophore-mediated biocontrol agents for several plant pathogens (Dube, 1992; Van Peer *et al*., 1990; Scher and Bake, 1982). Moreover, lytic enzymes produced by bacteria were also suspected to be involved in the growth inhibition of the pathogens (Buchenauer, 1998). In the present study, the *P. putida* which belong to the group of fluorescent Pseudomonads, which is proved to produce different antifungal metabolites might have involved in suppression of *F. oxysporum*, *C. fimbriata* and *S. rolfsii*.

Earlier findings indicated that the AM fungal association in host plants induced the local and systemic resistance to disease of crop plants (Pozo *et al*., 2002) by stimulating the production of root exudates, phytoalexins and phenolic compounds (Morandi, 1996 and Singh *et al*., 2000). There are reports which suggest that application of *Glomus* spores enhanced lignin and phenolic contents which induced systemic resistance in plants reducing the disease incidence. The exudates released by strawberry roots colonized by *Glomus etunicatum* and *Glomus monosporum* inhibited the sporulation of *Phytophthora fragariae* under *in vitro* conditions (Norman and Hooker, 2000). Also it was reported that application of *Glomus etunicatum* reduced *Fusarium* wilt incidence by 40% in tomato (Ozgonen *et al*., 2001). These earlier findings suggested the role of AM fungi on reducing the disease incidence in many crops.

Our study proved that the *Glomus* spore associated *P. putida* has strong antagonistic activity against *F. oxysporum*, *C. fimbriata* and *S. rolfsii.* Panneerselvam *et al*. (2013) reported that inclusion of mycorrhiza associated bacteria *i.e. P. putida* is a prerequisite while applying AM fungi for better colonization and growth promotion of sapota. Hence, application of AM fungi along with its associated bacteria may be more effective in disease suppression rather than inoculation of AM fungi alone. The information generated from this study may be useful for the researchers to explore the mycorrhiza associated bacteria for management of plant diseases in future. Indepth studies are needed in these lines to understand the interactions between mycorrhiza and its associated bacteria for plant disease management.

Acknowledgements

The authors gratefully thank the Director and Head, Division of Soil Science & Agrl. Chemistry, ICAR-Indian Institute of Horticultural Research for the support and infrastructure facilities. The financial grant received from AMAAS project sponsoring agency is gratefully acknowledged.

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