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ORIGINAL PAPER

Pseudomonads: major antagonistic endophytic bacteria to suppress bacterial wilt pathogen, *Ralstonia solanacearum* in the eggplant (*Solanum melongena* L.)

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Abstract Endophytic bacteria of eggplant, cucumber and groundnut were isolated from different locations of Goa, India. Based on in vitro screening, 28 bacterial isolates which effectively inhibited *Ralstonia solanacearum*, a bacterial wilt pathogen of the eggplant were characterized and identified. More than 50% of these isolates were *Pseudomonas fluorescens* in which a vast degree of variability was found to exist when biochemical characteristics were compared. In greenhouse experiments, the plants treated with *Pseudomonas* isolates (EB9, EB67), *Enterobacter* isolates (EB44, EB89) and *Bacillus* isolates (EC4, EC13) reduced the wilt incidence by more than 70%. All the selected isolates reduced damping off by more than 50% and improved the growth of seedlings in the nursery stage. Most of the selected antagonists produced an antibiotic, DAPG, which inhibited *R. solanacearum* under in vitro conditions and might have been responsible for reduced wilt incidence under in vivo conditions. Also production of siderophores and IAA in the culture medium by the antagonists was recorded, which could be involved in biocontrol and growth promotion in crop plants. From our study we conclude that *Pseudomonas* is the major antagonistic endophytic bacteria from eggplants which have the potential to be used as a biocontrol agent as well as plant growth-promoting rhizobacteria. Large scale field evaluation and detailed knowledge on antagonistic mechanism could provide an effective biocontrol solution for bacterial wilt of solanaceous crops.

Keywords Endophytic bacteria · *Ralstonia solanacearum* · *Pseudomonas* · *Bacillus* · *Enterobacter* · Eggplant · Bacterial wilt · Antagonism

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Introduction

Endophytic bacteria live in plant tissues without doing substantive harm or gaining benefit other than residency (Kobayashi and Palumbo 2000). Hallmann et al. (1997) defined an endophyte as any microorganism that resides inside the plant without regard to the specific tissue colonized and these bacterial endophytes can be isolated from surface-disinfected plant tissue or extracted from internal plant tissue. Although endophytes were already described in the past century, they have received considerable attention only in the past decade when their capacity to protect their hosts against biotic factors was recognized. Endophytes enter plant tissue primarily through the root zone; however, aerial portions of the plants such as flowers, stems, cotyledons may also be used for entry (Kobayashi and Palumbo 2000). Once inside the plant tissue, endophytic bacteria may either remain localized at the point of entry or spread throughout the plant (Hallmann et al. 1997). These microorganisms can reside within the cells (Jacobs et al. 1985), in the intercellular spaces or in the vascular system (Bell et al. 1995). The capability of colonizing host tissue has made endophytes valuable for agriculture as a tool to improve crop performance.

Bacterial wilt caused by *Ralstonia solanacearum* (Smith) Yabuuchi is one of the important and also a major constraint in the production of eggplant and many other

crops in tropical, subtropical and warm temperate regions of the world (Buddenhagen and Kelman 1964; Hayward 1991). This pathogen is present worldwide and has a wide host range including several hundreds of susceptible species in at least 50 different plant families, which makes the pathogen most destructive and most difficult to control (Kelman et al. 1994; Hayward 1991). Eggplant cultivation in Goa is commonly affected by wilt and the popular local cultivar (Agassaim) is highly susceptible to this disease, with incidence ranging from 30 to 100% (Ramesh 2006). Conventional management strategies like crop rotation, adjusting the date of planting, cultural methods and soil treatment are not effective (Chellemi et al. 1997). Resistant cultivars are either location-specific or generally not preferred by growers due to low consumer preference. Thus, control of *R. solanacearum* seems to be difficult due to lack of universal control treatments (Hayward 1991), and to date no effective control method exists for bacterial wilt disease.

Recent studies have indicated that biological control of bacterial wilt disease could be achieved using antagonistic bacteria. Different bacterial species viz. *Alcaligenes* spp. and *Kluyvera* spp. (Assis et al. 1998), *Pseudomonas fluorescens, P. alcaligenes*, *P. putida*, *Flavobacterium* spp. and *Bacillus megaterium* (Reiter et al. 1998), *B. pumilus* (Benhamou et al. 1998) and *Microbacterium* spp., *Clavibacter michiganensis*, *Curtobacterium* spp. and *B. subtilis* (Zinniel et al. 2002) have been reported as endophytes and were inhibitory to plant pathogens. Toyota and Kimura (2000) have reported the suppressive effect of some antagonistic bacteria on *R. solanacearum*. Moreover, Ciampi-Panno et al. (1989) have proved the use of antagonistic microbes in the control of *R. solanacearum* under field conditions. Endophytic bacteria colonize an ecological niche similar to that of plant pathogens, especially vascular wilt pathogens, which might favour them as potential candidates for biocontrol agents and plant growth-promoting agents (Ramamoorthy et al. 2001). The present study aims at isolation, characterization of potential antagonistic endophytes against *R. solanacearum* and evaluating their biocontrol efficiency in the eggplant (*S. melongena* L.).

Materials and methods

Endophytic bacteria and *R. solanacearum*

Plant samples were collected by removing the healthy, vigourous plants along with roots form various locations in Goa, India. Endophytic bacteria were isolated by modifying the method described by Bhowmik et al. (2002). Surface sterilized stem/root pieces were homogenized using sterile pestle and mortar with 3 ml of phosphate buffer. Different aliquots of (100, 200, 400, 600 µl) of the tissue extract were plated onto KB medium (King et al. 1954) and incubated at 28 ± 2°C for 24–48 h for the development of colonies. After confirming the endophytic nature of the bacterial isolates, they were further purified and preserved at −78°C in the culture collection for further studies. *Ralstonia solanacearum* was isolated from a freshly wilted eggplant by streaking a loopful of flowing ooze containing the bacteria onto sterile TZC agar plates (Kelman 1954) and the plates were incubated at 28 ± 2°C for 48 to 72 h. After incubation the virulent *R. solanacearum* colonies were selected, purified and preserved at −78°C for further studies.

Antagonism of endophytic bacteria against *R. solanacearum* under in vitro conditions

In the agar diffusion test, 600 µl of 48-h-old-grown *R. solanacearum* strain Rs21-O (approximately 10^{10} c.f.u./ml) was seeded into 1 l of KB agar medium. With a sterile cork borer 3 wells of 7 mm diameter were prepared by removing agar discs from the solidified medium. 20 µl of 48-h-old-grown endophytic bacterial suspension (approximately 3.5×10^{10} c.f.u./ml) was added to each well. Inhibition zone around the well (radius in mm), over growth zone (radius in mm) of the antagonistic bacteria if any were recorded by measuring from the outer edge of the well after 48 h. All the 144 endophytic bacterial isolates were screened for antagonism against the pathogen. In the streaking method, the bacterial isolate and pathogen were streaked perpendicular to each other on KB agar plates without touching at the meeting end. Growth inhibition of pathogen at the meeting point was recorded after 48 h. Screening was also performed by agar diffusion method using the culture filtrate obtained by centrifuging the 48-h-old-grown cultures at 10,000 rev/min for 10 min. All the experiments were conducted at 28 ± 2°C.

Characterization and identification

Morphological characterization of each isolate and biochemical characterization of selected isolates were carried out as per the standard method described elsewhere. Clustering of the isolates was carried out using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA). Based on the similarity coefficient and other biochemical characters, the isolates were identified as described by *Bergey's Manual of determinative bacteriology* (Holt et al. 1994). The most effective six isolates (EB9, EB67, EB44, EB89, EC4 and EC13) were identified based on 16S rRNA sequencing.

Evaluation of antagonistic efficacy of the endophytic bacteria against *R. solanacearum* under in vivo conditions

Seed treatment and nursery experiments

Seeds of eggplant (*S. melongena* L.) cultivar Agassaim were treated with the 48-h-old-grown culture (approximately 3.5×10^{10} c.f.u./ml) of the selected isolates for 30 min and were shade-dried at 28 ± 2°C for 1 h. The treated seeds (150) were sown in pots containing standard pot mixture (red earth: sand: farmyard manure = 2:1:1) in a greenhouse. Observations were recorded on germination percentage in the beginning; root length; shoot length and wet weight of the seedlings after 15 and 25 days of sowing by removing three seedlings from each replication. Incidence of damping off was also recorded in each treatment.

Seedling treatment during transplanting under greenhouse conditions

Two experiments were conducted simultaneously under greenhouse conditions using 30-days-old seedlings obtained from the above nursery experiment. In experiment one, five healthy seedlings from the nursery were again treated with the same isolates by dipping the seedlings in 48 h-old-grown cultures (approximately 3.5×10^{10} c.f.u./ml) for 20 min. The treated seedlings were planted in the pots containing standard pot mixture and drenched with the 48-h-old-grown culture (10 ml/plant) of respective antagonistic bacterial isolates. Three replications with five seedlings each were maintained for each antagonistic bacterial treatment. One week after transplanting, the seedlings were challenge-inoculated with 5 ml of *R. solanacearum* strain RS21-O (approximately 1.2×10^6 c.f.u./ml) by pouring the bacterial suspension around the root zone. Experiment two was conducted in a similar way as described above with only the difference being treating and inoculating 10 seedlings per replication. The incidence of wilt was recorded periodically over a period of time up to 6 weeks. The data were analysed statistically and the mean comparisons were carried out using a least significant difference (LSD) test ($P < 0.05$). Biological control efficacy (BCE) was calculated as described by Guo et al. (2004).

Estimation of indole acetic acid (IAA) and siderophore produced by the endophytic bacteria

Bacterial isolates were grown in tripticase soya broth with tryptophan (100 µg/ml) for 30 h. The presence of IAA in the culture filtrate was quantified (Gorden and Paleg 1957) and expressed as µg/ml. Bacterial strains were grown in the KB broth for 3 days for siderophore production. Siderophore produced by antagonistic bacteria was estimated as described by Reeves et al. (1983) and expressed as µg/ml.

Extraction and analysis of 2,4-diacetylphloroglucinol (DAPG)

For extraction of DAPG, the different strains were cultivated on the pigment production medium for 4 days at 150 rev/min at 30°C. Extraction of DAPG was carried out as described by Viswanathan (1999). The extracts were separated on thin layer chromatography (TLC) on 250 µm thick silica gel (200 mesh grade) and acetonitrile/methanol/water (1:1:1 by v/v) solvent system was used for separation of compounds.

Antimicrobial effect of DAPG on *R. solanacearum*

The extracted DAPG was used for assessing its efficiency in inhibiting *R. solanacearum* strain Rs21-O under in vitro conditions. Sterile filter paper discs (5 mm diam) were dipped in the DAPG extract for 1 min and air dried inside the laminar flow chamber. The air-dried discs were then placed on *R. solanacearum* seeded medium as described previously by inoculating 600 µl *R. solanacearum* strain Rs21-O (approximately 10^{10} c.f.u./ml). Observation on the development of for inhibition zone (radius in mm) around the disc was recorded after 48 h of incubation.

Results and discussion

Endophytic bacteria

A total of 144 endophytic bacteria were isolated from three crops, the details of which are presented in Table 1. More endophytic bacteria were isolated from stem tissue than from root tissue in case of eggplant. In the cucumber however, almost equal numbers of endophytes were isolated from root and stem tissue. More than 60% endophytes from these crops were Gram-negative. Studies reported that both Gram-positive and Gram-negative bacterial endophytes have been isolated from several tissue types in numerous plant species and several different bacterial species have been isolated from a single plant (Kobayashi and Palumbo 2000). Zinniel et al. (2002) reported more Gram-positive endophytes than Gram-negative endophytes from corn and sorghum. Significant variation in the types of indigenous bacteria isolated from diverse host plant species depends on host specificity, geographical distribution, plant age and tissue type (Kobayashi and Palumbo 2000).

Table 1 Isolation of endophytic bacteria and morphological characterization

Crop	No. of endophytes	Fast growers (%)	Pigment production (% of isolates)			Gram reaction (% of isolates)	
			Fluorescent	Non-fluorescent	No pigment	Gram +ve	Gram −ve
Eggplant (*Solanum melongena* L.)	109	18.3 (20)	14.7 (16)	29.4 (32)	55.9 (61)	35.8 (39)	64.2 (70)
Cucumber (*Cucumis sativus* L.)	23	34.8 (8)	52.1 (12)	30.4 (7)	17.5 (4)	13.0 (3)	87.0 (20)
Groundnut (*Arachis hypogaea* L.)	12	25.0 (3)	0	33.3 (4)	66.7 (8)	16.7 (2)	83.3 (10)

Values in the parentheses indicate the number of bacterial isolates

Antagonism of endophytic bacteria against *R. solanacearum*

Assessment of the ability of the endophytic bacteria to inhibit the growth of *R. solanacearum* is the first step towards the selection of potential biocontrol agents. The antagonistic ability of the endophytic bacteria towards *R. solanacearum* was assayed in vitro by the agar diffusion method and also by the streaking method. Results indicated that 16 fluorescent and 18 non-fluorescent isolates from eggplant, 12 fluorescent, two non-fluorescent isolates from cucumber and four non-fluorescent isolates from groundnut inhibited the growth of the pathogen. In the streak method, inhibition of *R. solanacearum* at the meeting point of the antagonist and the pathogen was noted. Further, the inhibition zone caused by culture filtrates of endophytic bacteria ranged between 1.5 and 10.0 mm in radius. Based on the effectiveness, 28 isolates were selected for further studies. Results of the 13 selected promising isolates are given in Table 2. Although, there is general agreement that suppression in vitro does not necessarily relate to the same in field conditions (Kloepper 1993), it is a commonly followed method in the initial screening against bacterial (Meenakumari et al. 2003) and fungal (Viswanathan et al. 2003) pathogens. According to Cook and Sequeira (1991) *R. solanacearum* is a poor competitor outside the plant system, giving opportunity for biological control.

In recent years interest in biological control of plant diseases has been significantly increased worldwide and several biocontrol agents against plant diseases have been introduced. Numerous plant growth promoting rhizobacteria (PGPR) have been reported which promote plant growth and also control plant diseases (Viswanathan and Samiyappan 2002; Bashan and de Bashan 2002). When cell-free culture filtrates were used, diffusible metabolites produced by the isolates inhibited *R. solanacearum* growth indicated the role of secondary metabolites in the pathogen suppression in vitro. Xiao and Kisaalita (1998) have reported that *Pseudomonads* exert their beneficial effects in part by producing yellow-green, fluorescent siderophores called pyoverdins, under iron-deficient conditions.

Characterization and identification of endophytic bacteria

The majority of the selected isolates (21 out of 28) were Gram-negative bacteria. Among Gram-negative bacteria, most of the isolates (18) were found to be species of *Pseudomonas*; two isolates (EB44, EB 89) were identified as species of *Enterobacter* and one isolate (EB9) as *Burkholderia cepacia*. Seven isolates (EB10, EB13, EB56, EB66, EB70, EC4 and EC13) were identified as species of *Bacillus* (Table 2). Partial sequence data of 16S rRNA from the most effective six isolates (EB9, EB67, EB44, EB89, EC4 and EC13) were submitted to GenBank (Accession Nos. FJ194525 to FJ194530). All the Gram-negative isolates were categorized into four phylogeny groups based on the biochemical tests when 75% similarity co-efficient was considered. Majority of the identified *P. fluorescens* isolates of biovar I, III, IV and V, *P. putida* and other *Pseudomonas* spp. were in group one. Group two comprised one isolate of *Pseudomonas* spp. (EB20) and one isolate of *Enterobacter* spp. (EB44), group three comprised of *P. fluorescens* biovar V (EB43) and group four comprised one isolate of *Burkholderia cepacia* (EB9) (Fig. 1). Studies focusing on finding plant growth-promoting bacteria have frequently found success with bacterial genera *Pseudomonas* and *Bacillus*. (Merriman et al. 1974). Viswanathan et al. (2003) have reported isolation of *P. fluorescens* from sugarcane and Reiter et al. (2002) have reported isolation of fluorescent *Pseudomonas* from potato plants. Biochemical characterization and identification fairly yielded a correct identification as reported by Zinniel et al. (2002). EB44 and EB89 earlier identified as *Pseudomonas* were later confirmed as species of *Enterobacter* based on 16S rRNA sequence analysis. This kind of difference in identification is evident from the reports of Shah et al. (1998) where the isolates of *Pseudomonas* were later confirmed as *Enterobacter* based on detail characterization. Other *P. fluorescens* isolates from this study were later confirmed by PCR amplification of 16 s rRNA-ITS region (Jaxon 2007).

Table 2 Inhibition of *R. solanacearum* by promising endophytic bacteria and their identification

Isolates	Inhibition of *R. solanacearum* (radius in mm)			Identified as
	24-h-old culture[a]	Streak method[b]	Culture filtrate[a]	
EB9	16.0		2.5	*Burkholderia cepacia*
EB44	10.3	+	2.5	*Enterobacter* spp.
EB52	10.6	+	7.5	*Pseudomonas fluorescens* biovar IV
EB53	13.0	+	4.5	*Pseudomonas* spp.
EB66	15.6	+	6.4	*Bacillus cereus*
EB67	16.3	+	8.5	*Pseudomonas* spp.
EB69	19.3	+	10.0	*Pseudomonas fluorescens* biovar V
EB87	5.3		6.5	*Pseudomonas fluorescens* biovar I
EB89	5.6		5.3	*Enterobacter cloacae*
EC2	20.0	+	7.6	*Pseudomonas fluorescens* biovar III
EC4	14.3		6.5	*Bacillus* spp.
EC13	6.6		4.0	*Bacillus* spp.
EC21	19.6	+	7.0	*Pseudomonas fluorescens* biovar III

[a] Inhibition zones are mean of 12 values (Three replications and four plates/replication). Inhibition zone was measured as radius from the outer edge of the well

[b] "+" Indicates the pathogen inhibition at the meeting point

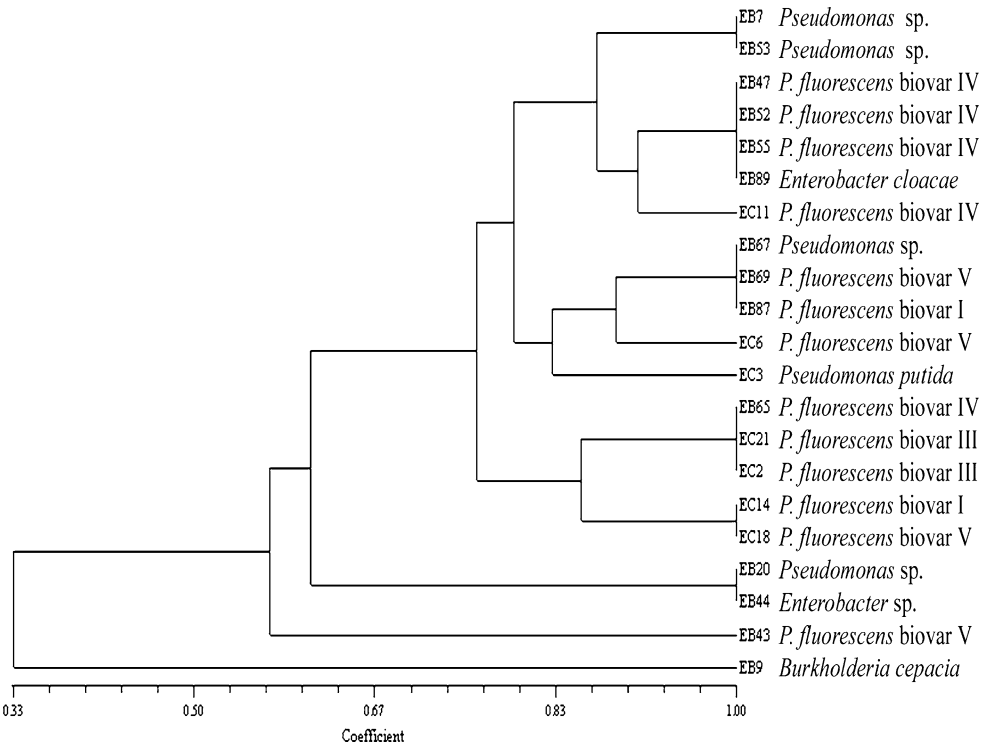

Fig. 1 Dendrogram constructed by unweighted pair group method with arithmetic average (UPGMA) clustering using NTSYSpc software v 2.02i (Applied Biostatistics Inc. USA) showing the phylogenetic relationships of promising Gram negative endophytic antagonistic bacteria based on biochemical characters. EB44, EB67, EB89, EB9 and EB69 were identified close to the species based on 16S rRNA sequencing. Clustering is done at 75% similarity co-efficient

Testing the efficacy of endophytes under in vivo conditions

At nursery stage, the incidence of damping off was reduced by more than 50% by almost all the antagonists. EB44, EB55, EB66, EB87, EB 89, EC2, EC3, EC4, EC6 and EC14 recorded less diseased seedlings (<5%) compared to the untreated control (42.7%). All the effective isolates increased the shoot length, root length and wet weight of the seedlings in nursery consistently over a period up to 45 days. However the maximum improvement was recorded in EB7, EB13, EB20, EB47, EB52, EB53 and EB70 treatments (Data not shown). Fluorescent *Pseudomonas* promotes plant growth by producing and secreting auxins,

gibberellins and cytokinins (Xiao and Kisaalita 1998) and indole-3-acetic acid (Suzuki et al. 2003).

Greenhouse conditions

The effect of selected isolates on disease suppression was tested by seed treatment at the time of sowing and seedling treatment during transplanting. In experiment 1, there was no wilt incidence in EB9, EB44, EB67, EB87, EC4, and EC13 after 2 weeks and in EB9, EB67 ad EC13 after 3 weeks of pathogen inoculation. In experiment 2, EB69, EB 87 and EC13 did not show any wilting after 2 weeks and after 3 weeks none of the treatments were completely free from wilt incidence. However, after 6 weeks of pathogen inoculation, at least 20% wilt was noticed in all the treatments of both the experiments. EB9, EB52, EB67, EC4 and EC13 from experiment 1 reduced the wilt incidence by 80% and EB13, EB44, EB53, EB66, EB67, EB69 and EB89 form the experiment 2 reduced the wilt incidence by at least 70% when compared to the control. The mean of both experiments indicated that the plants treated with EB9, EB44, EB67, EB89, EC4 and EC13 showed reduced incidence of the disease by 70% compared to the untreated control (Table 3).

Chen et al. (1995) have reported that application of endophytic bacteria by stem injection in cotton plants reduced root rot caused by *Rhizoctonia solani* and vascular wilt caused by *Fusarium oxysporum*. These bacteria move upward and downward form the point of application and by colonizing the internal root tissues, can exclude the entry of pathogen in the vascular stele. Thus endophytic bacteria can out-compete the vascular plant pathogens. So establishing beneficial bacterial populations in the rhizosphere seems to be a key for obtaining a healthy microfloral balance within the plants. Under field conditions, the effectiveness of the biocontrol agent depends on various factors like time of application, age of the plant, strength of the biocontrol agent formulation, mode of application and many other factors (Botelho and Mendonca-Hagler 2006). In host range studies, endophytes obtained from the monocots corn and sorghum colonized in their hosts and perished in other monocots and dicots. This might be due to the development of an evolutionary niche within the plant by the bacteria (Zinniel et al. 2002). However, in our studies other than the bacteria from eggplant, endophytes from cucumber also performed well in suppressing the pathogen. This may be due to the fact that crops in complementary rotations can share same or similar endophytic populations and the possibility exists of utilizing beneficial relationships between plant and endophytes over successive crops to develop a sustainable crop production system.

Production of secondary metabolites

DAPG extract was separated on TLC and the R_f values of the spots were calculated. Bacterial isolates produced

Table 3 Incidence of wilt in eggplant treated with 48-h-old-grown antagonistic bacteria and challenge-inoculated with *R. solanacearum*

Treatments	Experiment 1[a]				Experiment 2[b]				Mean of two experiments	
	2 Weeks	3 Weeks	6 Weeks	BCE (%)	2 Weeks	3 Weeks	6 Weeks	BCE (%)	Wilt (%)	BCE (%)
EB9	0.0	0.0	20.0	80.0	10.0	13.3	33.3	66.7	26.7	73.3
EB44	0.0	20.0	40.0	60.0	6.7	16.7	20.0	80.0	30.0	70.0
EB52	20.0	20.0	20.0	80.0	20.0	23.3	50.0	50.0	35.0	65.0
EB53	20.0	40.0	40.0	60.0	20.0	30.0	30.0	70.0	35.0	65.0
EB66	20.0	40.0	60.0	40.0	3.3	10.0	23.3	76.7	41.7	58.3
EB67	0.0	0.0	20.0	80.0	10.0	10.0	30.0	70.0	25.0	75.0
EB69	20.0	40.0	60.0	40.0	0.0	3.3	20.0	80.0	40.0	60.0
EB87	0.0	20.0	40.0	60.0	0.0	20.0	50.0	50.0	45.0	55.0
EB89	20.0	40.0	40.0	60.0	3.3	20.0	20.0	80.0	30.0	70.0
EC2	40.0	60.0	60.0	40.0	20.0	30.0	46.7	53.3	53.3	46.7
EC4	0.0	20.0	20.0	80.0	6.7	20.0	33.3	66.7	26.7	73.3
EC13	0.0	0.0	20.0	80.0	0.0	20.0	36.7	63.3	28.3	71.7
EC21	60.0	60.0	80.0	20.0	30.0	40.0	46.7	53.3	63.3	36.7
Streptomycin	0.0	0.0	20.0	80.0	10.0	20.0	30.0	70.0	25.0	75.0
Control	100.0	100.0	100.0	0.0	100.0	100.0	100.0	0.0	100.0	0.0
LSD ($P < 0.05$)	13.0	16.6	17.8	–	12.8	11.5	10.9	–	–	–

Mean of three replications, values are arc sine transformed before analysis

[a] Five seedlings were treated with antagonistic bacteria and challenge inoculated with *R. solanacearum*

[b] Ten seedlings were treated with antagonistic bacteria and challenge inoculated with *R. solanacearum*

Table 4 TLC analysis of DAPG extracts, in vitro inhibition of *R. solanacearum* by DAPG extract, IAA and siderophore production

Isolates	TLC analysis of DAPG extract R_f values		Inhibition of *R. solanacearum* by DAPG extract[a]	IAA (μg/ml)[b]	Siderophore production (μg/ml)[c]
	0.70–0.79	0.80–0.89			
EB9		0.82	0.00	39.0	166.4
EB44		0.89	1.85	32.9	
EB52		0.86	1.20	58.2	22.8
EB53		0.86	1.10	38.7	
EB66	0.71		0.80	36.5	
EB67		0.85	2.00	55.5	118.6
EB69		0.81	1.75	38.5	170.7
EB87	0.79		0.00	36.3	248.7
EB89	0.77		1.80	34.7	
EC2		0.87	2.40	42.6	41.5
EC4	0.77		1.05	29.3	59.2
EC13	0.72		1.45	51.2	
EC21	0.75	0.83	2.45	52.4	59.9
Control	–	–	0.0	0.0	0.0
LSD ($P < 0.05$)			0.545		

[a] Inhibition zone was measured as radius from the outer edge of the well was mean of two replications

[b] IAA was estimated in culture filtrate and expressed as μg/ml using analytical grade IAA as standard

[c] Dihydroxy type of siderophores was estimated in culture filtrated and expressed as μg/ml using dihydroxybenzoic acid as standard

compounds of R_f values around 0.84–0.89 were found to reduce bacterial wilt in greenhouse studies. When the DAPG extracts were used in bioassay, the extracts from those isolates produced larger inhibition zones (Table 4). All the isolates produced IAA in the culture medium (21–68 μg/ml) and EB7 produced the highest amount. Dihydroxyphenol-type siderophores are produced by most of the endophytic bacteria; EB87 produced the highest amount (248 μg/ml) (Table 4). One of the mechanisms by which *P. fluorescens* suppresses plant pathogens is the production of antibiotics such as 2, 4-diacetylphloroglucinol (DAPG), pyrrolnitrin pyoluteorin, and phenazine-1-carboxylate. Raaijmakers and Weller (2001) reported the existence of wide genotypic diversity of DAPG-producing *Pseudomonas* species which could be exploited for the identification of superior biocontrol agents against plant pathogens. *Pseudomonas fluorescens* strain Hv37a exhibited antibiosis in vitro against broad-range phytopathogenic bacteria, fungi and suppressed the disease development of *Pythium ultimum* on cotton (Kloepper 1993). Production of siderophores is another mechanism by which endophytic biocontrol agents suppress the pathogens indirectly by stimulating the biosynthesis of other antimicrobial compounds by increasing availability of minerals to the biocontrol agent in addition to iron chelation (Duffy and Defago 1999). TLC analysis of secondary metabolites showed that each isolate produced different types of metabolites indicating the array of compounds present in it.

In our study, the isolates which produced DAPG metabolites of 0.80–0.89 R_f value inhibited the pathogen both under in vitro and greenhouse conditions. The reported R_f value of DAPG is 0.78 (Yuan et al. 1998) and differences of up to 40% between observed and published R_f values may be encountered in TLC experiments unless the same absorbents and preparative methods are used (Burkhead et al. 1994). This leads to the assumption that DAPG may act as one of the important mechanisms to reduce the bacterial wilt in eggplant in combination with other mechanisms such as siderophore production, induced resistance. Detailed studies on different mechanisms including ISR is being carried out at our centre to understand bacterial wilt suppression by antagonistic bacteria. The challenge ahead on this aspect is development of proper formulations, field application of potential antagonists and development of a consortium of antagonists which possess different mechanisms.

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