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Biological control of bacterial wilt in tomato involves 2,4-DAPG based antagonism

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ABSTRACT: *Rhizobacteria producing 2,4-diacetylphloroglucinol (2, 4-DAPG) play a significant role in reducing soilborne pathogenic activity in the rhizosphere of crop plants. In the present study, we conducted experiments to evaluate the biocontrol potential of four phlD⁺ Pseudomonad strains (s188, s218, s245 and s288) for suppression of bacterial wilt disease caused by Ralstonia solanacearum and plant growth promotion ability on mature tomato plants. In vivo assay showed that the plants inoculated with strain s188 recorded the highest increment in plant biomass, percent wilt reduction and inhibition of pathogen in rhizosphere soil, thus, proving to be a superior isolate for development as biocontrol agent. Further, to understand the mechanism of antagonism involving role of 2, 4-DAPG in suppression of bacterial wilt disease, 2,4-DAPG- negative mutant strain, s188MT, was generated by transposon mutagenesis. This mutant had lost the ability to inhibit the growth of wilt pathogen, R. solanacearum, in vitro. In planta comparison of biocontrol efficacy as well as plant growth promotion ability of the mutant strain with that of parent strain revealed substantial loss in both the traits in case of the mutant, suggesting a major role of 2, 4-DAPG in s188. The phlD⁺ Pseudomonad strain s188 has proven to be consistently efficient as biocontrol agent of bacterial wilt in tomato as well as plant growth promoting rhizobacteria (PGPR), hence can be incorporated in integrated disease management package for control of bacterial wilt after investigation in field trial.*

Key words: *Bacterial wilt, DAPG, PGPR, R. solanacearum, tomato*

INTRODUCTION

The bacterial wilt disease of tomato caused by *Ralstonia solanacearum* is one of the most important diseases responsible for significant losses in tomato production (Nguyen *et al.*, 2010). In India, the disease is destructive in most of the tomato growing states causing yield losses to the extent of 90% (Nazeem *et al.*, 2011). The causal agent *Ralstonia solanacearum* affects more than 30 plant species, the most susceptible being tomato, potato, eggplant and pepper. Management of bacterial wilt in tomato and in other crops has been difficult with no commercial pesticide available. Even though integrated management, including cultural practices, crop rotation and use of resistant cultivars provide some limited success, the disease still threatens commercial tomato production in India and worldwide (Chandrashekara *et al.*, 2010, 2012)

Pseudomonads are well characterized plant growth promoting rhizobacteria (PGPR) that have been long recognized as promising alternative for effective disease management. The primary mechanism involved in disease suppression by Pseudomonads is production of secondary metabolites that are inhibitory to soil borne pathogens. Among these metabolites, 2,4 -DAPG, a polyketide compound, has received worldwide attention due to its broad-spectrum antiviral, antifungal, antibacterial and antitumor activities. There are numerous reports documenting 2,4-DAPG producers being successfully evaluated for suppression of various diseases like take-all of wheat caused by *Gaeumannomyces graminis* (de Souza *et al.*, 2003), black root rot of tobacco caused by *Thielaviopsis basicola* (Keel *et al.*, 1992), bacterial leaf blight of rice caused by *Xanthomonas oryzae* pv *oryzae* (Velusamy *et al.*, 2006) etc. Evidence for an important role of 2,4-DAPG in plant protection also comes from studies on 2,4 DAPG-negative mutants of Pseudomonads and non producing strains into which 2, 4-DAPG biosynthetic plasmids have been transferred (Schnider-Keel *et al.*, 2000; Jorge *et al.*, 2003, Zhou *et al.*, 2012). Considering the growing importance of 2,4-DAPG producers in sustainable agriculture, we screened 297 wilt antagonistic bacteria in our previous work and identified 42 DAPG producers on the basis

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of amplification of phlD gene, which is the highly conserved gene, essential for synthesis of DAPG. Out of these 42, the four most effective *phlD*$^+$ bacteria in terms of plant growth and wilt disease suppression in young tomato plants were selected and identified as Pseudomonas sp based on their partial 16S rDNA sequence (Srinivasamurthy *et al*., 2012). We sought to extend the previous studies by testing these promising PGPRs on mature tomato plants for suppression of bacterial wilt and plant growth promotion. We also sought to assess the mechanism of action of the most efficient PGPR and confirm if DAPG is involved in wilt suppression.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains and their growth media used in this study are listed in Table 1. The *phlD*$^+$ plant growth promoting bacterial strains s188, s218, s245 and s288 were identified as *Pseudomonas* spp (Gene accession number HM196840, HM196831, HM196832, HM196825 respectively) on the basis of 16S rRNA sequencing in our previous study. *Pseudomonas fluorescens* (BAA-477) was used as standard reference strain for DAPG production.

Table 1
The details of bacterial strains used in the study

Bacterial strains	*Medium*	*Characteristics*	*Source*
Pseudomonas spp (s188, s218, s245, s288)	King's B Agar / Luria Agar	Chl^S (50 µg/ml) Nal^R (25 µg/ml), *phlD*$^+$	NRCPB, New Delhi, India
s188MT, s188MT2	-do-	Chl^S (50 µg/ ml) Kan^R (50 µg/ml), Nal^R (25µg/ml), *phlD*$^-$	This study
Pseudomonas fluorescens (BAA-477)	-do-	Chl^S (50 µg/ ml) Nal^R (25 µg/ml), *phlD*$^+$	ATCC, LGC Prochem India, Bangalore, India
R. solanacearum (RSPAL)	CPZ agar (1%TTZ)	Chl^R (50 µg/ ml) Tet^R (150 µg/ ml) Nal^S (25 µg/ml), *phlD*$^-$	ITCC, National Chemical laboratory (NCL), Pune, India
E. coli S17.1 (pSUP5011)	Luria Agar	pBR325 derivative containing Tn5-mob Kan^R (50 µg/ml), Nal^s (25 µg/ml)	Simon *et al*. (1983a)

^{s}Sensitive ^{R}Resistance

Glass house experiment

A glass house study was conducted to evaluate the biocontrol and plant growth promoting potential of four *phlD*$^+$ bacteria to suppress bacterial wilt disease (*R. solanacearum*) on mature tomato plants grown in field soil. Bacterial wilt susceptible tomato variety *Arka vikas* obtained from IIHR, Bangalore was used in the study. The sandy loam soil (pH 6.0) collected from cultivated field of Indian Agricultural Research Institute (IARI), New Delhi was dried, large size sand particles were removed and mixed with sand (10:1). The 5 kg soil mixture was filled in pot (8 inches diameter), sterilized at 121.5^{0}C for 1hr (sterilization was repeated for three times with gap of two days) and used for experiments. This soil was made wilt sick by inoculation with bacterial wilt causal agent *Ralstonia solanacearum* and population was maintained 10^8 cfu g^{-1} through repeated inoculation prior to transplantation of tomato seedlings. Four week old tomato seedlings raised by using sterile plant growth mixture (sand : saw dust : cocopeat (1 :1 : 2) were uprooted from seedling trays and roots soaked in respective *phlD*$^+$ test bacterial culture (10^8 cfu ml^{-1}) for 10 min and transplanted to pots containing wilt sick soil. Three plants per pot was maintained in all the treatments with four replications each in a growth chamber with 270 µEm^{2}s^{-1} light intensity (12:12h day-night period), 80% relative humidity at 30-35^{0}C temperature. Treatments T1 to T4 were inoculated with *phlD*$^+$ bacterial isolates (s188, s218, s245 and s288 respectively). Treatment T5 was inoculated with *phlD*$^+$ reference strain of *Pseudomonas fluorescens* (BAA-477). Treatment T6 was inoculated with only the pathogen *R. solanacearum* (negative control). The treatment T7 was an absolute control with neither pathogen nor *phlD*$^+$ bacteria inoculated. The experiment was conducted two times with completely randomized design.

Plant growth parameters, bacterial wilt incidence and rhizosphere microbial population

The efficacy of *phlD*$^+$ antagonistic bacteria for promotion of plant growth and suppression of wilt

disease was measured at 60 days after transplanting (DAT). The plant height was measured from the base of the plant to the base of the fully opened top leaf of each replication and average was expressed in centimeters per plant. Then the plants were uprooted to measure the dry weight after oven drying at 60°C to a constant weight. The data was recorded and compared with untreated absolute control treatment T7 to assess growth promotion.

Disease incidence was recorded by counting the proportion of wilted plants to total plants per pot. Wilt reduction was calculated according to Aliye *et al*. (2008) as :

PR= $[(P_C-P_T) / P_C]$ x100%, Where PR is percent reduction, P_C and P_T are percentage values of control and the treatment group respectively.

The pathogen population in tomato rhizospheric soil was enumerated by serial dilution plate technique using casamino tetrazolium chloride agar medium (tetracycline 150 µg ml^{-1} and chloramphenicol 50µg ml^{-1} respectively) and population of *phlD*$^+$ bacteria using Luria Agar supplemented with antibiotic Nalidixic acid (25 µg ml^{-1})). Plates were incubated at 28^{0}C for 48 h and colony counts expressed in terms of log cfu g^{-1}.

Tn5 Mutagenesis and screening for phlD negative mutant strain

Mutagenesis was carried out by mating the recipient strain *Pseudomonas* spp s188 with the donor *E.coli* strain 17.1 harboring the suicidal plasmid pSUP5011::Tn5-Mob (Simon *et al*., 1983) as described by Das et al. (2006). The recipients of transposon were selected on Luria agar plate containing both kanamycin sulphate (50 µg ml^{-1}) and nalidixic acid (25 µg ml^{-1}). The transconjugants thus selected were further screened for mutants with defective antagonism against wilt pathogen *R. solanacearum* under *in vitro* conditions by dual inoculation culture technique (Lemessa and Zeller, 2007). The 2, 4-DAPG producing reference strain *P. fluorescens* (BAA-477) was used as positive control.

Evaluation of mutant strain in glass house

A glass house study was conducted to determine the possible role of 2, 4-DAPG in suppression of the wilt pathogen. *In planta* assay was conducted on wilt susceptible tomato variety, Arka vikas with five treatments. T1 and T2 were inoculated with *phlD*$^+$ and *phlD*$^-$ PGPR strains (s188 and s188MT respectively). Treatment T3 was inoculated with *phlD*$^+$ reference strain, *Pseudomonas fluorescens* (BAA-477). Treatment T4 was negative control with only pathogen inoculation. The treatment T5 was the absolute control without inoculation of pathogen or *phlD*$^+$ bacteria. Raising tomato seedlings, preparation of wilt sick soil, PGPR inoculation, transplantation of tomato seedlings, observations of plant growth parameters (Plant height, total dry weight), wilt incidence and microbial rhizosphere population was carried out as explained in the earlier sections. The observations were recorded at 60 DAT.

Statistical analysis

All the data obtained in the present study were subjected to analysis of variance (ANOVA) (single factorial method) using (AGDATA and AGRES software) 1994 Pascal Intel software solutions, version 3.01. When a significant F test was obtained at P<0.001, the separation of the treatment means was accomplished by Fisher's protected least significant difference (LSD).

RESULTS

Evaluation for plant growth promotion and disease suppression

The four *phlD*$^+$ bacterial antagonist's s188, s218, s245 and s288 were evaluated for their plant growth promoting potential as well as wilt suppression in tomato plants grown till maturity in greenhouse (60DAT).

The growth of tomato plants as measured by height and dry weight was significantly increased in all the PGPR treated plants compared to that of their respective control (P<0.001). Highest plant height at 60DAT (67.16 cm) was recorded in the treatment T1 inoculated with s188 followed by treatment T3 inoculated with s245 (62.17 cm) (Fig.1). Accordingly,

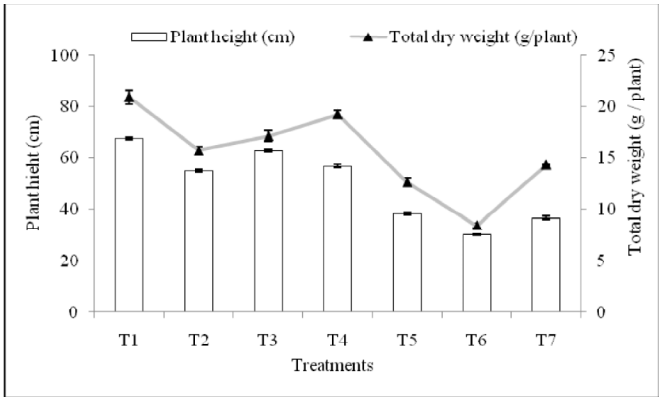

Figure 1: Efficacy of antagonistic *phlD*+ PGPRS (s188, s218, s245, s288, BAA) on plant height and total dry weight of tomato plants under green house conditions at 60 DAT.

a significant increase in dry weight by 82.43and 67.56% was recorded in the treatments T1 and T3 respectively relative to the noninoculated absolute control treatment.

Evaluation of the four antagonists for biocontrol of mature tomato plants in greenhouse was done by measuring two parameters viz. wilt symptoms in tomato plants and enumeration of pathogen population from tomato rhizosphere. Results indicated that s188, s218 and s245 significantly ($P<0.001$) reduced wilt incidence by 82, 63 and 65% respectively as compared to control at 60DAT (Fig.2). These treatments (T1, T2, T3) also maintained significantly lower pathogen population in the rhizosphere (3.77 to 4.82 cfu g^{-1}) relative to the pathogen alone control (T6) (7.30 cfu g^{-1}) (Fig.2). Significantly high population density (5.71 cfu g^{-1}) of *phlD*$^+$ bacteria (s188) in treatment T1 was recorded in wilt sick soil as compared to other *phlD*$^+$ inoculated treatments (5.02 to 5.45 log cfu g^{-1}) (Fig. 2).

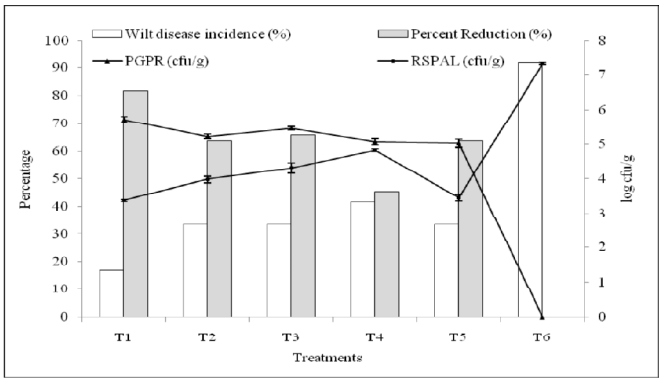

Figure 2: The biocontrol efficacy of *phlD*$^+$bacteria (s188, s218, s245, s288, BAA) expressed as wilt incidence, percentage wilt reduction, rhizosphere population dynamics of *phlD*$^+$bacteria (s188, s218, s245, s288, BAA), pathogen *R. solanacearum* in tomato plants under green house conditions at 60 DAT

The overall results of wilt suppression and plant growth promotion of mature tomato plants indicated Pseudomonad strain s188 to be the most effective PGPR as the plants inoculated with it had significantly high plant height, dry weight, low pathogen population as well as high rhizosphere competence.

Transposon mutagenesis and isolation of phlD$^-$ mutant

Tn5 was introduced into s188 using the suicide plasmid pSUP5011 containing Tn5-*Mob* from *E. coli* S17.1 at a transposition frequency of 2×10^{-4} per donor. Screening 4500 transconjugants for loss of antagonism against *R. solanacearum* by *in vitro* dual inoculation assay identified two mutants (Fig. 3).

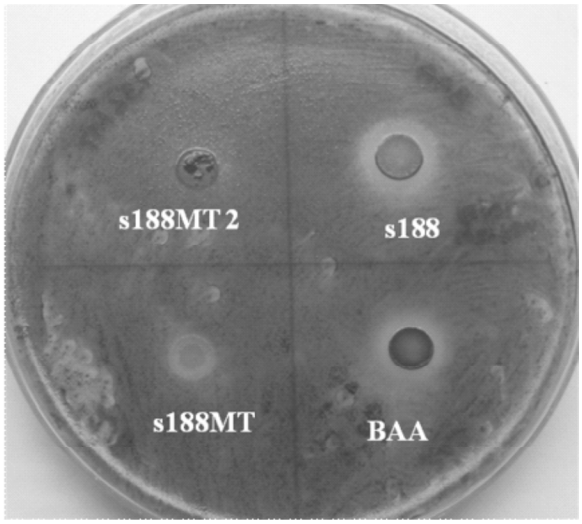

Figure 3: *In vitro* antagonistic assay of *phlD* mutant (s188MT) and parental (s188) *phlD*$^+$ bacteria against *R. solanacearum* using CPZ agar medium at 48h after incubation.

PCR based screening of the mutants for *phlD* gene amplification revealed the mutant s188MT to be defective in DAPG as the characteristic 629bp fragment could not be amplified.

Evaluation of DAPG mutant strain for PGP and biocontrol efficiency

The plant growth promotion (PGP) efficiency of DAPG mutant strain s188MT monitored by measuring plant biomass (dry weight and plant height) at 60DAT showed significant drop compared to the parent strain s188 (Fig. 4). However, there was no significant difference between the uninoculated control and mutant s188 treated plants.

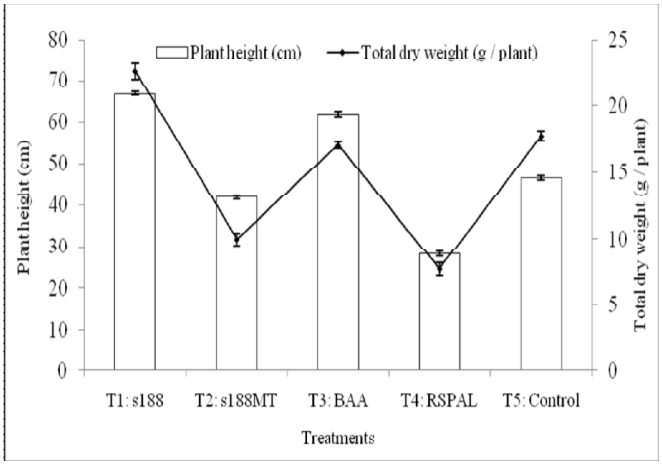

Figure 4: Efficacy of *phlD*+ bacteria (s188, BAA), mutant strain (s188MT) and *R. solanacearum* (RSPAL) on plant height and total dry weight of tomato plants under green house condition at 60 days after transplanting.

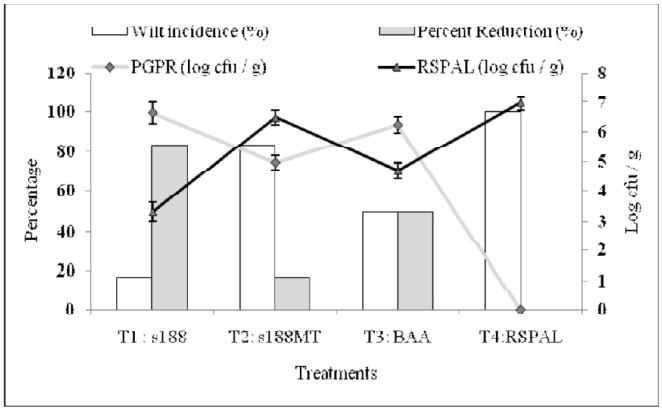

Figure 5: Comparison of biocontrol efficacy of *phlD*+ bacteria (s188, BAA), with mutant strain (s188MT) and pathogen *R. solanacearum* (RSPAL) as depicted by rhizospheric population densities, percentage wilt disease and wilt reduction in tomato plants at 60 DAT under green house condition

Further, the results for assessment of biocontrol efficiency (Fig. 5) showed that the plants treated with mutant strain had higher pathogen population in rhizosphere (6.47 log cfu g^{-1}) which was comparable to the plants treated with pathogen alone as control. The wild type strain s188 treated plants suppressed the wilt incidence by 83.33 % while the mutant strain defective in DAPG production showed minimal reduction (16.66%).

DISCUSSION

PGPRs are an important functional group of beneficial bacteria used for control of soilborne pathogens and plant growth promotion. Phl or 2,4-DAPG producing PGPRs attract special attention owing to their broad spectrum of antimicrobial properties. In this investigation, four *phlD*$^+$ PGPRs were evaluated for plant growth promotion and suppression of bacterial wilt on mature tomato plants with an objective of selecting the most efficient antagonist and identifying role of 2,4-DAPG in wilt suppression. The antagonistic bacteria reported to be effective *in planta* grown in plant growth mixture in our previous study have been further evaluated for their performance in natural soil conditions in the current study before labeling them as biocontrol agents. It is because in the natural conditions, physical and chemical parameters of soil like temperature, pH, carbon, nitrogen, iron, zinc etc have a great influence on colonization ability, density of population, antibiotic production and potential of suppression of pathogen by inoculant PGPR (Shanahan *et al.,* 1992; Weller *et al.*, 2002).

The four PGPRs tested on mature tomato plants grown in wilt sick soil in greenhouse, *in vivo*, proved to be considerably efficient though they differed in the degrees of plant growth promotion and antagonism against the wilt pathogen *Ralstonia solanacearum.* The plants inoculated with strain s188 showed the highest increment in plant biomass and inhibition to pathogen, thus proving to be the superior isolate for development of biocontrol agent. Suppression of pathogen activity in rhizospheric soil by inoculation of *phlD*$^+$ bacteria may be responsible for increase in plant growth parameters. The high population density of *phlD*$^+$ bacteria recorded in rhizosphere of tomato plants in the present study indicated their competitive ability which in turn contributed to suppression of disease causing plant pathogen. This is in congruence with earlier reports correlating competitive ability of bioinoculants to better plant microbe interaction which in present work is aimed for efficient disease suppression (Notz *et al.*, 2002; Ryan *et al.*, 2004; Rai *et al.*, 2012).

In our previous study, s188 was tested to be positive for synthesis of HCN and siderophores besides 2,4-DAPG which raises a possibility of involvement of these metabolites in wilt suppression. Hence, in this study, we looked for association between absence of DAPG production and changes in plant growth promotion and protection provided by s188.

We employed transposon mutagenesis to generate *phlD* mutants as *phlD* is the key biosynthetic gene essential for synthesis of the precursor molecule monoacetylphloroglucinol (MAPG). The mutant showed substantial loss in its antagonistic activity both *in vitro* as well as *in planta* assay. The *phlD*$^+$ bacterial strain s188 showed antagonistic activity against *R. solanacearum* under *in vitro* assay and also shown promotion of plant growth under green-house experiments with reduction of wilt disease incidence. These results further supported the role of DAPG in wilt suppression by s188. Generation of mutants defective in these secondary metabolite productions to study the mechanism of disease suppression by PGPRs has been successfully demonstrated in few other studies (Velusamy *et al.*, 2006; Lanteigne *et al.*, 2012; Maketon *et al.*, 2012; Weller *et al.*, 2012).

In this study, we have identified DAPG as the likely compound responsible for the Pseudomonad strain s188's ability to limit the growth of *Ralstonia solanacearum in vitro* and under soil conditions. In conclusion, the Pseudomonad strain s188 has proven to be consistently efficient in the suppression of bacterial wilt in tomato and has a strong potential to be part of an integrated disease management package

for control of bacterial wilt. However, validation under variety of field conditions will be a prerequisite towards its implementation as reliable biological control strategy.

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