Characterization of siderophore producing bacterial strain *Pseudomonas fluorescens* Mst 8.2 as plant growth promoting and biocontrol agent in wheat

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A set of 28 *Pseudomonas* bacterial strains were examined for siderophore production by chrome azurol sulphonate (CAS) assay. Among the 28 strains tested, 14 were found to be siderophore producers. These strains were evaluated for their biocontrol potential against *Rhizoctonia solani* using various dual culture assays. The role of siderophores in the inhibition of *R. solani* was confirmed by iron chloride (FeCl\(_3\)) experiment. The eight strains that showed best antagonism were found to have the potential of nitrogen fixation, phosphate solubilization, indole acetic acid, hydrolytic enzymes, hydrogen cyanide and antibiotics production. Spectrochemical analysis of siderophores indicated that all the bacterial strains produce catecholate siderophores. Our data demonstrated that bacterial strain Mst 8.2 produces more than one antifungal agents but the siderophore production is the key mechanism involved in the antagonism. Bacterial strains MS-3y, Mst 8.2 and Mst 7.4 were the most effective with more than 70% disease reduction in plant growth of wheat. The complete 16S rRNA gene sequence analysis demonstrated that Mst 8.2 is a *Pseudomonas fluorescens* strain. In conclusion, Mst 8.2 with its multi-mechanisms of defense has excellent potential to be used as successful biocontrol agent against *R. solani* root rot disease in wheat.

**Key words:** Bioantagonism, rhizobacteria, *Pseudomonas*, wheat, siderophore.

**INTRODUCTION**

Environment friendly control of plant diseases is a pressing need for agriculture in the 21st century. Biological control using antifungal rhizobacteria to suppress plant diseases offers a powerful alternative to the use of synthetic chemicals (Emmert and Handelsman, 1999). Different studies have demonstrated the ability of certain bacteria to suppress diseases caused by soil and seed-borne plant pathogens (Whipps, 2001; Dobbelaere et al., 2003). Rhizobacteria usually do not rely on single mechanism of promoting plant growth (Glick et al., 1999). In addition to nitrogen fixation and plant hormones production e.g. indole acetic acid, gibberellic acid and ethylene, plant growth promoting rhizobacteria (PGPR) are also able to provide the plant with sufficient iron in iron-limiting soils (Compant et al., 2005), or other important minerals, e.g. phosphate and zinc (Gull et al., 2004). Different mechanisms have been identified in bio-antagonism of fungal plant diseases including competition for space or nutrients (e.g iron), production of antifungal secondary metabolites (e.g HCN), and secretion of hydrolytic enzymes such as chitinases and glucanases (Mayak et al., 2004).

*Rhizoctonia solani* is one of the most important soil borne fungal pathogens which develop both in cultured and non cultured soils. Its incidence during the seedling
stage is serious, resulting in damping-off and root rot. Root rot diseases caused by *Rhizoctonia* spp., results economic losses to legumes, vegetables, fruits, cereals and cotton (Sneh et al., 1991). Different fungicides have been used to control *Rhizoctonia* diseases; however, the control of *Rhizoctonia* spp. on seedling of rice, mung bean, potato and many vegetables by antagonistics: *Bacillus subtilus* F29-3, *Pseudomonas fluorescens*, *Rhizobium* and *Azotobacter* strains, has also been reported (Cook et al., 2002). There is a dire need for potent biocontrol agents for such a wide host range of pathogens to replace the harmful fungicides and fumigants.

Despite abundance of iron in the earth's crust, it is largely unavailable for microbial and plant assimilation. Soil microorganisms and even some plants have developed mechanisms that ensure iron availability in an environment that provides iron “Fe$^{3+}$” at only about $10^{-18}$ M. This strategy involves the secretion of low molecular weight iron chelating compounds called siderophores that show a considerable structural diversity and can be classified according to their main chelating groups (Nielsen and Sorenson, 2003). Many Pakistani soils (>90%) are Fe deficient due to high pH (Ahmad et al., 1996). The use of siderophore producing bacteria can help to improve iron deficiency, plant growth and the yield of economically important crops (Westover et al., 1997).

Siderophores apart from their role in active transport of iron may act as growth antagonists by means of sequestering iron from the environment, restricting the growth of pathogens (Berg et al., 2002). The study presented here was aimed at identifying rhizobacterial strains which could help to control *Rhizoctonia* diseases, in particular root rot of wheat. The rhizobacteria were characterized for their growth promoting traits (nitrogen fixation, indole-3-acetic acid (IAA) production and P-solubilization) and production of defensive secondary metabolites [hydrogen cyanide (HCN), antibiotics, hydrolytic enzymes and siderophores]. The role of siderophores in inhibitory capacity of these biocontrol bacteria producing more than one antimicrobial compound against *R. solani* was also evaluated. The complete 16S rRNA gene of a highly promising strain was sequenced for identification purpose.

Understanding the mechanisms of disease control may help to control soil borne diseases efficiently and could help to identify biocontrol agents that not only suppress disease but provide plant growth potential that is required in commercial agriculture.

**MATERIALS AND METHODS**

**Bacterial strains, media and growth conditions**

The bacterial strains used in this study were obtained from Biofertilizer Resource Centre (BIRcen) culture collection (National Institute for Biotechnology and Genetic Engineering (NIBGE) Faisalabad, Pakistan). They were grown on Luria Bertani (LB) medium at 28 ± 2°C for 48 to 72 h to obtain pure cultures and stored at 4°C in culture tubes and Petri dishes (Maniatis et al., 1982). Glassware was cleaned with 6 M HCl or 6N HNO$_3$ to avoid contamination with iron.

Reference siderophore producing strains *Pseudomonas putida* B10 and *P. salomonii* CFBP2220 were kindly provided by Dr. Jean Marie Meyer (University of Louis Pasteur, Strasbourg Cedex, France). The pathogenic fungal strain of *Rhizoctonia solani* was obtained from Fungal Culture Bank (University of Punjab Lahore, Pakistan). *R. solani* cultures were stored at 4°C in tubes containing potato dextrose agar (PDA) medium. The pathogen was identified by phenotypical observations using standard mycological literature.

**Pathogenicity test of *R. solani***

The pathogenicity of selected fungus to wheat (*Triticum aestivum* cv. INQ-91) was performed by modification of the method of Cook (1993). Fungal suspension (20 μl) at 10$^6$ colony forming unit (CFU/ml) was deposited at the leaf surface of wheat plants. Sterile water (20 μl) was used as control. Brown discoloration around the inoculation spot within 72 h was considered as positive reaction.

**Pre-evaluation of antagonistic properties of *Pseudomonas* strains**

Bacterial strains were tentatively identified as *Pseudomonas* species based on their growth on specific Cetrimide *Pseudomonas* agar medium. *Pseudomonas* strains were assayed for their inhibitory activity against the soil-borne pathogenic fungus *R. solani* by three dual culture methods.

**Round circle method**

One 10 mm disk of a pure culture of *R. solani* was placed at the center of petri dish containing PDA. A circular line, made with a 6 cm diameter petri dishes (Maniati et al., 2001). The bacterial suspension was deposited around the inoculation spot. The inhibition of radial fungal growth as a clear zone between the bacterial and fungal colonies. Inhibition of growth was calculated using the following formula:

% Inhibition = [(1 – fungal growth) / control growth] × 100

**Spot inoculation method**

*Pseudomonas* strains were cultured in LB agar plates and a block of 6-mm disk was cut in the center of each plate. A 6 mm disk of *R. solani* was placed in the cut LB agar plates and 24 h old cultures of *Pseudomonas* strains were inoculated on two sides of fungal disk as spots. *R. solani* was also raised on LB agar (Arora et al., 2001). The plates were incubated at 28 ± 2°C for 5 days to measure inhibition of radial fungal growth as a clear zone between fungal and bacterial colonies. Percent inhibition was determined by the reduction in fungal growth compared to control that was inoculated with a bacterial strain. Duncans Multiple Range Test (Duncan, 1955) was performed (<0.05).

**Isosceles right triangle method**

Agar blocks (2 mm) from a fresh culture of *R. solani* were tested in a dual culture on PDA and Kings B (KB) media against
Pseudomonas strains for inhibition of growth. To standardize our technique, a template was made from cardboard in the shape of an isosceles triangle with the sides 5.8 cm in length and 4 cm from the angle to the base. Individual bacterial colony was inoculated along the hypotenuse of the triangle (Baligh et al., 1999). Fungal inoculum was placed in the right angle so that the plug was at a maximum distance of 4 cm from the bacteria streak. The plates were incubated at 28 ± 2°C. The zone of inhibition between the bacteria and the leading edge of the fungal colony was measured after 4 days. Duncans Multiple Range Test (Duncan, 1955) was performed (< 0.05) for statistical evaluation in this dual culture assay.

Physiological and biochemical characterization of bioantagonistic bacteria

Bioantagonistic bacterial isolates were assayed for their plant growth promoting potential (production of IAA, nitrogenase activity and P-solubilization) and production of defensive metabolic compounds (siderophores, HCN, hydrolytic enzymes and antibiotics) that could inhibit R. solani.

A smear of each bacterial isolate was tested for Gram reaction using the standard procedure (Vincent, 1970). Pseudomonas strains were tested for IAA production by colorimetric method (Okon et al., 1977) using nitrogen free medium (NFM). Trypsophan (100 mg/L) was added as the precursor of IAA. To estimate the nitrogen fixing ability, pure bacterial colonies were inoculated onto semi-solid NFM in vials and incubated at 28 ± 2°C for 48 h. Nitrogenase activity was measured as described by Yasmin et al. (2004). Acetylene (10% v/v) was injected into the vials. After incubation for 16 h at 28 ± 2°C, gas samples (100 µl) were analyzed for the presence of ethylene using a gas chromatograph (Thermoquest, Trace G.C. Model K, Rodono Milan, Italy), a porapack Q column, and a H₂-flame ionization detector.

Pikovskai’a’s medium was used to screen for phosphate solubilization (Gull et al., 2004). Bacteria able to form clear zones on tricalcium phosphate agar plates were considered phosphate solubilizers.

Siderophore production by bacterial strains was evaluated using universal chrome azurol S assay (Schwyn and Neilands, 1987). Cyanide production by bacterial strains was detected with a saturated potassium ferrocyanide solution (0.1 mg/ml) (Casanovas et al., 1995). Petri dishes containing tryptic soy agar (TSA) (10%) supplemented with 4.4 g/L of glycine were incubated at 28 ± 2°C for 3 days. A piece of filter paper impregnated with 0.5% picric acid and 2% sodium carbonate was placed in the lid of each Petri dish. A change in colour from yellow to orange brown on the filter paper indicated cyanide production.

The ability to hydrolyze gelatin and starch was examined by plating bacteria on nutrient agar containing 2% starch or 1% gelatin and incubating for 48 h at 28 ± 2°C as described by Marten et al. (2000). Starch hydrolysis was assayed after overlaying Petri dishes with Lugol’s solution. A positive reaction was shown by a colorless zone “starch, blue colour” around the bacterial colonies. To determine the ability to hydrolize gelatin, the plates were incubated with a saturated ammonium sulfate solution for 5 min; hydrolysis was detected by clear zones around bacterial colonies.

Bacterial colonies were screened for chitinase production (B1, 4-glucosamine polymer degradation) by plating on 1% chitin agar (CA) plates. Clear halos indicating enzymatic degradation were measured after 5 days of incubation at 28 ± 2°C (Marten et al., 2000). Production of reducing sugar or β-1, 3-glucanase activity was observed by using CA plates with β -1, 3 glucan (5g/L) (laminarin) as the carbon source (Marten et al., 2000). Cellulase, pectinase, mannnase and xylanase production was determined as described by Andro et al. (1984) and Samanta et al. (1989). Protease production by Pseudomonas strains was detected by streaking each bacterial isolate on skim milk agar (SMA) medium. 50 ml of sterilized skim milk was mixed at 55°C with 50 ml of one fifth volumes of TSA and 4% agar, and incubated for 3 days at 28 ± 2°C. Clear zones indicate casein degradation (Maurhofer et al., 1995).

Resistance to ampicillin, tetracycline and chloramphenicol, each at concentration of 10, 50 and 100 µg/ml was determined. Clear halo zones around filter paper disks were interpreted as indicators of organisms resistant to these antibiotics (Yasmin et al., 2004).

Bacterial antibiotic production detection assays

Volatile antibiotics

100 µl of a bioantagonistic bacterial suspension (5 × 10⁹ CFU/ml) were placed at the center of one half of a Petri dish containing Kings B medium, and a disk (10 × 5 mm) of four day old culture of R. solani was placed at the center of another Petri dish containing PDA (Montealegre et al., 2003). Both half plates were placed face to face preventing any physical contact between the pathogen and bacterial suspension and were sealed to prevent loss of volatiles formed. Plates were incubated at 28 ± 2°C for 48 h and the growth of pathogen was measured and compared to controls in which sterile distilled water was used in place of the Pseudomonas culture. Duncan Multiple Test (Duncan, 1955) was <0.05.

Diffusible antibiotics

PDA plates, covered with a cellophane membrane, were inoculated with 100 µl of bacterial suspension (5 × 10⁹ CFU/ml). After incubation for 72 h at 28 ± 2°C, the membrane with the bacterial growth was removed, and the plates were inoculated with a disk (10 × 5 mm) of R. solani. Plates were further incubated at 28 ± 2°C for 48 h and the growth of the pathogen was measured (Montealegre et al., 2003). Controls were run with sterile distilled water replacing the bacterial suspension. Results are expressed as means of percent inhibition of growth of R. solani. Duncan Multiple Test was <0.05.

Role of siderophores in the inhibition of R. solani in vitro

Bacterial strains that showed strong antagonistic activity against R. solani were tested to evaluate the role of siderophores in fungal growth inhibition. Each strain was spotted on one side of a plate of Kings B media supplemented with FeCl₃ at 0, 5, 10, 25 and 50 µg/ml. A disc of PDA (5 mm in diameter) of R. solani culture was placed on the other side of the plate and incubated at 28 ± 2°C for three days. Percent inhibition was calculated using the following formula:

% Inhibition = [(1 – Fungal growth/control growth) × 100

To verify the sensitivity of R. solani to iron deprivation, 40 µl of a solution (0.1 mg/ml) of ethylenediamine-di-orthohydroxyphenyl acetic acid (EDDA) was added in a well 4 mm in diameter on one side of Kings B agar plate (instead of bacterial culture) and the R. solani culture was placed as a disc of PDA 5 mm diameter on the other side of the same plate after 2 days and incubated at 28 ± 2°C for 5 days. In the control plate, deionized water (adjusted to pH 9 with NaOH) was used instead of EDDA (Becker and Cook, 1988).

Quantitative determination of bacterial siderophores

CAS-bioassay

The eight best antagonistic bacterial strains were evaluated for siderophore production by the universal siderophore CAS assay.
(Schwyn and Neillands, 1987). The bacterial cultures were incubated for 48 h in an incubator shaker at 250 rpm and 28 ± 2°C and then centrifuged for 15 min in a Beckman GPR tabletop centrifuge set at 3000 rpm (1,620 × g) at 4°C. Bacterial culture supernatant (1 ml) was mixed with 1 ml CAS shuttle solution. A reference was prepared with the same medium used for culturing the bacterial strains but not inoculated with the bacterium. The samples and reference absorbance “A” was measured at λ of 630 nm after incubation for 1 h at room temperature. The percentages of siderophores were calculated by subtracting the sample absorbance from the reference (Millages et al., 2002). Siderophore units were defined as % siderophore unit = [(A1 - A2 / A1)] × 100. Percentages of siderophore units less than 10 were considered as negative and in this case no change in the blue color of CAS shuttle solution was observed.

Specific colorimetric tests were used to detect the hydroxamate and phenolate types of siderophores in cell free supernatant from the bacterial culture. Preparation of samples was carried out as described by methods of Diarra et al. (1996). Supernatants from bacterial cultures to be tested for siderophore activity were prepared in the following manner. First, single pure colonies of bacteria from LB plates were grown in 50 ml of LB broth contained within 150 ml flasks. The cultures were incubated for 48 h in an incubator shaker at 250 rpm and 28 ± 2°C and bacterial growth was assessed at 660 nm. The supernatants were obtained after centrifugation (12,000 × g for 30 min at 4°C) and concentrated by freeze drying. Methanol was added and the mixture was stirred overnight at the room temperature and centrifuged to remove undissolved material. The yellow supernatant was evaporated to dryness and resuspended in 2 ml of water. The supernatants were made completely cell free by further passing through 0.2 μm pore size sterilizing filters.

Hydroxamate siderophores

The presence of hydroxamate type structure was determined by the method of Csaky (1948). The assay consisted digestion of samples containing hydroxamic acid in sulfuric acid for the detection of bound hydroxylamine. A pink color is indicative of a positive reaction and the intensity of the reaction depends upon the release of hydroxylamine. To 0.5 ml of culture supernatant which had been concentrated 10 fold by freeze drying, 0.5 ml of 6 M H2SO4 was added: the mixture was autoclaved in a glass-stoppered tube at 121°C for 30 min and allowed to cool, and 1 ml of 1% (w/v) sulfanilic acid in 30% (v/v) acetic acid and 0.5 ml of 1.3% (w/v) iodine (I2) in 30% (v/v) acetic acid were added. After 5 min at room temperature, excess I2 was eliminated by the addition of 1 ml of 2% (w/v) Na2AsO3 solution.

A solution of α-naphthylamine (0.3% [w/v] in 30% acetic acid [v/v]; 1 ml) was added and the total volume was increased to 10 ml with distilled water. After 30 min at room temperature, absorbance at 540 nm was recorded. The assay is quantitative for hydroxamic acids. Hydroxylamine hydrochloride was used as the positive hydroxamate control. A blank was prepared by the addition of reagents to 0.5 ml of distilled water.

Catecholate siderophores

Catecholate type siderophores structures were detected by colorimetric test of Arnow (1937) in which catechol gives a yellow color when reacted with nitrous acid and changes to an intense orange red when the medium is made strongly basic. Since catecholate type siderophores are derivatives of 2, 3-dihydroxybenzoic acid (2, 3-DHBA), this compound was used as standard for this assay. 1 ml of the culture supernatant was decanted to a test tube and reagents were added in the following order, with mixing after each addition: 1 ml of 0.5 M HCl, 1 ml of nitrite-molybdate reagent, 1 ml of 1 M sodium hydrosolate, and distilled water to increase the volume to 5 ml. The absorbance was recorded at 510 nm. All the experiments were repeated three times with 3 replicates.

Biocontrol assay on wheat plants

The plant growth promoting activity of biocontrol bacteria was evaluated under controlled conditions in a growth room. Sterile plastic pots (6.45 cm in diameter and 10 cm deep) were twothird filled with 250 g of local silt loam soil that had been sterilized with moist heat at 60°C for 4 h and then air dried. Each pot was inoculated with two 1 cm diameter agar plugs taken from the edge of growing R. solani mycelia. The soil was then moistened with water applied from the top and inoculated beneath clear plastic sheeting to prevent drying for one week. This allowed time for the pathogen to colonize the pasteurized soil from the source of inoculum provided (Berg et al., 2000).

Seeds were inoculated by dripping 1 ml suspension of bacterial cells (106 to 109 cells/ml) on to seeds before covering them with soil. For inoculation of seeds, bacterial cultures were grown in LB broth for 24 h at 28 ± 2°C. The cell pellets were obtained by centrifugation at 6000 × g for 5 min, washed twice with sterile distilled water and resuspended in 0.1 M MgSO4 (pH 7) to give an absorbance of 0.5 at 660 nm. The negative control consisted of 0.1 M MgSO4 solution (Bakker et al., 2003).

Three wheat seeds per pots were planted to a depth of 1 cm, covered with 1 cm thick layers of non inoculated soil, watered from the top and incubated in a growth room at 20 to 25°C. Pasteurized soil of the same quantity per pot and incubated in the same manner but without pathogen inoculum served as control checks. After emergence, plants were watered twice weekly with 5 ml of 1/4 strength Hoagland solution (Arnon and Hoagland, 1940). The experiment was laid out in Completely Randomized Design (CRD) with 11 treatments and 4 replicates. Three replicates were used for final data collection while a fourth replicate was used to assess fungal growth by uprooting the seedlings.

The occurrence of disease was assessed at the time of appearance of the tops “plant stunting” and the roots “girdled or severely by Rhizoctonia lesions. The incidence of seminal root rot, crown root rot and disease intensity were determined 25 to 30 days after planting. The 2nd and 3rd leaf of each plant was assessed for Rhizoctonia lesions and percent diseased leaf area of each was recorded.

The leaf spot disease index was determined at 28 days after planting (DAP), according to a disease rating scale from 0 to 5 in which 0= no lesion, 1= 1 to 20% leaf area with lesions, 2= 20 to 40% of leaf area with lesions, 3= 40 to 60% of the leaf area with lesion, 4= 60 to 80% of the leaf area with lesion and 5= completely dead. With this scale, the percentage area with lesions reflected the combined severity of R. solani (Raupach and Kloeper, 1998).

Analysis of 16S rRNA gene sequence

The complete 1.5 Kb 16S rRNA region (Escherichia coli positions 8 to 1526) of isolate Mst 8.2 was amplified using primers P1 (50 pmol/μl) as forward primer (5'-CGGGATCCAGAGTTTGATCCTGGTCAGAACGCT-3') and P6 (50 pmol/μl) as reverse primer (5’GGGATCTACGCGTACCTGGTACGACCTCACCC-3’). Reaction mixture (25 μl) was prepared for full length 16S rRNA gene amplification and initially denatured at 94°C for 2 min followed by 30 cycles consisting of denaturation at 94°C for 30 s, primer annealing at 55°C for 60 s and primer extension at 72°C for 3 min and a final extension at 72°C for 10 min in a thermal cycler.

The polymerase chain reaction (PCR) product was cloned into...
Pathogenicity test

Pathogenicity test showed that the selected R. solani fungal suspension at 10^8 CFU/ml could induce brown discoloration around the inoculation spot within 72 h. These results suggested that the fungal strain is pathogenic and can be used for antagonistic tests against bacterial strains.

Pre-evaluation of antagonistic properties of bacteria

Out of 14 siderophore producing strains, dual culture assays led to the selection of 8 best antagonistic bacteria which proved their inhibitory capability by all 3 methods tested (Table 1). In the round circle assay, no physical contact was observed between any of the bacteria tested (Table 1). In the spot inoculation test, inhibition of fungal growth (mm) was compared with others in the Gene Bank databases using the NCBII BLAST (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi).

RESULTS AND DISCUSSION

The present study was mainly conducted to assess the antifungal potential and biochemical variation in the production of secondary metabolites by Pseudomonas isolates. Fluorescent Pseudomonas have drawn attention worldwide because it stimulate plant growth by producing secondary metabolites such as siderophores, antibiotics, volatile substances (ammonia and hydrogen cyanide) plant hormones and hydrolytic enzymes (Westover et al., 1997).
PDA medium (Table 1). All the bacterial strains were more inhibitory on KB than on PDA, except 4.1.1.A, 4.2.1.A, Z.4.2 and Z.2.7 which exhibited less antagonistic response on KB medium (Table 1). On KB medium, Mst 8.2 strain displayed the greatest inhibition against *R. solani* with a zone of 3.1 cm length.

The results of dual culture studies showed that antagonism to soil-borne root pathogen varied with the type of media and the secondary metabolites production. The variability of inhibition among *Pseudomonas* strains against *R. solani* on the different media LB, KB and PDA illustrate the importance of nutrition on the efficacy of biological control microorganisms. The suppression of disease on KB medium was significant as compared to PDA indicating the provision of better nutritional requirements for *Pseudomonas* on KB medium (Baligh et al., 1999). The results of triangular assay indicate that the distance or physical contact between pathogenic fungi and antagonistic bacteria is an important criterion for successful biological control. Cattelan et al. (1999) suggested that a successful biocontrol agent should be medium-independent so that it can perform better under field conditions. The variation of the antagonistic activities of the bacterial strains on different media might be due to the chemical modification of the antifungal compounds in response to different nutrients. These results are in accordance with Jayaswal et al. (1990), Baligh et al. (1999) and Amer and Utkhede (2000), who reported antagonistic variation of *Pseudomonas* strains on corn meal agar (CMA), Kings B agar and PDA. Different methods of dual antagonistic tests reveal that the formulation and method of applying the antagonistic bacteria in soil may improve their efficacy as a biological control against soil borne diseases (Kazempour, 2004).

**Physiological and biochemical characterization of bioantagonistic bacteria**

Antagonistic bacterial strains were characterized on the basis of their morphological and biochemical characteristics (Table 2). All the bacterial isolates were gram-negative, motile rods and fast-growing. The production of IAA by six rhizobacteria not only contributes to increased root length and nutrient uptake by plants but is also involved in antifungal activity indicating its contribution in biocontrol enhancement. Six bacterial strains showed nitrogenase activity. Siderophore-producing biocontrol strains fix more nitrogen than nonsiderophore producers. Streit and Phillips (1996) reported that this might be due to sufficient iron supply under iron-limited conditions for the nitrogenase enzyme. Thus, knowledge of how rhizobacteria acquire iron should help further our understanding of these economically important symbioses (Glick et al., 1999).

Four strains MS-3y, Z5, Z11, Z.2.7 were shown to have P-solubilization potential. P-solubilizing bacterial strains could be potent biocontrol agents because they promote lateral root development and nutrient uptake (Gull et al., 2004). A high proportion of rhizospheric bacteria are intrinsically resistant to antibiotics. A PGPR resistant to high levels of antibiotics might have an ecological advantage for survival in the rhizosphere when introduced as a biocontrol inoculum (Dobereiner and Baldani, 1999).

*Pseudomonas* bacteria produce siderophores which facilitate iron uptake. Siderophore-producing bacterial strains showed a violet coloration on CAS blue media, demonstrating the production of catecholate siderophores (Figure 2). No strains changed the blue color of CAS agar medium.
Table 2. Physiological and biochemical traits of *Pseudomonas* strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>IAA</th>
<th>ARA</th>
<th>P-Solubilization</th>
<th>Siderophore (CAS)</th>
<th>HCN</th>
<th>Gelatin hydrolysis</th>
<th>Starch hydrolysis</th>
<th>Antibiotic resistance</th>
<th>Chitinase</th>
<th>Protease</th>
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<tr>
<td>MS-3y</td>
<td>+</td>
<td>+</td>
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<td>+++</td>
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<td>+++</td>
<td>+++</td>
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<td>+</td>
<td>Tetracycline (100 µg/ml)</td>
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<td>+++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>Chloramphenicol (100 µg/ml)</td>
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Siderophore activity *in vitro*: + represents < 5 mm wide violet zone, ++ represents 5 to 10 mm wide violet zone, +++ represents > 10 mm wide violet zone.

Cyanogenesis: + represents brown pigmentation on margins of filter paper; ++ represents brown pigmentation on half portion of filter paper; +++ complete brown pigmentation on filter paper.

Gelatin and starch hydrolysis *in vitro* plate assay: + represents hydrolysis; - represents no hydrolysis.

Antibiotic resistance *in vitro* assay: + represents resistance; - represents no resistance.

Hydrolyzing enzymes, *in vitro* plate assay: –, no hydrolysis; +, hydrolysis. All isolates were negative for glucanase, cellulase and pectinase production.

Figure 2. Violet pigmentation shows catecholate siderophore production on chrome azuoral sulphonate (CAS) agar by different bacterial strains.

To orange indicating no hydroxamate siderophore production. Arnow and Csaky tests revealed that catecholate siderophores were produced at a higher rate than hydroxamate siderophores (Table 3). Mst 8.2, 3.1.1.C, Z5, Z11 and Z.2.7 bacterial strains produced more than 50 µg/ml of catecholate siderophores, whereas more than 10 µg/ml of hydroxamate siderophores were produced by MS-3y, Mst 8.2, Mst 7.4, Z5 and Z11. This distinct response of color change “violet or orange” is related to structural differences in the type of siderophores secreted (Milagres et al., 1999). The two major groups of siderophores, hydroxamate and catechol yield highly colored Fe (III) complexes. At neutral pH, the hydroxamate complexes are reddish orange and catechol complexes are reddish violet. It has been shown that disease reduction involving siderophore-mediated competition is an important antagonistic interaction that results in the exclusion of fungal pathogens from the rhizosphere due to reduction in the availability of iron for spore germination and hyphal growth (Rachid and Ahmed, 2005).

Five strains MS-3y, Mst 8.2, Mst 7.4, 3.1.1.C and Z.2.7 were found to be intense hydrogen cyanide producers giving strong orange red pigmentation (Table 2). An isolate capable of cyanide production could be a better biocontrol agent because cyanide acts as an inducer of plant resistance (Berg et al., 2002). HCN producing microorganisms are regarded as beneficial when they suppress unwanted components of a microbial community (Lugtenberg et al., 2001).

Two *Pseudomonas* isolates Mst 8.2 and Mst 7.4 were able to hydrolyze gelatin, and four strains MS-3y, Mst 8.2, Mst 7.4 and 3.1.1.C displayed starch hydrolysis ability and thus differed from the other isolates (Table 2). The ability to hydrolyse starch indicates that the strains have the potential to produce amylase and the capability of using a complex carbon source, while the ability to hydrolyse gelatin hydrolysis suggests the
production of specific proteinases which aid in the defensive mechanisms of bacterial strains (Marten et al., 2000).

For three strains, endochitinolytic activity could be detected; whereas 6 isolates showed activity of proteases (Table 2) indicating that these enzymes could be involved in antagonism against R. solani because chitinase and β-1, 3-glucanase have been correlated with induced resistance (Dahiya et al., 2006). None of these isolates produced glucanase, cellulase, hemicellulase (Mannase and xylanase) or pectinase, which suggests that antagonism against R. solani by these enzymes could be excluded. Mst 8.2 and 3.1.1.C were resistant to all tested antibiotics (ampicillin, tetracycline and chloramphenicol) while strains Mst 7.4 and MS-3y showed resistance against only two antibiotics.

Mst 8.2 was the antagonistic bacterial isolate that showed the best inhibitory effect on the growth of R. solani through the production of volatile and diffusible antibiotics. The percent inhibition of antagonistic isolate Mst 8.2 was 42 at 72 h culture (Table 4). The production of antibiotics has been demonstrated to be a widespread mechanism exerted by microorganisms to control a wide variety of phytopathogens (Thomashow and Weller, 1996; Hass and Defago, 2005).

Role of siderophores and iron deprivation in the inhibition of R. solani in vitro

Most of the bacterial strains that inhibited R. solani on Kings B were inhibitory on Kings B + FeCl₃ at 10 to 25 µg/ml. Furthermore, the bacterial strains Mst 8.2, Mst 7.4 and 3.1.1.C were still somewhat inhibitory to R. solani on Kings B amended with FeCl₃ at 50 µg/ml (Table 5). The addition of FeCl₃ to Kings B at 50 µg/ml eliminated the inhibitory effect of siderophores produced by bacterial strains Z2, Z5 and Z11. It is apparent from the data (Table 5) that different mechanisms might be responsible for the inhibition of R. solani but siderophore production might be the main and sole biocontrol mechanism. Reduction of antagonism by the addition of iron indicated

Table 3. Detection of different types of siderophores in the bacterial low-molecular mass fractions.

<table>
<thead>
<tr>
<th>Strains</th>
<th>CAS (%)</th>
<th>Hydroxamate (µg ml⁻¹)</th>
<th>Catechol (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ms-3y</td>
<td>91.1</td>
<td>12.2c</td>
<td>49.8d</td>
</tr>
<tr>
<td>Mst 8.2</td>
<td>92.6</td>
<td>15.5a</td>
<td>54.3a</td>
</tr>
<tr>
<td>Mst 7.4</td>
<td>90.3</td>
<td>14.3b</td>
<td>52.2bc</td>
</tr>
<tr>
<td>3.1.1 C</td>
<td>87.2</td>
<td>5.9a</td>
<td>52.9ab</td>
</tr>
<tr>
<td>Z2</td>
<td>89.0</td>
<td>7.9d</td>
<td>49.7d</td>
</tr>
<tr>
<td>Z5</td>
<td>90.1</td>
<td>15.1ab</td>
<td>52.1bc</td>
</tr>
<tr>
<td>Z11</td>
<td>86.6</td>
<td>14.2b</td>
<td>52.2bc</td>
</tr>
<tr>
<td>Z.2.7</td>
<td>89.8</td>
<td>5.1ef</td>
<td>49.8d</td>
</tr>
<tr>
<td>LSD</td>
<td>NS</td>
<td>1.275</td>
<td>6.205</td>
</tr>
</tbody>
</table>

¹The production of siderophores was determined as siderophore units (%) using CAS assay; ²Assessed using Csaky and Arnow assays. NS indicates a non significant analysis of variance.
Table 5. Role of ferric chloride in antagonistic activity of bacterial strains against *R. solani*.

<table>
<thead>
<tr>
<th>Strains</th>
<th><em>R. solani</em> growth on kings B medium with FeCl₃ (µg ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Ms.3y</td>
<td>+</td>
</tr>
<tr>
<td>Mst 8.2</td>
<td>+</td>
</tr>
<tr>
<td>Mst 7.4</td>
<td>+</td>
</tr>
<tr>
<td>3.1.1 C</td>
<td>+</td>
</tr>
<tr>
<td>Z2</td>
<td>+</td>
</tr>
<tr>
<td>Z5</td>
<td>+</td>
</tr>
<tr>
<td>Z11</td>
<td>+</td>
</tr>
<tr>
<td>Z.2.7</td>
<td>+</td>
</tr>
</tbody>
</table>

*indicates approximate size of the zone of inhibition on King’s B: + = ≤1.0 cm; ++ = 1 to 2 cm; +++ ≥ 2.0 cm; +/- = no clear inhibition zone; - = no zone.

Table 6. Biocontrol bacteria suppress the incidence of *Rhizoctonia* root rot in wheat.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Percentage of roots with lesions</th>
<th>*Leaf spot (disease index)</th>
<th>**HRLH Disease intensity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Root rot seminal</td>
<td>Root rot crown</td>
<td></td>
</tr>
<tr>
<td>T₁</td>
<td>Untreated control</td>
<td>0.00f</td>
<td>0.00f</td>
</tr>
<tr>
<td>T₂</td>
<td>Fungicide</td>
<td>67.7a</td>
<td>47.0a</td>
</tr>
<tr>
<td>T₃</td>
<td>Ms-3y</td>
<td>10.4b</td>
<td>2.7a</td>
</tr>
<tr>
<td>T₄</td>
<td>Mst 8.2</td>
<td>18.4c</td>
<td>7.7b</td>
</tr>
<tr>
<td>T₅</td>
<td>Mst.7.4</td>
<td>16.1d</td>
<td>7.3d</td>
</tr>
<tr>
<td>T₆</td>
<td>3.1.1.C</td>
<td>16.3d</td>
<td>3.4d</td>
</tr>
<tr>
<td>T₇</td>
<td>Z2</td>
<td>18.2c</td>
<td>4.7cd</td>
</tr>
<tr>
<td>T₈</td>
<td>Z5</td>
<td>21.5b</td>
<td>5.9b</td>
</tr>
<tr>
<td>T₉</td>
<td>Z11</td>
<td>19.6b</td>
<td>5.6b</td>
</tr>
<tr>
<td>T₁₀</td>
<td>Z.2.7</td>
<td>18.1c</td>
<td>4.9cd</td>
</tr>
<tr>
<td>T₁₁</td>
<td></td>
<td>17.7d</td>
<td>3.3d</td>
</tr>
</tbody>
</table>

*Disease index (number of lesions) assessment was based on appearance of tops (plant stunting) and the roots (*Rhizoctonia* lesions) by direct seeding according to a disease rating scale from 0 to 5 (0 = healthy to 5 = dead) based on degree of disease infection per plant. Plants that did not collapse and showed non symptoms of root rot were considered healthy. The experiment was performed with three replications.** HRLH: Highest lesion height / highest plant height × 100, based on highest point reached by *Rhizoctonia* lesions relative to highest point of the plant.

that siderophores and other defensive metabolites were inhibitory to fungal growth and showed a cumulative effect of different defensive mechanisms (Bultreys and Gheysen, 2000).

Biocontrol assay on wheat plants

Bacterial strains were evaluated for their biocontrol potential under growth room. All the bacterial strains showed strong antagonism towards *R. solani* and improve the growth of wheat plants (Table 6). Mst 8.2 and Mst 7.4 bacterial strains showed biocontrol activity by reducing seminal root rot by 77 and 75% and crown root by 93% in comparison to controls (T₂) with pathogenic fungus only.

Plants growth was stunted in treatment T₂, T₆ and T₉ because of the occurrence of *Rhizoctonia* root rot. Most plants rated 1 (trace to 20% leaf area with lesions) or 2 (>20% to < 40% leaf area with lesions). MS-3y, Mst.8.2, Mst.7.4 and 3.1.1.C were found to be the best siderophores producing PGPR strains (Table 6) that induced not only resistance against root rot but also improved the plant growth as compared to healthy control. These results support the fact that siderophore-producing *Pseudomonas* rhizobacteria rapidly colonize plant roots of several crops and this colonization can result in significant growth, dry weight and yield increases (De Boer et al., 2003).

16S rRNA sequence analysis

Sequencing of 16S rRNA gene was used to identify the best antagonistic isolate Mst 8.2. The sequence of the 16S rRNA gene (accession number DQ916132) revealed
that the closest phylogenetic neighbour of Mst 8.2 is \textit{P. fluorescens} “g193359813” showing 99% nucleotide sequence identity. On the bases of the observations of yellow and green fluorescence on Kings B and Glucose asparagines (GASN) media respectively by the bacterial culture along with molecular identification, Mst 8.2 was recognized as \textit{P. fluorescens}. The GASN agar was used to test for the presence of green fluorescent \textit{Pseudomonas} that is the characteristic of \textit{pyoverdin} siderophore producer \textit{P. fluorescens} strains (Bultrays and Gheyesen, 2000).

Evidence for the production of catecholate or hydroxamate siderophores by different bacterial species has been shown previously (Christensen et al., 1994; Hohlneicher et al., 1995; Howard et al., 2000) but the production of structurally different and potent antifungal catecholate siderophore by \textit{P. fluorescens} strain Mst 8.2 against \textit{R. solani} in wheat is being reported for the first time. The utilization of multi-mechanism of defense showed that this strain could be an effective biocontrol agent for wheat crop.

Conclusions

The characterization and screening of plant growth-promoting bacterial strains has helped in the selection of Mst 8.2 “with the highest production of siderophores and other antimicrobial compounds” as a potent strain against \textit{R. solani}. Our data demonstrated that the Mst 8.2 produces more than one antifungal compounds and the siderophore production is the key mechanism involved in the antagonism against pathogenic fungus \textit{R. solani} in wheat crop.

Thus, it can be concluded that a single PGPR strain can produce more than five different kinds of defense compounds to compete pathogens and a biocontrol agent possessing multi-mechanism systems of defense, can antagonize root pathogens in a better way. This basic research will minimize the chances of failure of biocontrol activity under field conditions and these tools will allow the isolation of improved strains and more efficient bioformulation to control fungal pathogens.

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REFERENCES


