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Biocontrol of bacterial wilt of tomato caused by *Ralstonia solanacearum* by isolates of plant growth promoting rhizobacteria

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Abstract

A pathogenic *Ralstonia solanacearum*Tom5 was isolated from a wilted tomato plant. It was biochemically characterized and found to belong to biovar IIIA. From a collection of bacterial isolates from rhizosphere of plants growing in the local field five were selected which were antagonistic to the pathogen Tom5. These were identified as *Pseudomonas aeruginosa*T1, *Pseudomonas sp.BH25*, *Pseudomonas* sp.AM12, *Pseudomonas* sp.AM13 and *Pseudomonas putida*R6. All the strains had properties of plant growth promoting rhizobacteria. These were assessed for their potential to biocontrol the manifestation of the pathogen on tomato by evaluating seedling emergence, vigour of the germinated seedlings and survivability of the seedlings following their transplantation. Among the strains *Pseudomonas* sp. BH25 was found to be promising to combat the pathogenic effect of *R. solanacearum*Tom5 in bioassays. In the present study, the pathogen *Ralstonia solanacearum*Tom5 caused only 40% seedling emergence as compared to 76% in the control, while combination of the antagonist BH25 with the pathogen Tom5 (Tom5:BH25 at 1:10) improved the percentage of the seedling emergence and the value (75%) was almost similar to that of the control. Combination of BH25 with the pathogen also improved the fresh weight, dry weight and vigour index [(mean root length + mean shoot length) x percentage of emergence of seedlings] of the seedlings as compared to those in the pathogen treated ones and their values were almost similar to those of the control. Vigour index of the seedlings was reduced from 935 in the control to 237 in the Tom5 treated ones and the value was restored to 878 by combining a 10-fold high concentration of BH25 with the pathogen Tom5 during inoculation. It was concluded that biocontrol organisms could be isolated from rhizosphere and applied to bacterial wilt infested field to combat the disease infestation.

Keywords: Tomato, bacterial wilt, *Ralstonia solanacearum*, plant growth promoting rhizobacteria, biocontrol, seedling emergence, vigour index.
Abbreviations: ACC_1-aminocyclopropane-1-carboxylate, AYG_Ammonium sulfate yeast extract glucose, DW_Dry weight, ES_ Emergence of seedling, FW_Fresh weight, IAA_Indole-3-acetic acid, MRL_Mean root length, MSL_Mean shoot length, PGPR_Plant growth promoting rhizobacteria, TZC_2,3,5-triphenyl tetrazolium chloride, VI_Vigour index.

Introduction

Rhizosphere is the region surrounding a root and is affected by the root itself (Handelsman and Stabb, 1996). It is rich in microbial population and is a dynamic and complex environment. Deleterious microorganisms living in the rhizosphere and interacting with the plant roots may cause development of plant diseases. PGPR, which exert a beneficial effect on the plant they colonize, on the other hand, interact with the plant roots as well as with other microorganisms in the rhizosphere. Some of the PGPR are antagonists to recognized root pathogens and may result in prevention of development of plant diseases (Cook et al., 1995). Biocontrol is the use of the disease-suppressive PGPR to keep the level of deleterious microorganisms under control or below a threshold limit. This suggests the introduction of biocontrol agents from outside in the rhizosphere to achieve disease suppression. Bacterial wilt caused by *Ralstonia solanacearum* is a devastating disease of crops (Hayward, 1991). It occurs widely in tropical and subtropical regions of the world (Kelman, 1998) causing severe losses in yield. The disease affects crops such as tomato, eggplant, potato, tobacco and pepper as well as other important crops like banana, peanut and ginger. Approximately 450 crop species were reported as hosts of this pathogen (Swanson et al., 2005). Attention has been paid to minimize the disease infestation through cultural practices, development of resistant varieties and use of chemicals, but all have a limited success. Furthermore, use of chemicals has its adverse effects on the environment and the non-target organisms. *R. solanacearum* is a genetically diverse (Poussier et al., 1999) soil-borne plant pathogen (Hayward, 1994). The genetic diversity of the pathogen often overcomes the resistance of the crop (Wang et al., 1998). As such it is recommended to identify the race and biovar of the organism from the affected area to select and introduce promising biocontrol PGPR for the identified pathogen to suppress its disease manifestation.
Several studies have been made to control bacterial wilt of tomato with exogenous application of PGPR (Hass and Defago, 2005; Aliye et al., 2008; Xue et al., 2009; Nguyen and Ranamukhaarachchi, 2010). It is important to evaluate PGPR antagonistic to the pathogen and incorporate them into successful disease management as biocontrol agent. A key feature of such organisms is their ability to adjust to the rhizosphere and to aggressively colonize the host roots (Dunne et al., 1997). Therefore, it was recommended that to

achieve greater efficiency of biocontrol agents they should be isolated from the environment where they would be required to function (Cook, 1993). Some of the naturally antagonistic microorganisms isolated successfully against *R. solanacearum* are *Bacillus* species and *Pseudomonas fluorescens* (Anuratha and Gnanamanickam 1990, Xue et al., 2009), *Stenotrophomonas maltophilia* (Messiha et al., 2007), *Streptomyces setonii* (Lemessa and Zeller, 2007). The objective of the present study was to isolate PGPR from the rhizosphere of plants growing in the same locality as of the bacterial wilt infested tomato field wherefrom the pathogen *R. solanacearum* was isolated and to screen their antagonistic activity against the pathogen as well as to study their biocontrol potential to manage the wilt disease.

Results and discussion

Isolation of Ralstonia solanacearum from diseased plants

Stem pieces of wilted tomato plants when dipped in water showed a continuous white streaming of bacterial ooze. From this suspension bacterial colonies were isolated on TZC medium. After 48 hours of incubation at 30^{o}C a viscous colony with pink centre and white border developed on the plate was selected as that of a putative *R. solanacearum*. The colony was soluble in 3% KOH solution which eliminated any possible confusion of the organism with other wilt causing pathogen of tomato. The colony was streaked on TZC medium and a bacterial isolate *R. solanacearum*Tom5 was picked up and stored.

Biochemical characterization of Ralstonia solanacearum Tom5

For a confirmation biochemical study of the organism *R. solanacearum*Tom5 was carried out. The study revealed that it had catalase and oxidase activities. It hydrolyzed urea but not starch and did not liquefy gelatin. It had the ability to utilize citrate and to reduce nitrate. It was negative for phenylalanine deaminase, lysine decarboxylase and arginine dihydrolase activities. It was methyl red test negative but Voges Proskauer test positive. The isolate could use the disaccharides cellulose, lactose and maltose and sugar alcohols sorbitol and mannitol but not dulcitol. On the basis of biochemical tests and utilization of sugar and sugar alcohols *R. solanacearum*Tom5 was grouped under biovar IIIA. *R. solanacearum* belongs to the rRNA homology group II under the subclass Proteobacteria (Buddenhagen and Kelman, 1964) and has five races based on its host range and five biovars based on differences of oxidation/utilization of certain carbon sources (Palleroni and Duodoroff, 1971). Chakraborty et al. (1994) found the occurrence of *Pseudomonas solanacearum* (now *R. solanacearum*) biovar III in tomato, eggplant and potato in West Bengal, India. The results of the present study are in agreement with their findings.

Isolation and characterization of rhizobacteria antagonistic to Ralstonia solanacearumTom5

About six hundred discrete colonies of bacteria were isolated by dilution plating of rhizospheric soil of plants growing in the local farms as well as of stem washing of rotten tuberose stick. The isolates were assessed for their ability to inhibit the growth of *R. solanacearum* by cross streaking. The results led to the selection of the isolates T1, BH25, AM12, AM13 and R6 showing discernible growth inhibition of Tom5 (Table 1) as antagonists of the pathogen. The results were further confirmed by assaying the culture filtrate of the isolates grown in Potato dextrose broth by cup assay on a lawn of Tom5. The culture filtrates exhibited zone of growth inhibition of *R. solanacearum*Tom5 around the cups (Table 1). Biochemical characterization of the selected antagonists identified them as species of *Pseudomonas* (Table 2). This was corroborated by 16S rDNA sequencing of the isolates and their BLAST analysis. The analysis revealed that 16S rDNA of the isolate T1 had 99% similarity with that of *Pseudomonas aeruginosa,* of the isolate BH25 had 95% similarity with that of *Pseudomonas* species, of AM12 had 99% similarity with that of *Pseudomonas putida, Pseudomonas fluorescens* and of *Pseudomonas brassicacearum,* AM13 had 99% rDNA sequence similarity with that of *Pseudomonas putida, Pseudomonas brassicacearum,* and of *Pseudomonas* sp. and 98% similarity with that of *Pseudomonas fluorescens* and *R6* had 98% rDNA sequence similarity with that of *Pseudomonas putida* and of *Pseudomonas* sp. Anuratha and Gnanamanickam (1990) attempted biological control of bacterial wilt caused by *R. solanacearum* in India with antagonistic bacteria. Shekhawat et al. (1993) observed that biological management of bacterial wilt with several bacterial and actinomycete strains were possible. A large number of PGPR were reported to promote plant growth and to control plant diseases (Basan and de Basan, 2002). Although, different bacterial species, e.g., *Alcaligenes* sp. and *Kluyvera* sp. (Assis et al., 1998), *Bacillus* sp. and *Erwinia* sp. (Trigalet et al., 1993) were reported to inhibit plant pathogens, different species of fluorescent *Pseudomonas* contributed significantly in the biocontrol of plant diseases.

PGPR activity of the antagonists and role of their siderophore production in the antagonism

All the antagonists could use ACC as the sole nitrogen source for their growth (Table 3) indicating them as PGPR. Although all the strains produced IAA the strain *Pseudomonas* speciesBH25 produced the highest level of it. The strains T1, BH25 and R6 solubilized insoluble phosphate in Pikovskaya's medium as well as in AYG medium. Only the strain BH25 reduced nitrate and the strain AM12 produced HCN, whereas the strains T1 and BH25 reduced nitrite. It seemed possible that the strains BH25 and T1 having appreciable PGPR activity could serve as good biocontrol agents. PGPR are able to grow using ACC as the sole nitrogen source (Glick, 1995). Growth of all the strains using ACC as the sole source of nitrogen, in the present study, proved them to be PGPR. PGPR include a diverse group of free-living soil bacteria that can stimulate the growth of plants by one or more of a number of different mechanisms (Glick et al., 1995). The attributes of the PGPR isolated in the present study include their siderophore production, phosphate solubilization and production of the phytohormone IAA. The widely recognized mechanisms of biocontrol mediated by PGPR were analyzed to be by (1) competition for an ecological niche or a substrate, (2) production of inhibitory chemicals, and (3) induction of systemic resistance (ISR) in host plants to a broad spectrum of pathogens (Bloemberg and Lutenberg, 2001). Of these siderophore(s) produced by PGPR having higher affinity for iron than those produced by pathogenic microorganisms may be a necessary attribute for biocontrol (Loper and Henkels, 1999). To assess the role of siderophore production by the antagonists in biocontrol, culture filtrates of the selected antagonists grown in potato dextrose broth were assayed on

Table 1. Cup assay of culture filtrate of antagonist isolates grown in the absence or presence of iron (100 µm)in potato dextrose medium on a lawn of *Ralstonia solanacearum*Tom5.

Antagonist strain	Iron free culture filtrate*	100 µM iron supplemented culture filtrate
T1	+	+
BH25	+	+
AM12	+	+
AM13	+	+
R6	+	-

*+ indicates presence and – indicates absence of zone of inhibition

Table 2. Biochemical tests of the antagonist isolates.

Character	Antagonist isolate*				
	T1	BH25	AM12	AM13	R6
Catalase	+	+	+	+	+
Oxidase	+	+	+	+	+
Starch hydrolysis	-	+	-	-	-
Gelatin liquefaction	-	-	-	-	-
Urea hydrolysis	+	+	+	+	+
Lysine decarboxylase	-	-	-	-	-
Arginine dihydrolase	-	-	-	-	-

*+ indicates presence and – indicates absence of the character

the lawn of *R. solanacearum*Tom5. The broth was made iron free by treatment with hydroxyquinoline (Rosenberg, 1979). In one set of experiment iron free broth was supplemented with $FeCl_3$ to a final concentration of 100 µM to inhibit the production of siderophore in the culture. The culture filtrates of all the strains, except that of R6, grown either in the presence or absence of iron inhibited the growth of *R. solanacearum*Tom5 (Table 1) indicating that siderophore was not responsible for the growth inhibition by the strains. The culture filtrate of the strain R6, however, grown in the presence of iron did not inhibit the growth of Tom5 (Table 1) indicating that the growth inhibitory principle of the strain R6 may be a siderophore.

Plant infectivity test for disease suppression by the antagonists

In the first method of plant infectivity test, percent ES of tomato (var. Pusa Ruby susceptible to bacterial wilt) was evaluated following treatment with *R. solanacearum* Tom5 (10^7 cells/ml) or an antagonist (10^7 cells/ml) and the value was compared to that of the untreated control. After sowing the seeds on water agar, ES was complete on the 14th day and no further ES was noted after that (data not shown). As such all the data were collected on the 14th day after sowing the seeds. Data in Table 4 show that *R. solanaceraum*Tom5 treatment reduced percent ES from the tomato seeds drastically to 40% from 76% in the control. MRL, MSL, FW, and DW also became reduced significantly by treatment with the pathogen in respect to those in the control experiment. Measurement of vigour index (VI = [(mean root length + mean shoot length) x percentage of emergence of seedlings] provided a final reflection of the potential of the treatments. VI of the seedlings was reduced significantly from 935 in the control to 237 in the Tom5 treated ones. However, following treatment with an individual antagonist, except T1, the results were not significantly different from that of the control (Table 4). When any of the antagonists was combined with *R. solanacearum*Tom5 in a ratio of 1:1 the deleterious effect of the pathogen became noticeably reduced and percent ES, MRL, MSL, FW, DW and the VI improved over those of the Tom5 treated ones, reflecting an alleviation of the pathogenic effect of Tom5 by the antagonist. A further improvement in all the parameters was noted when the concentration of the antagonist was increased and the ratio of the pathogen to

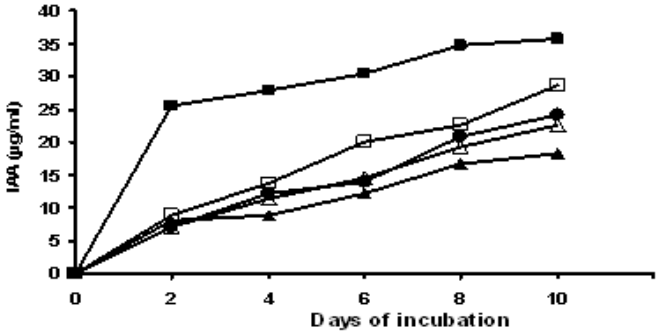

Fig 1. Production of indole-3-acetic acid by PGPR strains antagonistic to *Ralstonia solanacearum*Tom5. ■, BH25; □, AM13; ●, R6; Δ, AM12; ▲, T1.

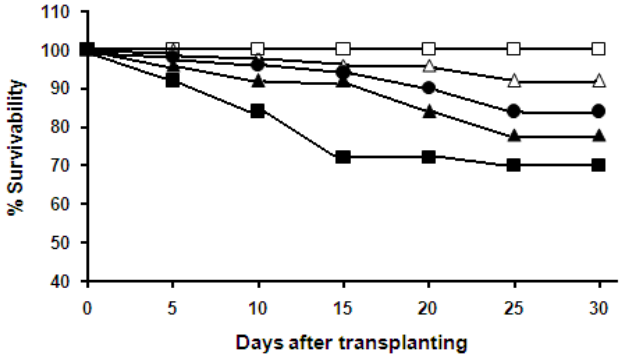

Fig 2. Percent survivability of tomato seedlings after transplantation to sand following inoculation with Tom5 alone (10^7 cells/ml) or in combination with the antagonist BH25 in different proportions. □, Control; ■, Tom5; Δ, BH25; ▲, Tom5:BH25 (1:1); ●, Tom5:BH25 (1:5).

antagonist became 1:5. When the concentration of the antagonist was still increased in respect of Tom5 and the ratio of Tom5 to antagonist was 1:10 the deleterious effect of the pathogen was practically absent (Table 4). Of the five antagonists selected to study their biocontrol effect the strain *Pseudomonas* sp.BH25 was observed to be the best one among them. Treatment with *Pseudomonas* sp. BH25 at a

Table 3. PGPR activity of the antagonist strains.

Activity	Antagonist strains*				
	*Pseudomonas aeruginosa*T1	*Pseudomonas* sp.BH25	*Pseudomonas* sp. AM12	*Pseudomonas* putida AM13	*Pseudomonas putida*R6
Growth on ACC	+	+	+	+	+
Siderophore production	+	+	+	+	+
Phosphate solubilization	+	+	-	-	+
IAA production	+	+	+	+	+
HCN production	-	-	+	-	-
Nitrate reduction	-	+	-	-	-
Nitrite reduction	+	+	-	-	-

*+ indicates presence and – indicates absence of the activity

Table 4. Effect of treatment of *Ralstonia solanacearum*Tom5 or its antagonist either alone or in a combination on tomato seedling emergence and health of seedlings.

Treatments	*Emergence of seedling (ES) (%)	Mean root length (MRL) (cm)	Mean shoot length (MSL) (cm)	Vigour index (VI)	*Fresh weight (FW) (g)	*Dry weight (DW) (g)
Control	76 ^{abc}	7.8 ^{b}	4.5 ^{a}	935.1 ^{b}	4.66 ^{a}	0.167 ^{a}
Tom5	40 ^{i}	3.5 ^{n}	2.5 ^{g}	237.1 ^{j}	3.25 ^{f}	0.105 ^{d}
BH25	81 ^{a}	8.2 ^{a}	4.6 ^{a}	1043.7 ^{a}	4.20 ^{ab}	0.171 ^{a}
T1	67 ^{cde}	6.7 ^{defg}	4.5 ^{a}	755.0 ^{d}	4.19 ^{ab}	0.161 ^{ab}
AM12	78 ^{ab}	7.0 ^{cde}	4.5 ^{a}	897.3 ^{b}	4.03 ^{bcde}	0.161 ^{ab}
AM13	79 ^{ab}	7.0 ^{cde}	4.5 ^{a}	913.3 ^{b}	4.14 ^{abc}	0.159 ^{ab}
R6	73 ^{abc}	6.4 ^{h}	4.5 ^{a}	796.0 ^{cd}	3.79 ^{bcdef}	0.165 ^{a}
Tom5: BH25 (1:1)	60 ^{efg}	4.6 ^{m}	3.1 ^{ef}	462.0 ^{gh}	3.28 ^{f}	0.115 ^{cd}
Tom5: BH25 (1:5)	70 ^{bcd}	7.0 ^{cde}	3.6 ^{bcd}	746.3 ^{d}	3.69 ^{bcdef}	0.133 ^{bc}
Tom5: BH25 (1:10)	75 ^{abc}	7.3 ^{c}	4.4 ^{a}	877.9 ^{b}	4.10 ^{abcd}	0.173 ^{a}
Tom5 : T1 (1:1)	51 ^{gh}	3.7 ^{n}	2.9 ^{f}	341.2 ^{i}	3.29 ^{f}	0.125 ^{cd}
Tom5 : T1 (1:5)	56 ^{fgh}	5.9 ^{ij}	3.6 ^{bcd}	533.7 ^{fg}	3.37 ^{f}	0.138 ^{bc}
Tom5 : T1 (1:10)	58 ^{fgh}	6.3 ^{h}	3.9 ^{b}	592.1 ^{ef}	3.40 ^{f}	0.156^{ab}
Tom5 : AM12 (1:1)	56 ^{fgh}	5.1 ^{l}	3.0 ^{f}	454.6 ^{gh}	3.27 ^{f}	0.162 ^{ab}
Tom5 : AM12 (1:5)	60 ^{efg}	6.2 ^{hi}	3.3 ^{de}	575.2 ^{ef}	3.33 ^{f}	0.162 ^{ab}
Tom5:AM12 (1:10)	71 ^{bcd}	6.7 ^{efg}	3.7 ^{bc}	740.1 ^{d}	3.49 ^{ef}	0.166 ^{a}
Tom5 : AM13 (1:1)	54 ^{fgh}	5.6 ^{jk}	2.9 ^{f}	460.5 ^{gh}	3.32 ^{f}	0.165 ^{a}
Tom5 : AM13 (1:5)	62 ^{def}	6.8 ^{def}	3.4 ^{cd}	640.5 ^{e}	3.51 ^{def}	0.169 ^{a}
Tom5: AM13 (1:10)	78 ^{ab}	7.1 ^{cd}	3.9 ^{b}	855.2 ^{bc}	3.57 ^{cdef}	0.173 ^{a}
Tom5 : R6 (1:1)	49 ^{h}	5.4 ^{kl}	2.8 ^{f}	403.1 ^{hi}	3.28 ^{f}	0.157 ^{ab}
Tom5 : R6 (1:5)	52^{gh}	5.9 ^{ij}	3.0 ^{f}	464.6 ^{gh}	3.38 ^{f}	0.159 ^{ab}
Tom5 : R6 (1:10)	60 ^{efg}	6.5 ^{gh}	3.1 ^{ef}	583.3 ^{ef}	3.45 ^{ef}	0.161 ^{ab}

*Values are means of 100 seeds/seedlings. The highest value in a column is denoted with a superscript letter 'a' and the subsequent values in decreasing order are denoted with letters in the alphabetical list in ascending order. Values with the same superscripted letter(s) are not different at 5% level of significance, while values with different letters are significantly different. Table value of F at 21, 44 (P=0.05)= 1.80; Calculated value of F for ES= 15.13, MRL=98.75, MSL= 47.15, VI=65.00, FW=4.911, DW=7.48.

ten-fold concentration of *R. solanacearum* Tom5 increased the percentage of ES to 75 which is statistically not different from that in the control (76%). It may be noted that BH 25 also produces the highest level of IAA among the antagonists (Fig 1). In the second method percent survival of aseptically grown seedlings was examined following inoculation with *R. solanacearum* Tom5 alone or in combination with an antagonist by root dipping method and transplantation. Fig 2 shows that following inoculation with *R. solanacearum* Tom5 survivability of the seedlings decreased by almost 30% on the 25th day of transplantation in respect of control. Treatment with *Pseudomonas* BH25 alone caused only a marginal (about 8%) decrease in the survivability of the seedlings. However, inoculation with *Pseudomonas* BH25 at increasingly higher concentration mixed with the pathogen increased the survivability of seedlings. When the concentration of the strain BH25 was increased to five-fold that of Tom5 about 90% of the seedlings survived. Seed inoculation with a biocontrol strain is an effective method used in biological control of diseases. Rath and Wolf (1992) reported that inoculation with *P. fluorescens* and *Streptomyces* resulted in an increase in field emergence of sugar beet seedlings. Homma et al. (1997) found suppression of sugar beet damping-off by seed bacterization with *Stenotrophomonas* sp. in a paper-pot system. It was reported that some strains of *P. fluorescens* actively suppressed disease occurrence of tomato bacterial wilt when introduced to plant rhizosphere by root dipping (Aino et al., 1993). It was suggested that some of the *P. fluorescens* strains were incorporated into roots of tomato seedlings, probably through physically injured sites or normal openings and colonized in the tissues. As such in the present work the root tips of the seedlings were excised in root dipping method of inoculation to facilitate the entry of the antagonistic bacteria. Results of Fig 2 also support that presence of an antagonistic strain such as *Pseudomonas* sp.BH25 in the rhizosphere increases the survivability of the seedlings alleviating the detrimental effect of the pathogen.

Materials and Methods

Isolation and characterization of Ralstonia solanacearum

Wilted tomato plants were collected from local fields in the State of West Bengal, India. The stems of the plants were cut into two inch pieces. Lower end of the pieces was immersed in sterile water and kept for ten minutes to allow the bacteria to ooze out. An aliquot of this bacterial suspension was then transferred to Nutrient broth and allowed to grow for overnight at 30^{o}C. The bacterial culture was diluted (10^{-4} to 10^{-8} dilution) and plated onto TZC agar medium (Kelman, 1954) and incubated for 48 hours at 30^{o}C. From amongst a large number of colonies one showing irregular viscous appearance with pink centre and white border was selected and purified. The purified isolate was named as Tom5. The colony of Tom5 was also tested for its solubility in 3% KOH. A discrete pure colony of Tom5 was preserved in sterile water for further study. A series of biochemical tests (catalase, oxidase, phenylalanine deaminase, lysine decarboxylase and arginine dihydrolase activities, hydrolysis of starch, liquefaction of gelatin, utilization of urea and citrate, nitrate reduction, methyl red and Voges Proskauer tests) was carried out for characterization of the isolate *R. solanacearum* Tom5 (Holding and Collee, 1971; Palleroni, 1984). Biovar of the *R. solanacearum*Tom5 was determined on the basis of their ability to utilize disaccharides and oxidize hexose alcohols using sugar and sugar alcohols discs (Himedia) on basal medium (Hayward, 1964).

Isolation of antagonists to Ralstonia solanacearumTom5 and their identification

Rhizospheric soil of some field grown crops were suspended in sterile water and allowed to settle down. The supernatant was decanted and dilution plated on Potato Dextrose Agar (PDA) medium. The individual colonies developed were screened for their antagonistic activity against *Ralstonia* isolate Tom5 by cross streaking on PDA medium. Five discrete bacterial isolates named as T1, BH25, AM12, AM13 and R6 out of more than six hundred were selected based on discernible growth inhibition of Tom5 by them using the methods of cross streaking and cup assay of their culture filtrates on a lawn of Tom5. The selected antagonists were characterized through a battery of biochemical tests (catalase and oxidase activities, hydrolysis of starch, liquefaction of gelatin, utilization of urea, reduction of nitrate and nitrite, lysine decarboxylase and arginine dihydrolase activities). The biochemical tests were carried out following the methods as described (Holding and Collee, 1971; Palleroni, 1984).The antagonists were finally characterized by their 16S rDNA sequencing and BLAST analysis.

Plant growth promoting activities of the antagonists

Plant growth promoting properties of the selected antagonists were determined by their growth on ACC as the sole source of nitrogen (Glick et al., 1995), IAA production (Bric et al., 1991), phosphate solubilization (Halder et al., 1990a, b), HCN production and production of siderophore (Schwyn and Neilands, 1987). For assay of phosphate solubilization Pikovskaya's medium, pH 6.8 (Pikovskaya, 1948) as well as AYG medium (Halder et al., 1990a) was used with tricalcium phosphate and hydroxyapatite respectively as the source of insoluble phosphate. The antagonists were assayed for their production of siderophore on chrome azurol sulfone (CAS) agar plate (Schwyn and Neilands, 1987). The role of siderophore, if any, in the process of antagonism was assessed by studying growth inhibition of *R. solanacearum*Tom5 by the antagonists grown under iron (100 µM) supplemented and depleted conditions.

Plant infectivity test

Plant infectivity test was carried out using two different methods. In the first method surface sterilized seeds of a susceptible tomato variety (var. Pusa Ruby) were soaked in a suspension of *R. solanacearum*Tom5 (10^{7}cells/ml) and antagonistic bacteria mixed in a predetermined ratio. The soaked seeds were then sown on water agar. Seeds treated with no bacterial inoculum, only pathogen as well as with only antagonist (10^{7}cells/ml) as inoculum were also sown in control experiments. ES from seeds was then monitored at a regular interval. The percentage of ES, root and shoot length and FW and DW of the seedlings were recorded at 14 days after sowing. In the second method, tomato seedlings were grown on sterile sand and uprooted carefully at the four-leaved stage and the roots were cleaned thoroughly with water. The tips of the roots were surgically removed to create an injury and the roots were then dipped for three hours in the culture suspension of Tom5 (10^{7}cells/ml) and an antagonistic bacteria mixed in a predetermined ratio. Control experiments with seedlings treated with no bacterial inoculum, only pathogen as well as with only antagonist suspension (10^{7}cells/ml) as inoculum were also carried out. The seedlings were then transplanted to sterile sand mixed with the same culture suspension and watered with Hoagland's nutrient solution (Hoagland and Arnon, 1950) as and when required. Survivability of the seedlings was monitored at a regular interval.

Statistical analysis

The biochemical characterization studies for the pathogen and antagonists were done twice with three replications. The plant infectivity studies were made under complete randomized block design (CRD) with each treatment replicated thrice. The observed data were statistically analyzed using analysis of variance following least significance difference at 5% level of significance and the mean values were compared by Duncan Multiple Range Test (DMRT) using SPSS (Statistical Package for the Social Sciences, later modified to Statistical Product and Service Solutions) software (version 10). The highest value in a column is denoted by superscript letter 'a' and subsequent values in deceasing order are noted in the alphabetical list in ascending order. The same superscript letter(s) in a column designated that the values are not significantly different.

Conclusion

The present study showed that the bacterial wilt of tomato is caused by *R. solanacearum* biovar IIIA under conditions of West Bengal, India (new alluvial zone). The work also indicated that PGPR isolates antagonistic to *R. solanacearum* could be isolated from local fields and used to combat the deleterious effects of *R. solanacearum*. Thus, it is hoped that the inoculant antagonists isolated from local fields will manage the bacterial wilt disease of tomato more efficiently in the field.

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