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Morphological and biochemical characterization of antagonist *Pseudomonas* isolates

N. SRINIVASA[1], H. RYMBAI*, A.M. RAJESH[1], P. GANESHAMOORTHI[1], B. RAMANUJAM[2] AND K.R. YATHISH[3]
Deprtment of Fruit Science, ASPEE College of Horticulture and Forestry, Navsari Agricultural University, NAVSARI (GUJARAT) INDIA (E-mail address: rymbaihort@gmail.com)

Abstract : The morphological and biochemical characterization of phylloplane and rhizosphere *Pseudomonas* isolates collected from different places were determined. The *Pseudomonas* isolates antagonistic activity against *Alternaria solani,* were also tested to determine their capacity to inhibit fungal infection on tomato. The result showed that among the seven isolates of *Alternaria solani* on potato dextrose agar, isolate HES AL-1 recorded maximum growth coupled with distinct sporulation and colony characteristics. Based on morphological and biochemical characterization of the twelve bacterial antagonists, ten genuses belong to *Pseudomonas* while two were of Flourescent *Pseudomonas.* It was also confirmed that the growth of all the seven isolates of *Alternaria solani* was significantly inhibited by the antagonists *Pseudomonas* in potato dextrose agar (PDA) conditions. Out of twelve *Pseudomonas* isolates, five isolates *viz.*, S4B7P (27.74), S1B8P (27.33), S2B10P (25.47), S3B3PF (23.07) and S1B1P (19.69) which showed higher mean inhibition percentage on all the isolates of *Alternaria solani* were identified as potential antagonistic against *Alternaria solani*. Further, in pot culture under green house conditions, among the five promising isolates of *Pseudomonas* isolates, S1B8P isolate and S3B3PF isolate expressed the minimum mean per cent disease incidence (PDI) in all the three methods of inoculation. While, among the three methods of inoculation tested, pre inoculation of *Pseudomonas* antagonists followed by *Alternaria solani* showed the minimum PDI (20.68). Therefore, foliar application of *Pseudomonas* antagonists before inoculation with pathogens may reduce the incidence of early blight in tomato.

Key Words : Morphological and biochemical characterization, *Pseudomonas* antagonists, *Alternaria solani,* Tomato

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INTRODUCTION

Tomato (*Lycopersican esculentum* Mill) a native of South America, belongs to the family Solanaceae and is one of the most importance vegetables in the world. Among vegetables, tomato ranks next to potato in the world acreage and first among processing crop (Anonymous, 2012). In India, it occupies an area of about 8.6 lakh ha with an annual production of 165.2 lakh tones (Anonymous, 2011). The total global area under tomato annually is 45.82 lakh ha with production of 11505.1 lakh tones (Anonymous, 2011). However, this crop suffers from several diseases like damping off, wilts, leaf curl, leaf spots, early blight or fruit rot and buckeye rot (Balanchard, 1992). Among which, early blight and fruit rot caused by *Alternaria solani* (Ell and Martin) is the world's most catastrophic diseases incurring loss both at pre and post harvest stages of tomato. The disease appears at all stages of the crop and causing losses up to 50-86 per cent in fruit yield (Mathur and Shekawat, 1986). At present, the strategy for management of the disease is based mainly on fungicidal application. Chemical fungicides used in management of these diseases are not only costlier but also harmful to the environment, beneficial organisms and human beings. Hence, in order to minimize the fungicidal application,

*** Author for correspondence**
[1]Division of Plant Pathology, Indian Agricultural Research Institute, NEW DELHI, INDIA
[2]Plant Pathology, Project Directorate of Biological Control, Hebbal, BENGALURU (KARNATAKA) INDIA
[3]Directorate of Maize Researh, Indian Council Agricultural Research, NEW DELHI, INDIA

biocontrol using antagonistic organisms may offer great alternative potential for the management of plant diseases without any adverse effect on environment. Bacterial antagonists like species of *Pseudomonas* spp. are extensively used in control of several plant diseases of crop plants (Chattannavar *et al*., 1988; Mathivanan *et al*., 2000; Prasad and Kulsresta, 2002). However, very limited studies were conducted with regard to the biological control of foliar plant pathogens like *Alternaria* spp. which causes extensive damage on tomato. Keeping these in view, the present work was to develop biocontrol strategies for leaf blight and leaf spot disease of tomato caused by *Alternaria solani* was taken up with an objective to identify the potential *Pseudomonas* isolates as antagonists against *Alternaria solani* using dual culture technique.

MATERIALS AND METHODS

Collection, isolation and identification of *Alternaria* species:

Collection of Alternaria spp. :

A systematic study was undertaken to isolate native isolates of *Alternaria* spp. in tomato from different locations *viz.,* Hessarghatta, Devanahalli, Doddaballapur, Hoskote, Ramanagar (Bangalore rural district), Chikkaballapura (Kolar district) and Hosur (Tamil Nadu) during 2007-09 at the Project Directorate of Biological Control (PDBC), Bangalore, India.

Isolation of Alternaria spp. *from tomato* :

Several isolates of the fungus causes early blight of tomato were isolated from leaves by following standard tissue isolation technique on Potato dextrose agar (PDA) medium. The infected leaves collected from tomato showing typical symptoms of *Alternaria* were washed in running tap water and cut into small pieces measuring about 2mm and surface sterilized with sodium hypochlorite (5%) solution for two minutes followed by dip in sterilized water. Such bits were transferred to Petridishes containing 15 ml potato dextrose agar {potato (200 g), dextrose (20 g), agar (20 g) and distilled water (1000ml)} and incubated at 24 $\pm 1^0$C for seven days. Pure cultures of *Alternaria* spp. were obtained by hyphal tip isolation method.

Identification of Alternaria spp. :

Seven individual cultures grown on PDA were identified based on the diversity in growth and cultural characters such as colony colour, margins and topography morphological, mycelial character and spore production of *Alternaria solani* isolates and was purified by single spore isolation according to Sorauer, 1896 by Common Wealth Mycological Institute, Kew, Suney, England (Ellis, 1997). All these isolates were assigned with code numbers (Table A).

Pathogenicity testing of Alternaria solani isolates :

The pathogenicity of the isolated strain was tested by following standard method according to Amaresh (1997). Tomato seeds of local variety US-618 were surface sterilized with sodium hypochlorite solution (1%) for 3 minutes and sown in earthen pots containing sterilized soil. They were allowed to grow for a month. Prior to inoculation, the plants were predisposed to 95 per cent humidity for 24 hr. Thereafter, they were inoculated with a spore suspension (10^4 spores/ml) of the HES AL-1 isolate of *Alternaria solani*, using an atomizer. After inoculation, the plants were exposed to the same conditions for 24 hr. Suitable control plants were maintained by spraying sterile distilled water. Symptoms appeared after five days. The organism was reisolated from these artificially infected leaves and the culture obtained was compared with the original culture for confirmation.

Maintenance of culture of Alternaria solani isolates :

The seven isolates of *Alternaria solani* isolated from tomato were maintained at 4 ^{0}C in refrigerator and subcultured periodically at an interval of 30 days during the course of the study.

Collection of bacterial antagonists :

Isolation of bacterial antagonists from phylloplane :

The leave samples of healthy tomato crop were collected from different locations for isolation of phyllosphere bacteria. Ten grams of the leaf sample was transferred to 90 ml sterile water blank in a 250 ml flask and shaken for twenty minutes at 250 rpm in a rotary shaker to dislodge microorganisms adhering to the leaf surface. The phyllosphere washings were plated on Nutrient Agar (Beef extract 3g; Bacto-peptone 5g; Glucose 5g; NaCl 5g; Agar 15g; Tap water 1000ml; pH 7.0-7.2) to isolate bacterial antagonists. The plates were incubated at 30° C for 48 hr in an incubator. Bacterial colonies that emerged from

Table A : List of isolates of *Alternaria solani* isolated from seven different places

Sr. No.	Districts	Places	Isolate code
1.0	Bangalore Rural	Hessarghatta	HES AL-1
2.	Bangalore Rural	Devanahalli	DHL AL-2
3.	Bangalore Rural	Doddaballapura	DBR AL-3
4.	Bangalore Rural	Hoskote	HSK AL-4
5.	Hosur (Tamil Nadu)	Hosur	HSR AL-5
6.	Bangalore Rural	Ramanagar	RNR AL-6
7.	Kolar	Chikkaballapura	KLR AL-7

these plates were subcultured on Nutrient Agar plates and maintained as pure cultures. All these isolates were assigned with code numbers based on their place of collection.

Isolation of bacterial antagonists from rhizosphere :

Rhizosphere soil of tomato plants were collected from Hosur district (Tamil Nadu) at 15cm depth. Ten milligram of rhizhoshere soil was transferred to 90 ml sterile water blank in a 250 ml flask and shaken for twenty minutes at 250 rpm in a rotary shaker to dislodge microorganisms adhering to the rhizosphere root surface. One ml of rhizosphere soil suspension was spread uniformly on Nutrient agar plates and incubated at 30 °C for 48 h in an incubator. Bacterial colonies that emerged from these plates were purified and subcultured on Nutrient agar slants and maintained as pure cultures. The isolates obtained were assigned with code number based on their place of collection.

Characterization and identification of the bacterial antagonists :

Twelve isolates of bacteria isolated from phylloplane and rhizosphere of tomato plants collected from different locations were characterized and identified upto generic level based on morphological tests and biochemical tests.

Morphological characters :

Bacterial isolates were examined for colony morphology, fluorescence under UV, Gram reaction and cell shape as per the standard procedures given by Anonymous (1957) and Barthalomew and Mittewer (1950).

Biochemical tests :

The gelatin liquefaction ability of bacterial isolates was examined by procedure of Blazevic and Ederer (1975). The hydrolysis of starch was determined based on the ability of the isolates to hydrolyse starch (Eckford, 1927). In casein hydrolysis, the triplicate plates of skim milk agar with test cultures were incubated at 30°C for two days and then observed for clear zones around the colony against a black background as given by Seeley and Vandemark (1970). The acid and gas production of the bacterial isolates were also tested according to Seeley and Vandemark (1970).

In vitro screening of Pseudomonas antagonists against different isolates of Alternaria solani by dual culture method:

In vitro screening of P*seudomonas* spp. antagonists against seven isolates of *Alternaria solani* by following dual culture method was carried out on Potato dextrose agar medium, for identifying potential antagonists against *Alternaria solani*. Fifteen ml of PDA medium was poured into sterile Petriplates. An agar disc of (Six mm) seven days old culture of *Alternaria solani* was placed at one side of the Petriplate at a distance of 2 cm from the periphery. Simultaneously, test bacterial isolate was streaked in a 4 cm line opposite to the disc of *Alternaria solani* at a distance 4 cm. A control was maintained where only *Alternaria solani* was grown. The inoculated dual and monoculture plates were incubated for eight days at 27±1°C. After the period of incubation, the colony diameter of *Alternaria solani* was recorded both in the control plate and the dual culture plate. The radial growths of *Alternaria solani* were recorded in dual culture and monoculture plates after 8 days of incubation. The measurements were used to calculate the percentage of inhibition of *Alternaria solani*.

Five *Pseudomonas* isolates *viz.,* S1B1, S1B8, S2B10, S3B3 and S4B7 which showed maximum inhibitory effect on seven isolates of *Alternaria solani* in the dual culture test were selected as promising *Pseudomonas* antagonists for further evaluation in the pot culture.

Green house evaluation of the promising isolates of Pseudomonas spp. *against Alternaria solani infection in tomato* :

Pot culture experiments were conducted in a glass house at the Project Directorate of Biological Control (PDBC), Bangalore to study the effect of five promising isolates of *Pseudomonas* on infection of *Alternaria solani* in tomato seedlings.

Raising of tomato seedlings :

Tomato seedlings (Var. US-618) of one month old were raised in the clay pots (9 inch diameter) containing potting mixture of red loamy soil: FYM: river sand (2:1:1).

Preparation of Alternaria solani inoculums :

Fast growing and highly sporulating isolate of *Alternaria solani* (HESAL-1) was grown on PDA for 15 days in Petriplates. The mycelial cum spore suspension was prepared by flooding the plates with sterile water and thorough scraping of the colony. The spore concentration in the suspension was estimated by using Haemocytometer and adjusted to the spore load of 10^4 spores/ml and used for inoculation in the pot culture studies.

Preparation of inoculum of Pseudomonas spp. :

The five promising *Pseudomonas* isolates of bacteria *viz.,* S1B1, S1B8, S2B10, S3B3 and S4B7 were selected for pot culture studies based on their *in vitro* antagonistic effect on *Alternaria solani*. These isolates were grown in Nutrient broth in a laboratory shaker at 150 rpm for two days. After two days the culture broth was centrifuged at 7000 rpm and the bacterial pellets obtained after centrifugation was suspended in 100 ml of phosphate buffer. The bacterial load was adjusted to $1x10^6$/ml and Triton-X100 was added at 0.01per cent to the bacterial suspension and used as inoculum in the pot culture studies.

Inoculation of Alternaria solani and *Pseudomonas antagonists in tomato seedlings* :

The spore suspensions of *Alternaria solani* and *Pseudomonas* antagonists were sprayed with atomizer on both sides of the leaf surface. The control plants were sprayed with sterile water containing 0.01% Triton-X100. Three methods of inoculation of pathogen and antagonists were employed in this experiment (Table B).

In method with pre inoculation of *Alternaria solani* and followed by inoculation with *Pseudomonas* antagonists after 24 hrs. The suspension of *Alternaria solani* (5ml of spore suspension containing with the spore dose of 1^{04} spores/ml) was sprayed on each tomato plant 24 hrs prior to spraying of selected *Pseudomonas* antagonistic isolates (5ml of spores suspension containing with the spore dose of $1x1^{06}$ cells/ml). In pre inoculation of *Pseudomonas* antagonists and followed by the inoculation with *Alternaria solani* after 24 hrs. The selected *Pseudomonas* bacterial suspension was sprayed on each tomato plant 24 hrs prior to spraying of *Alternaria solani***.** In simultaneous inoculation of *Alternaria solani* and *Pseudomonas* antagonists, both *Alternaria solani* and selected *Pseudomonas* antagonist were sprayed simultaneously on tomato leaves.

The effect of antagonists on disease development/ intensity was monitored at 15 DAS and 30 DAS after inoculation treatment are imposed. The disease intensity in

Table B : Treatment details in pot culture studies with *Pseudomonas* antagonists

Pre inoculation of *Alternaria solani* and followed by inoculation with *Pseudomonas* antagonists after 24 hrs.	
T_1	Foliar spray of *Alternaria solani* (10^4 spores/ml) and followed by foliar spray of isolate S1B1 (1x 10^6 cells/ml) after 24 hrs.
T_2	Foliar spray of *Alternaria solani* (10^4 spores/ml) and followed by foliar spray of isolate S1B8 (1x 10^6 cells/ml) after 24 hrs.
T_3	Foliar spray of *Alternaria solani* (10^4 spores/ml) and followed by foliar spray of isolate S2B10 (1x 10^6 cells/ml) after 24 hrs.
T_4	Foliar spray of *Alternaria solani* (10^4 spores/ml) and followed by foliar spray of isolate S3B3 (1x 10^6 cells/ml) after 24 hrs.
T_5	Foliar spray of *Alternaria solani* (10^4 spores/ml) and followed by foliar spray of isolate S4B7 (1x 10^6 cells/ml) after 24 hrs.
T_6	Uninoculated control (no pathogen, no antagonist)
T_7	Inoculated control (only pathogen, no antagonist)
Pre inoculation of *Pseudomonas* antagonists and followed by the inoculation with *Alternaria solani* after 24 hrs.	
T_1	Foliar spray of isolate S1B1 (1x 10^6 cells/ml) followed by foliar spray of *Alternaria solani* (10^4 spores/ml) after 24 hrs.
T_2	Foliar spray of isolate S1B8 (1x 10^6 cells/ml) followed by foliar spray of *Alternaria solani* (10^4 spores/ml) after 24 hrs.
T_3	Foliar spray of isolate S2B10 (1x 10^6 cells/ml) followed by foliar spray of *Alternaria solani* (10^4 spores/ml) after 24 hrs.
T_4	Foliar spray of isolate S3B3 (1x 10^6 cells/ml) followed by foliar spray of *Alternaria solani* (10^4 spores/ml) after 24 hrs.
T_5	Foliar spray of isolate S4B7 (1x 10^6 cells/ml) followed by foliar spray of *Alternaria solani* (10^4 spores/ml) after 24 hrs
T_6	Uninoculated control (no pathogen, no antagonist)
T_7	Inoculated control (only pathogen, no antagonist)
Simultaneous inoculation of *Alternaria solani* and *Pseudomonas* antagonists	
T_1	Foliar spray of isolate S1B1 (1x 10^6 cells/ml) and *Alternaria solani* (10^4 spores/ml) simultaneously
T_2	Foliar spray of isolate S1B8 (1x 10^6 cells/ml) and *Alternaria solani* (10^4 spores/ml) simultaneously
T_3	Foliar spray of isolate S2B10 (1x 10^6 cells/ml) and *Alternaria solani* (10^4 spores/ml) simultaneously
T_4	Foliar spray of isolate S3B3 (1x 10^6 cells/ml) and *Alternaria solani* (10^4 spores/ml) simultaneously
T_5	Foliar spray of isolate S4B7 (1x 10^6 cells/ml) and *Alternaria solani* (10^4 spores/ml) simultaneously
T_6	Uninoculated control (no pathogen, no antagonist)
T_7	Inoculated control (only pathogen, no antagonist)

Table C: Grade scale (0 to 9) for measuring the disease intensity (Mayee and Datar, 1986)

Rating	Description
0.	No symptoms on the leaf
1.	Small circular, scattered, brown spots covering1 per cent or less of the leaf area
3.	Spots enlarging, dark brown in colour covering 1 to 10 per cent of leaf area and infection on the lower most leaves of the plant
5.	Spots enlarging, dark brown in colour covering 11 to 25 per cent of leaf area and infection covering half of the plant
7.	Spots dark brown coalescing, occupying 26 to 50 per cent of leaf area and covering one third of the plant
9.	Spots uniformly dark brown, coalescing, covering 50 per cent or more leaf area and severe infection on all leaves

each leaf was recorded based on the diseased area using grade Table C.

The Per cent disease index (PDI) was calculated by using the formula given by Wheeler (1969).

$$PDI = \frac{S}{N} x \frac{100}{M}$$

where,

– S= Sum of numerical disease ratings
– N= No. of plants observed
– M= Maximum disease rating

The treatments details of the experiment is given in Table B, with each treatment was replicated thrice.The data were subjected to completely randomized block design analysis and interpretation of the data carried out in accordance with Panse and Sukhatme (1985). The level of significance used in the F and t test was P=0.01.

RESULTS AND DISCUSSION

The results obtained from the present investigation as well as relevant discussion have been summarized under following heads :

Cultural characteristics of seven isolates of *Alternaria solani*:

The microscopic studies revealed considerable variations among the cultural characteristics of seven isolates of *Alternaria solani* on PDA (Table 1). Two isolates HES AL-1 and DBR AL-3 showed dark brown colony mycelia colour; isolate DHL AL-2 had light brown, isolates HSK AL-4, HSR AL-5 and KLR AL-7 appeared grayish white mycelia colour while isolate RNR AL-6 recorded only gray mycelia colour. Pigmentation of the substrate was almost similar to the colour of the mycelium. Regarding topography of colony, two isolates namely HES AL-1 and HSR AL-5 had raised fluffy growth in a Petri plate, whereas three isolates, DBR AL-3, HSK AL-4 and RNR AL-6 gave medium fluffy growth and two isolates, DHL AL-2 and KLR AL-7 showed flat mycelial growth. Similarly, four isolates *viz.*, DHL AL-2, HSK AL-4, HSR AL-5 and KLR AL-7 had smooth margin colony whereas the remaining three isolates, HES AL-1, DBR AL-3 and RNR AL-6 showed irregular margin colony.

Significant differences in growth and sporulation were also observed among the isolates collected from different locations (Table 3). Isolate HES AL-1 recorded maximum radial growth (79.00 mm), closely followed by DBL AL-2 (78.00 mm) and KLR AL-6 (77.00). While the minimum was observed in isolate RNR AL-6 (60.16 mm). These diversity in cultural characters such as colony colour, its margins and topography noticed among the isolates of the *Alternaria solani* were in accordance with Yunhui *et al.* (1994), Perez and Martinez (1995) and Babu *et al.* (2000).

Among the seven isolates, isolate HES AL-1 recorded the maximum growth on PDA coupled with distinct colony characteristics. Similar guidelines were used to differentiate

Table 1 : Colony characters of seven isolates of *Alternaria solani* on potato dextrose agar

Isolates	Colour of mycelia	Substrate color	Margin of colony	Topography of colony
HES AL-1	Dark brown	Light brown	Irregular	Raised fluffy growth
DHL AL-2	Light brown	Grayish white	Smooth	Flat mycelial growth
DBR AL-3	Dark brown	Brown	Irregular	Medium flat growth
HSK AL-4	Grayish white	Light grayish	Smooth	Medium raised growth
HSR AL-5	Grayish white	Light grayish	Smooth	Raised fluffy growth
RNR AL-6	Gray	Light brown	Irregular	Medium fluffy growth
KLR AL-7	Grayish white	Light grayish	Smooth	Flat mycelial growth

Table 2 : Growth and sporulation of seven isolates of *Alternaria solani* on potato dextrose agar

Sr. No.	Isolates	Colony diameter (mm)	Sporulation
1.	HES AL-1	79.00	MS
2.	DHL AL-2	78.00	MS
3.	DBR AL-3	67.50	SS
4.	HSK AL-4	66.66	SS
5.	HSR AL-5	64.16	SS
6.	RNR AL-6	77.00	SS
7.	KLR AL-7	60.16	MS
	S.E.±	(2.22)	
	C.D. at 1%	(9.35)	

MS – Moderately sporulated; SS – Sparsly sporulated

Table 3 : Morphological and bio chemical characterization of the potential antagonists

Name of the isolate	Morphological tests		Biochemical tests						Probable genus
	Colony morphology	Fluorescence under UV	Gram reaction and cell shape	Gelatin liquification	Starch hydrolysis	Casein hydrolysis	Acid production	Gas production	
S1B1	Creamy, slimy	-	-ve rods	-	+	+	+	-	*Pseudomonas*
S1B8	Whitish creamy, smooth circular	-	-ve rods	-	+	+	-	+	*Pseudomonas*
S2B2	Creamy, slimy, smooth	-	-ve rods	-	+	+	-	-	*Pseudomonas*
S2B10	Whitish creamy, smooth	-	-ve rods	-	+	+	-	-	*Pseudomonas*
S3B3	creamy, slimy circular	+	-ve rods	-	+	+	+	-	*Pseudomonas*
S4B7	Creamy, slimy, smooth	-	-ve rods	-	+	-	+	-	Fluorescent *Pseudomonas*
S5B5	Creamy, slimy	-	-ve rods	-	+	-	+	+	*Pseudomonas*
S6B6	Whitish cream, slimy	-	-ve rods	-	+	-	+	+	*Pseudomonas*
S7B7	Creamy, slimy, circular	+	-ve rods	-	+	-	+	+	*Pseudomonas*
S8B8	Creamy. slimy	-	-ve rods	-	+	-	+	+	Fluorescent
S9B9	Creamy, slimy, smooth	-	-ve rods	-	+	-	+	-	*Pseudomonas*
S10B10	Creamy, slimy	-	-ve rods	-	+	-	+	+	*Pseudomonas*

All tests were concucted in four replicates and were repeated thrice; '+' indicates positive reaction and '-' indicates negative reaction

Alternaria sp. isolates by different workers (Rotem, 1965; Mazzonnetto *et al.*, 1966) on potato dextrose agar. Isolate HES AL-1 was, therefore, selected as reference isolate for further studies.

Isolation and characterization of bacterial antagonists isolated from phylloplane and rhizosphere :

The results of the present study revealed that the twelve isolates of bacteria antagonists showed a wide variation in morphological and biochemical characters (Table 3). Three isolates namely S1B1, S5B5 and S10B10 showed creamy and slimy colony morphology in a Petri plate. Whereas two isolates S3B3 and S7B7 were creamy, slimy and circular shape; S2B2, S4B7 and S9B9 isolates showed creamy, slimy and smooth shape while, S1B8, S2B10, S6B6 isolates were whitish creamy, smooth and circular growth in a Petri plate. Among, the twelve isolates, two isolates, S3B3 and S7B7 were found to be fluorescent *Pseudomonas* while, the remaining ten isolates were found to be non-fluorescent *Pseudomonas.* It was interesting to observe that all the twelve isolates of bacterial antagonists showed Gram negative reaction and rod shape cell.

Similarly, a wide variation was noticed among the twelve isolates of bacterial antagonists regarding biochemical characters. The ten isolates which gave positive result in gelatin liquefaction were S1B1, S1B8, S2B2, S2B10, S3B3, S4B7, S5B5, S8B8, S9B9 and S10B10. While only two isolates *viz.*, S6B6 and S7B7 which did not shown positive result in gelatin liquefaction. All the twelve isolates showed a clear zone around the colony in starch agar plates. On casein hydrolysis, five isolates (S1B1, S1B8, S2B2, S2B10 and S3B3) exhibited clear zones around the colony against black background in skim milk agar plates. Whereas the other seven isolates (S4B7, S5B5, S6B6, S7B7, S8B8, S9B9 and S10B10) produced no clear zones around the colony. Regarding gas production, nine isolates (S1B1, S3B3, S4B7, S5B5, S6B6, S7B7, S8B8, S9B9 and S10B10) showed positive for gas production while the remaining three isolates (S1B8, S2B2, S2B10) showed no gas production. Similarly, six isolates *viz.*, S1B8, S2B2, S6B6, S7B7, S8B8 and S10B10 which changed the colour of medium to yellow were taken as positive for acid production whereas, the remaining six isolates (S1B1 S2B10, S3B3, S4B7, S5B5, S9B9) showed no yellow colour. Among them, ten isolates belonged to non-fluorescent *Pseudomonas* and the other two were of *P. fluorescens*. Thus, the large population of antagonists seems to be made of *Pseudomonas.* The results are in agreement with Hayward (1960) and Palieroni (1984), who characterized *Pseudomonas* spp. based on morphological and biochemical, tests. Therefore, based on these studies the isolates were identified as *Pseudomonas* spp. and assigned with code numbers as given in (Table 4).

***In vitro* evaluation of isolates bacterial antagonist against *Alternaria solani* :**

The results revealed a significantl reduction in radial

growth of *Alternaria solani* isolates due to *Pseudomonas* isolates over control (Table 5). The mean minimum radial growth in all the *Alternariasolani* isolates was observed with S4B7P (54.97 mm), followed by S1B8P(55.19 mm), S2B10P (56.59 mm), S3B3PF (58.33 mm) and S1B1P (61.02 mm) while the maximum radial growth was obtained in control. This inferred that the radial growth of *Alternaria solani* can be significantly reduced by inoculated with *Pseudomonas* species. The present finding is also inlined with the report of Adebola and Amadi (2010).

The antagonistic isolates also inhibited the growth of all the seven isolates of *Alternaria solani* significantly (Table 6). The maximum inhibition percentage on isolates HES AL-1, DHL AL-2, DBL AL-3, HSR AL-4, HSK AL-5, KLR AL-6 and RAM AL-7 of *Alternaria solani* was observed by S4B7P isolate (26.60), S1B8P isolate (32.49), S4B7P isolate (41.84), S1B8P isolate (22.60), S1B8P and S4B7P isolate (22.60), S1B8P isolate (34.03), S2B10P and S1B8P (24.09) and S2B10P isolate (21.75), respectively. Whereas, the minimum inhibition percentage on HES AL-1 (3.36), DHL AL-2 (13.44), DBL AL-3 (4.39), HSR AL-4 (10.31), HSK AL-5 (11.89), KLR AL-6 (9.91) and RAM AL-7 (9.54) isolates of *Alternaria solani* was recorded by batereial antagonist isolates S2B2P, S6B6P, S4B7P, S1B1P, S7B7PF, S9B9P and S10S10P and S6B6 recorded, respectively.

Among the twelve isolates of *Pseudomonas* spp. tested against *Alternaria solani*, the isolates S4B7P (27.74%), S1B8P (27.33%), S2B10P (25.47%), S3B3PF (23.07%) and S1B1P (19.69%) showed the maximum mean inhibitory effect on all

Table 4: List of phylloplane and rhizosphere *Peudomonas* isolates obtained from tomato plants from different locations

Sr. No.	Location	Districts	Part	Code no.
1.	Hessarghatta	Bangalore Rural	Phyllophan	*Pseudomonas* S1B1P
2.	Hessarghatta	Bangalore Rural	Phyllophan	*Pseudomonas* S1B8P
3.	Devanahalli	Bangalore Rural	Phyllophan	*Pseudomonas* S2B2P
4.	Devanahalli	Bangalore Rural	Phyllophan	*Pseudomonas* S2B10P
5.	Doddaballapura	Bangalore Rural	Phyllophan	*Pseudomonas* S3B3PF
6.	Hosur	Tamilnadu	Rhizosphere	*Pseudomonas* S4B7P
7.	Hosur	Tamilnadu	Phyllophan	*Pseudomonas* S5B5P
8.	Ramanagar	Bangalore Rural	Phyllophan	*Pseudomonas* S6B6P
9.	Chikkaballapura	Kolar	Phyllophan	*Pseudomonas* S7B7PF
10.	Hoskote	Bangalore Rural	Phyllophan	*Pseudomonas* S8B8P
11.	Doddaballpura	Bangalore Rural	Phyllophan	*Pseudomonas* S9B9P
12.	Rajanakunte	Bangalore Rural	Phyllophan	*Pseudomonas* S10B10P

Table 5: Effect of bacterial antagonists on radial growth (mm) of *Alternaria solani* isolates in dual culture at 8th day

Sr. No.	Bacterial isolates	HES AL-1	DHL AL-2	DBL AL-3	HSR AL-4	HSK AL-5	KLR AL-6	RAM AL-7	Mean
1.	S1B1P	55.83	58.66	44.83	60.83	70.33	65.83	70.83	61.02
2.	S1B8 P	51.66	50.00	45.00	52.50	57.16	62.50	67.50	55.19
3.	S2B2 P	62.33	64.83	59.16	57.16	62.50	70.00	68.33	63.47
4.	S2B10P	53.33	55.83	42.83	54.16	63.33	62.50	64.16	56.59
5.	S3B3 PF	56.66	53.83	47.16	55.16	57.16	64.16	74.16	58.33
6.	S4B7 P	47.33	54.33	44.00	52.50	57.50	63.33	65.83	54.97
7.	S5B5 P	60.33	64.16	57.16	55.33	70.83	69.16	71.66	64.09
8.	S6B6 P	54.66	64.16	66.33	56.66	65.50	68.33	74.16	64.26
9.	S7B7 PF	60.20	63.33	72.33	57.50	76.33	70.83	74.16	67.81
10.	S8B8 P	61.50	57.66	65.33	58.33	65.66	70.00	71.66	64.31
11.	S9B9 P	61.16	57.50	66.00	55.00	72.66	74.16	73.33	65.69
12.	S10B10P	59.16	62.00	66.33	54.16	58.50	74.16	70.00	63.47
13.	Control	64.50*	74.16*	75.66*	67.83*	86.66*	82.33*	82.00*	76.16*
	S.E.±	0.83	1.07	0.48	1.26	1.68	1.99	1.80	
	C.D. at 1%	3.21	4.12	1.85	4.85	6.47	7.66	6.92	

P=indicates *Pseudomonas;* PF=*P. Fluorescens;* BS=*B. Subtilis* , * Indicates radial growth of *Alternaria solani* in monoculture plates

Table 6: Effect of bacterial antagonists on per cent inhibition of *Alternaria solani* isolates in dual culture at 8th day

Sl. No.	Bacterial isolates	Isolates of *Alternaria solani*							Mean
		HES AL-1	DHL AL-2	DDL AL-3	HSR AL-4	HSK AL-5	KLR AL-6	RAM AL-7	
1.	S1B1P	13.43 (21.44)	20.88 (27.17)	40.74 (39.66)	10.31 (18.63)	18.83 (25.71)	20.04 (26.50)	13.63 (21.58)	19.69 (25.81)
2.	S1B8P	19.90 (26.48)	32.49 (34.64)	40.52 (39.53)	22.60 (28.38)	34.03 (35.69)	24.09 (29.37)	17.68 (24.86)	27.33 (31.27)
3.	S2B2P	3.36 (10.54)	12.56 (20.74)	21.79 (27.80)	15.71 (23.33)	27.84 (31.77)	14.95 (22.69)	16.67 (24.08)	16.13 (22.99)
4.	S2B10P	17.31 (24.57)	24.71 (29.30)	43.38 (41.19)	20.13 (26.62)	26.91 (31.24)	24.09 (29.37)	21.75 (27.79)	25.47 (30.01)
5.	S3B3PF	12.14 (20.36)	27.35 (31.45)	37.66 (37.85)	18.68 (25.56)	34.03 (35.69)	22.06 (28.00)	9.57 (17.76)	23.07 (28.09)
6.	S4B7P	26.60 (31.04)	26.73 (31.13)	41.84 (40.30)	22.60 (28.38)	33.64 (35.45)	23.08 (28.64)	19.70 (26.22)	27.74 (31.59)
7.	S5B5P	6.44 (14.16)	13.44 (21.44)	24.45 (29.63)	18.42 (25.40)	18.23 (25.22)	15.98 (23.45)	12.59 (20.73)	15.65 (22.86)
8.	S6B6P	15.25 (22.94)	13.44 (21.42)	12.33 (20.55)	16.44 (23.87)	24.41 (29.60)	17.00 (24.33)	9.54 (17.69)	15.49 (22.91)
9.	S7B7PF	6.65 (14.52)	14.59 (22.43)	4.39 (12.01)	15.21 (22.54)	11.89 (20.08)	13.96 (21.83)	9.56 (17.09)	10.89 (18.64)
10.	S8B8P	4.64 (12.20)	22.23 (28.12)	15.31 (22.98)	13.97 (21.76)	24.22 (29.47)	14.97 (22.56)	12.58 (20.54)	15.42 (22.51)
11.	S9B9P	5.17 (13.08)	22.45 (28.27)	12.77 (20.93)	16.44 (23.87)	16.22 (23.72)	9.91 (18.30)	10.57 (18.66)	13.36 (22.97)
12.	S10B10P	8.27 (16.66)	16.37 (23.83)	12.72 (20.88)	20.17 (26.57)	32.55 (34.72)	9.91 (18.04)	14.65 (22.29)	16.38 (23.28)
	Mean	12.06 (17.83)	19.11 (22.98)	27.87 (23.29)	15.25 (20.60)	22.70 (25.17)	16.22 (21.47)	15.35 (20.82)	18.37 (21.74)
	S.E.±	1.34 (1.34)	1.91 (1.43)	0.79 (0.66)	1.82 (1.52)	2.17 (1.75)	2.63 (2.17)	2.22 (1.97)	1.84 (1.55)
	C.D. at 1%	5.20 (5.19)	7.39 (5.54)	3.06 (2.58)	7.02 (5.88)	8.41 (6.78)	10.16 (8.41)	8.59 (7.62)	7.12 (6.00)

The figures in parentheses are angular (Arc sin) transformed values

Table 7 : Effect of promising *Psendomonas* antagonists on percent disease index (PDI) of *Alternaria solani* in tomato under green house conditions

Sl. No.	Bacterial isolates	Pre inoculation *A. solani* followed antagonist		Mean	Pre inoculation of antagonists followed by *A.solani*		Mean	Simultaneous inoculation of *A. solani* and antagonists		Mean
		15 DAI	30 DAI		15 DAI	30 DAI		15 DAI	30 DAI	
1.	S1B1P	16.97 (8.89)^{b}	33.25 (30.36)^{a}	25.11 (19.62)	16.98 (8.90)^{b}	22.01 (14.07)^{b}	19.49 (11.48)	18.70 (10.37)^{b}	23.14 (15.46)^{c}	20.92 (12.91)
2.	S1B8P	10.96 (3.70)^{c}	28.60 (22.96)^{b}	19.78 (13.33)	10.95 (3.69)^{c}	17.90 (9.62)^{c}	14.42 (6.65)	16.56 (.15)^{b}	27.16 (20.85)^{b}	21.86 (14.50)
3.	S2B10P	19.41 (11.11)^{b}	33.38 (30.36)^{a}	26.39 (20.73)	19.41 (11.11)^{b}	23.19 (15.55)^{b}	21.30 (13.33)	14.08 (5.96)^{c}	25.65 (18.75)^{b}	19.86 (12.35)
4.	S3B3PF	18.61 (10.37)^{b}	36.60 (35.55)^{a}	27.60 (22.96)	14.03 (5.92)^{b}	19.30 (11.11)^{c}	16.66 (8.51)	14.08 (5.96)^{c}	18.45 (10.07)^{d}	16.26 (8.01)
5.	S4B7P	12.72 (5.20)^{b}	27.58 (21.48)^{b}	20.15 (13.34)	12.71 (5.20)^{b}	26.47 (19.99)^{b}	19.59 (12.59)	18.05 (9.63)^{b}	27.14 (20.82)^{b}	22.59 (15.22)
6.	P.I.C	26.54 (19.99)^{a}	38.78 (39.25)^{a}	32.66 (29.62)	26.54 (19.99)^{a}	38.78 (39.25)^{a}	32.66 (29.62)	26.54 (19.99)^{a}	38.78 (39.25)^{a}	32.66 (29.62)
7.	U.I.C	0.00^{d}	0.00^{c}	0.00	0.00^{d}	0.00d	0.00	0.00^{d}	0.00^{e}	0.00
	Mean	17.53 (9.87)	33.03 (29.99)	25.28 (19.93)	16.77 (9.13)	24.60 (18.26)	20.68 (13.70)	18.00 (10.01)	26.72 (20.86)	22.36 (15.43)
	S.E.±	1.868 (1.736)	1.710 (2.652)	1.789 (2.194)	1.749 (1.575)	1.469 (1.814)	1.609 (1.694)	0.955 (0.998)	0.704 (0.960)	0.829 (0.979)
	C.D.at1%	8.073 (7.502)	7.389 (11.46)	7.731 (9.481)	7.560 (6.808)	6.350 (7.837)	6.955 (7.322)	4.129 (4.315)	3.044 (4.148)	3.586 (4.231)

The figures in parentheses are angular (Arc sin) transformed values, Mean values in each column with same superscript's do not differ significantly by DMRT (P=0.05)

the seven isolate of *Alternaria solani*. The present finding are in agreement with Niwas and Sharma, 1988; Ahmed and Saleh (1990); El-Abyad *et al*., (1993); Prasad and Kulshrestha (2002) who reported that the phylloplane bacteria exhibited the highest inhibition on mycelial growth of *Alternaria solani* and were found to be antagonistic to the sunflower foliar pathogen *Alternaria helianthi*. Babu *et al*. (2000) also reported the inhibition of *Alternaria solani* by 6 bacterial strains of *P. fluorescens* inhibited. Therefore, based on *in-vitro* evaluation, five antagonistic bacteria *viz.*, S4B7P, S1B8P, S2B10P, S3B3PF and S1B1P were identified as the most potential antagonists for further testing their efficacy under pot culture studies against *Alternaria solani*.

***In vivo* evaluation of *Pseudomonas* antagonists against *Alternaria solani* in pot culture under green house :**

The result indicated that the five promising *Pseudomonas* antagonists showed a significant decreased on per cent disease index at both 15 DAI and 30 DAI in all the three methods of inoculation (Table 7). After 24 hrs of pre-inoculation of *Alternaria solani* followed by *Pseudimonas* antagonists, among the five isolates of *Pseudimonas* tested, the pathogen inoculated control (32.66) showed the maximum mean per cent disease index (PDI). While, S1B8P isolate expressed the minimum mean PDI (19.78). In pre inoculation of *Pseudimonas* antagonists followed by *Alternaria solani* after 24 hrs, the pathogen inoculated control (32.66) recorded the maximum mean PDI. However, S1B8P isolate (14.42) showed the least mean PDI. In simultaneous inoculation of *Alternaria solani* and *Pseudimonas* antagonists, the maximum mean PDI was noticed in the pathogen inoculated control (32.66). While the minimum mean PDI was observed with S3B3PF isolate (16.26).

Among the three methods of inoculation tested, the pre inoculation of *Pseudomonas* antagonists followed by *Alternaria solani* showed the minimum PDI (20.68). Whereas, the maximum PDI (25.28) index was recorded in pre inoculation of *Alternaria solani* and followed by *Pseudomonas* antagonists. This indicated that inoculated with *Pseudomonas* antagonists prior to disease occurrence may further help in reduced disease incidence in tomato. The results obtained in the present investigation are in conformity with those of Babu *et al.* (2000); Romeiro *et al.* (2000) who obtained the inhibition of early blight (*Alternaria solani*) of tomato disease by 44-60 per cent in glasshouse by spraying of phylloplane bacterial antagonists.

Conclusion :

The present investigations revealed that there was a variation in growth and colony characters of seven isolates of *Alternaria solani* collected from different location. However, HEL AL-1 showed maximum growth and unique colony characters. The growth of seven *Alternariasolani* isolates were significantly reduced by the twelve antagonists *Pseudomonas* spp. under *in vitro* conditions, out of which five were identified as potential antagonists *viz.*, S4B7P, S1B8P, S2B10P, S3B3PF and S1B1P for further studying their antagonist behavioures in pot culture under green house. Further, under greenhouse, S1B8P and S3B3PF isoalates recorded the minimum PDI in tomato. While, among the inoculation methods pre-inoculation of antagonist followed by pathogen recorded the least PDI. Hence, our finding suggested that foliar spray of S1B8P and S3B3PF isolates can be effectively used for reducing the disease incidence of *Alternaria solani* and treatment of plant prior to disease occurrence is beneficial. Therefore, these two isolates of *Pseudomonas* spp. (S1B8P and S3B3PF) can be utilized as biocontrol agent against *Alternariasolani* in tomato through foliar application. Future assesment are needed to understand the mode of action and the complex process of biological control of *Alternaria solani*. More knowledge on the ecological behaviour of *Alternari asolani* and its antagonists is required to develop sound procedures for its control and eradication in infested fields.

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