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# Rhizosphere and endophytic bacteria for the suppression of eggplant wilt caused by *Ralstonia solanacearum*

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ABSTRACT

Forty eight endophytic bacteria and 101 rhizobacteria were screened for their antibacterial activity against *Ralstonia solanacearum*, causal agents of eggplant wilt. Among 22 effective antagonistic isolates, 18 were *Pseudomonas* spp. forming three groups based on biochemical characterization. Talc formulation of the antagonistic bacteria and non-formulated 24 h old grown antagonistic bacterial cells was evaluated in the greenhouse condition for the suppression of eggplant wilt. Talc formulation of two species of *Pseudomonas* (RBh41 and RBh42) completely suppressed the incidence of wilt up to 36 days of inoculation. Treatment with bacterial cells of *Pseudomonas mallei* (RBG4, ET17) and one *Bacillus* spp. (RCh6) reduced wilt incidence of 83% compared to control. Talc formulations of seventeen isolates of antagonistic bacteria were prepared and used for treating the nursery and seedlings during transplanting. Biocontrol efficiency of 100% was recorded in *Bacillus* sp. (RP7) treatment and 80% was recorded by EB69, RCh6 and RBG4 treatments during 2007–08. During 2008–09, EB69 recorded 100% biocontrol efficiency followed by RP7 (96%), RCh6 (93%). Yield increase of over 80% was recorded in RP6 and EB69 treatments followed by RBG4 treatment. EB69, RBG4 (*Pseudomonas* sp.) and reduced wilt over 65% and increased the yield (75%) consistently over the two years and hence these isolates could be considered for developing potential biocontrol agents with plant growth promoting characteristics for management of *R. solanacearum* in eggplant.



## 1. Introduction

*Ralstonia solanacearum* (Smith) Yabuuchi is an important soilborne bacterial plant pathogen which causes bacterial wilt, a widely distributed plant disease in tropical, sub tropical and warm temperate regions of the world (Hayward, 1991). This pathogen has an unusually wide host range of 200 plant species belonging to more than 50 families (Hayward, 2000). The host range includes solanaceous plants, leguminous plants, a few monocotyledons, tree, shrub hosts and certain ecotypes of the model plant *Arabidopsis thaliana* (Genin and Boucher, 2002). Some of its economically important hosts include tomato, pepper, potato, tobacco, banana, eggplant, cowpea, peanut, cashew, papaya and olive (Guo et al., 2004). Bacterial wilt is reported to be among the top five diseases (Elphinstone, 2005) and is a major yield constraint of eggplant (*Solanum melongena* L.) in coastal region of India, particularly in Goa (Ramesh, 2006).

Management of bacterial wilt in eggplant and in other crops has been difficult due to the diversity in *R. solanacearum* strains, their ability to survive in adverse soil conditions, worldwide distribution, varied hosts including asymptomatic hosts and efficient mechanism of invading host. Various strategies, including resistant varieties (Dalal et al., 1999), soil amendments (Islam and Toyota, 2004), soil solarization (Kumar and Sood, 2001), use of bio-fumigants (Pradhanang et al., 2003), transgenic resistant plant (Jia et al., 1999), plant growth promoting rhizobacteria (Guo et al., 2004), use of SAR inducers (Anith et al., 2004) had been developed with limited success in the bacterial wilt management.

Biological control has been accepted/emerged as one of the important methods in the management of soilborne plant pathogens. Potential biological control agents used to control *R. solanacearum* include avirulent strain of *R. solanacearum* in tomato (Dong et al., 1999), *Pseudomonas fluorescens* Migula in banana, brinjal and tomato (Anuratha and Gnanamanickam, 1990), *P. fluorescens* in eggplant (Ramesh, 2006), *Bacillus subtilis* (Ehrenberg) Cohn, *Pseudomonas cepacia* Palleroni and Holmes in tomato (Abdalla et al., 1999) and mycorrhizal fungi in Eucalyptus (Gong et al., 1999). Recently Xue et al. (2009) reported species of

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*Acinetobacter* and *Enterobacter* effectively reduced the incidence of bacterial wilt in tomato. A new concept, biological soil disinfestation (BSD) was reported as an effective method to control potato brown rot (Messiha et al., 2007).

Plant growth promoting rhizobacteria (PGPR) are potential agents for biological control of plant pathogens (Kloepper, 1993) in general and against *R. solanacearum* (Guo et al., 2004; Lemessa and Zeller, 2007). PGPR brings about disease suppression by various modes of action such as antagonism by producing antibiotics (Thomashow and Weller, 1988; Keel et al., 1992), chelating iron by siderophore production (Thomashow and Weller, 1988; Duffy and Defago, 1997), HCN production (Haas et al., 1991), competition for space and nutrients (Kloepper et al., 1988) and induction of systemic resistance (Wei et al., 1991, 1996).

Biological control agents or PGPR have been isolated mostly from naturally occurring rhizobacteria (Silveria et al., 1995). In recent years, a lot of attention was given to endophytic bacteria when their capacity to protect their hosts against biotic factors was recognized. Effectiveness of endophytic bacteria in suppression of bacterial wilt in eggplant was reported (Ramesh et al., 2009).

The present study was conducted with the objective of screening potential biological control agents or PGPR from rhizosphere and endophytic tissues of various crop plants and evaluating effective isolates in the field for bacterial wilt reduction in eggplant.

## 2. Materials and methods

### 2.1. Antagonistic bacterial strains and R. solanacearum

Endophytic and rhizobacteria were isolated from different crop plants (Table 1) of Goa, India. Endophytic bacteria were isolated from surface sterilized root and stem bits following the procedure described by Phadke (2007). The stem and root portion of collected plant samples were washed under tap water to remove adhered soil particles. Either roots or stem portion close to the root zone were cut with a sterile knife into approximately one cm pieces. The pieces were surface sterilized with 0.1% $HgCl_2$ solution for 1 min and washed twice with sterile distilled water. The surface sterilized pieces were suspended in 50 ml of sterile 0.1 M phosphate buffer (PB) (pH 7.0) for 5 min. The sterile pieces from PB were homogenized in a sterile pestle and mortar with 5 ml of PB. From this homogenate, aliquots of 100 μl and 200 μl were plated onto Kings medium B (King et al., 1954) and the plates were incubated at 28 ± 2 °C from 24 to 48 h for colony growth. Rhizosphere bacteria were isolated by serial dilution of the rhizosphere soil and *R. solanacearum* was isolated from freshly wilted eggplant. Freshly wilted eggplant plant was selected, removed from soil and was thoroughly washed to remove the adhering soil particles. Using a tissue paper the water was wiped out from the surface. A horizontal cut was made at collar region using a sterile knife and the upper portion was taken for isolation of bacteria. The cut-end surface was slightly dipped in the sterile water and the flowing bacterial ooze from vascular bundle was collected separately in sterile plate. A loopful of this flowing ooze containing the bacteria was streaked onto sterile TZC (Kelman, 1954) agar plate and the plates were incubated at 28 ± 2 °C for 48–72 h. After incubation the virulent *R. solanacearum* colonies (fluidal white/white with pink center) were selected and purified. *R. solanacearum* isolates were inoculated to eggplant to confirm their pathogenicity. All the purified bacterial isolates were maintained in glycerol stocks and stored at −80 °C in the culture collection of the Institute.

### 2.2. In vitro evaluation of bacterial isolates against R. solanacearum

In agar diffusion test, 24 h old grown *R. solanacearum* strain RS21 (O) was seeded in the King's B medium (0.25 μl $ml^{-1}$ at $10^{10}$ CFU $ml^{-1}$). The above strain was selected among the different strains from the culture collection as it was the more virulent strain on eggplant (caused 100% wilt when inoculated on eggplant) and collected from the geographical location where the field experiment was conducted. The strain was characterized as race 1, biovar 3 of *R. solanacearum*. With a sterile cork borer three wells of 7 mm diameter were prepared by removing the agar. 15 μl of the exponential growth phase bacterial culture (approximately $3.5 \times 10^{10}$ CFU $ml^{-1}$) was added in the wells and plates were incubated at 28 ± 2 °C for 24 h. Only medium was used as negative control in the experiment. Inhibition zone around the well (radius in mm), overgrowth zone (radius in mm) of the antagonistic bacteria over *R. solanacearum* inoculation zone if any were recorded by measuring from the outer edge of the well after 48 h. Three replications and four plates for each replication were maintained for each isolate. Further, cell free culture filtrate of antagonists was evaluated for their inhibition activity of *R. solanacearum*. The cell free culture filtrate was obtained by centrifuging the 48 h old grown cultures at 10,000 rpm for 10 min. The isolates which inhibited the *R. solanacearum* and showed overgrowth were selected for *in vivo* evaluation under greenhouse and field conditions.

### 2.3. Characterization and identification of antagonists

Twenty two isolates which effectively inhibited *R. solanacearum* were characterized in our laboratory based on morphological and biochemical properties. The biochemical tests include growth at 4 °C and 41 °C, 7% NaCl, pH 5.7, utilization of citrate, gelatin liquefaction, nitrate reduction and utilization of 13 carbon sources (Schaad, 1992). All the experiments were conducted at least three times with three replications for each isolate and for each test. Identification of isolates was done according to procedure described in Bergey's manual (Holt et al., 1994). Antibiotic resistance profiles of the bacteria were determined by testing against 15 antibiotics as per manufacturers (Himedia Laboratories, India) instructions. All Gram negative isolates were categorized into phylogenic groups based on biochemical tests using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA). The tree plot was constructed by unweighted pair group arithmetic average.

### 2.4. Evaluation of antagonistic bacteria against bacterial wilt under greenhouse conditions

Thirty days old seedlings of eggplant cv. *Agassaim* were planted in pots filled with standard pot mixture (red earth: sand: farmyard manure at 2:1:1). Talc formulation of the antagonists was prepared as described by Ramesh and Korikanthimath (2004) and the population of antagonistic bacteria in talc formulation was maintained at $3 \times 10^8$ CFU $g^{-1}$. Two experiments were conducted with 17 antagonists; in experiment one, plants were treated with talc formulation (5 g $plant^{-1}$). Talc formulation was mixed in water (5 g in 50 ml) and poured around the root zone. In the second experiment, plants were treated with bacterial cells from 24 h old grown cultures of antagonists (10 ml $plant^{-1}$) by pouring around the root zone. At the time of treatment the population of antagonistic bacteria in talc formulation was maintained at $3 \times 10^8$ CFU $g^{-1}$ and the population in the non-formulated cell suspension was $1.5 \times 10^6$ CFU $ml^{-1}$. After 10 days, the same treatments were repeated once again. *R. solanacearum* was grown in CPG broth and inoculated by drenching around root zone with 10 ml of inoculum ($1.2 \times 10^7$) after 20 days of second application of antagonistic bacterial treatment. In each experiment, streptomycin as positive control and PBS as negative control were maintained. For each treatment, two replications and in each replication four seedlings were maintained in a completely randomized design for both the