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Efficacy of talc-based formulation of *Pseudomonas fluorescens* on the management of leaf spot disease of *Stevia rebaudiana* Bertoni

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Abstract:
Stevia rebaudiana Bertoni, a natural sweetener contains two main sweetest compounds, stevioside (ST) and rebaudioside A (R-A), tasting about 300 and 450 times sweeter than sucrose, respectively. This commercially important plant also suffers a leaf spot disease caused by the fungus *Alternaria alternata* in various districts of Tamil Nadu, India. In the present study, ten isolates of fluorescent pseudomonads were evaluated for their ability to control leaf spot in stevia (*Stevia rebaudiana* Bertoni). These isolates were characterized as *Pseudomonas fluorescens* based on biochemical tests. Among these isolates, *P. fluorescens* isolate AUPF6 and AUPF5 showed the maximum inhibition of mycelial growth of *Alternaria alternata*. They also increased plant growth in stevia apart from reducing the leaf spot incidence under greenhouse condition. The isolates AUPF6 and AUPF5 were further tested for their ability to induce production of defense related enzymes and chemicals in plants. Earlier and increased activities of phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) were observed in *P. fluorescens* AUPF6 and AUPF5 pretreated stevia plants challenged with *Alternaria alternata*. Moreover, higher accumulation of phenolics was noticed in plants pretreated with *P. fluorescens* isolates AUPF6 and AUPF5 challenged with *Alternaria alternata* . Thus, the present study shows that in addition to direct antagonism and plant growth-promotion, induction of defense-related enzymes involved in the phenyl propanoid pathway collectively contributed to enhance resistance against the invasion of *Alternaria alternata* in stevia.

Keywords: *Stevia rebaudiana Bertoni, Alternaria alternata, leaf spot, Pseudomonas fluorescens, defense related enzymes.*

Introduction:

Stevia rebaudiana is a natural perennial herb, commonly referred to as honey leaf, candy leaf and sweet leaf. It grows upto 1m height and is widely cultivated for its sweet leaves [1]. Stevia is the world's only natural sweetener with zero calories, low carbohydrates and zero glycemic index. Stevia extract is known to be 300 times sweeter than sugar and has gained attention with the rise in demand for low-carbohydrate, low-sugar food alternatives and its use can be a boon to diabetics and weight losers [2]. Stevia sweeteners are becoming increasingly popular over the last several years because of consumer demand for all natural foods and beverages especially for ones low in calories. It is used as a sweetener even in confectionery, beverages, food industry and cosmetic industry [3].

Among the several constraints of Stevia cultivation, diseases are a major one. The diseases include Root rot disease caused by *Sclertoium rolfsii* [4], Leaf spot disease caused by *Alternaria alternata* [5]. Powdery mildew by *Erysiphe cichoracearum* DC, Damping-off by *Rhizoctonia solani* Kuehn. and Stem rot by *Sclerotium dephinii* Welch [6]. Among these diseases, leaf spot disease cause severe yield loss and stevioside content. Although fungicides have shown promising results in controlling the leaf spot disease, phytotoxicity and fungicide residues are major problems leading to environmental pollution and human health hazards. Development of fungicide resistance by *Alternaria* spp. further discourages its use for disease control [7]. Sanitation using sterile or clean water supplies, application of organic compost and regulation of watering and temperature during seedling growth contributed to the management of the disease to some extent. Thus, existing control measures are not effective for the control of damping-off disease. Biological control is an alternative approach to the chemical fungicides and it

may be a safe, effective and ecofriendly method for plant disease management. Soil has enormous untapped potential antagonistic microbes i.e. *Trichoderma* spp. and fluorescent pseudomonads which showantagonistic effects against soil-borne plant pathogenic organisms. The saprophytic pseudomonads associated with plants include *P. fluorescens, P. putida* and *P. aeruginosa*. The use of fluorescent pseudomonads is gaining importance for plant growth-promotion and biological control. Fluorescent pseudomonads control fungal pathogens through different modes of action such as competition for nutrients and space [8], antibiosis [9], production of siderophores [10] and lytic enzymes [11]. In addition, induction of resistance by fluorescent pseudomonads is an additional mechanism by which these bacteria protect several crop plants against pests and diseases [12]. The objectives of the present study are (i) to characterize and evaluate the fluorescent pseudomonads isolated from different crops grown in different regions of Tamil Nadu against leaf spot disease in Stevia under greenhouse and field conditions. (ii) to study the induction of various defense-related genes encoding proteins implicated in strengthening of plant cell walls by *P. fluorescens* in response to infection by *Alternaria alternata*

Materials and methods:

Isolation and characterization of microorganisms:

Ten isolates of fluorescent pseudomonads were isolated from rhizosphere soil of different crops grown in different parts of Tamil Nadu using King's medium B (KMB) [13]. The isolates were characterized based on standard biochemical tests [14]. The following tests were carried out: production of fluorescent pigment , gelatin liquefaction, nitrate reduction, arginine dihydrolase, levan formation, growth at 4 and 41 ◦C and different carbon sources utilization. Results of these tests were scored either as positive or as negative. Grouping was made with the aid of a determinative scheme developed by earlier workers [15]. *Alternaria alternata* was isolated from naturally infected Stevia and the pathogen was maintained on PDA medium. Pathogenicity was tested in Stevia plants by spraying the spore suspension of *A. alternata*.

In vitro* Screening of *Pseudomonas fluorescens* against *Alternaria alternate:

The antifungal activity of *P. fluorescens* isolates were tested by dual culture technique using PDA medium. A mycelial disc (9 mm) of seven days old culture of *A. alternata* was placed at one end of the Petri plate. The bacterial antagonists were streaked 1.0 cm away from the periphery of the plate just opposite to the mycelial disc of the pathogen. The plates were incubated at 28 ± 2°C. The growth of the pathogen towards the bacterial colony and inhibition zone were measured after 7 days of incubation and expressed in mm [16].

Development of talk based formulation of *Pseudomonas fluorescens:*

Ten gram of carboxy methylcellulose was mixed with 1 kg of talc powder and the pH was adjusted to 7.0 by adding 15 g of calcium carbonate. The mixture was then autoclaved for 30 minutes for two consecutive days. The *P. fluorescens* culture were grown in King's B medium (KMB) for 48 hours. 400 ml of the bacterial inoculum was then added to 1 kg of talc mixture and mixed well under sterile conditions. The product was dried under shade to bring the moisture content to less than 20 per cent. The formulation was packed in polythene bags, sealed and kept under room temperature [17].

Efficacy of fluorescent pseudomonads against leaf spot disease under greenhouse conditions:

Potting medium (red soil: sand: cowdung manure at 1 : 1 : 1 w/w/w) was autoclaved for 1 h on two consecutive days. The soil was placed in pots (15 cm diameter; 30 cm height). Rooted cuttings of Stevia were planted in the pots at 10 plants per pot. Propiconazole as foliar spray at 0.1 per cent was included as a standard treatment for comparison. The virulent strain of *Alternaria alternata*, mass multiplied in the PDA broth. The talc based formulations of *P. fluorescens* were applied on the plants as foliar spray at 0.2 percent concentration. Pathogen inoculated and pathogen un-inoculated controls (healthy) were maintained. Watering was done regularly and leaf spot disease incidence was recorded at 15 days after spraying. Four pots per replication were maintained. There were three replications and the pots were arranged in a randomized manner [18].

Induction of Defense Mechanisms and Experimental Design:

P. fluorescens, isolate AUPF6, was used in the induction of defense reactions in Stevia. The following treatments were included in the experiment: (1) Foilar spraying with *P. fluorescens* isolate AUPF6; (2) Foilar spraying with *P. fluorescens* isolate AUPF 6and challenge inoculated with *Alternaria alternata*; (3) plants inoculated with the pathogen 3 days after planting; and (4) non-treated plants. Rooted cuttings were planted in earthen pots filled with sterilized potting soil at 10 plants per pot. Three replications were maintained in each treatment; each replicate consisted of eight pots. The experiments were conducted using randomized block design. Four plants were sampled from each replication of the treatment separately (treatments were mentioned in the experimental design) and were maintained separately for biochemical analysis. Fresh leaves were washed in running tap water and homogenized with liquid nitrogen in a pre-chilled mortar and pestle. The homogenized root tissues were stored at −70 ◦C.

Estimation of phenylalanine ammonia lyase (PAL) activity:

Leaf samples (1 g) were homogenized in 3ml of ice cold 0.1M sodium borate buffer, pH 7.0 containing 1.4mM of 2-mercaptoethanol and 0.1 g of insoluble polyvenylpyrrolidone. The extract was filtered through cheesecloth and the filtrate was centrifuged at 16,000 *g* for 15 min. The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of l-phenylalanine to transcinnamic acid at 290 nm. Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1M borate buffer, pH 8.8 and 0.5 ml of 12mM l-phenylalanine in the same buffer for 30 min at 30 ◦C. The amount of *trans*cinnamic acid synthesized was calculated. Enzyme activity was expressed as nmol *trans*-cinnamic acid min−1 mg protein−1.

Assay of Peroxidase (PO):

Leaf samples (1 g) were homogenized in 2ml of 0.1M phosphate buffer, pH 7.0 at 4 ◦C. The homogenate was centrifuged at 16,000 *g* at 4 ◦C for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1% H2O2. The reaction mixture was incubated at room temperature (28 ± 2 ◦C). The changes in absorbance at 420 nm were recorded at 30 s intervals for 3 min. The enzyme activity was expressed as changes in the absorbance min−1 mg protein−1.

Assay of polyphenol oxidase (PPO):

Leaf samples (1 g) were homogenized in 2ml of 0.1M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 *g* for 15 min at 4 ◦C. The supernatant was used as enzyme source. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5).

To start the reaction, 200 µl of 0.01M catechol was added and the activity was expressed as changes in bsorbance at 495 nm min−1 mg protein−1.

Estimation of phenol:

Leaf samples (1 g) were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70 ◦C. One millilitre of the methanolic extract was added to 5ml of distilled water and 250 µl of Folin–Ciocalteau reagent (1 N) and the solution was kept at 25 ◦C. The absorbance of the developed blue colour was measured using a spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg catechol mg protein−1.

Native PAGE analysis:

The isoform profiles of PO and PPO were examined by discontinuous native polyacrylamide gel electrophoresis (native-PAGE). Leaf samples were collected on the third day after pathogen challenge, at which time the activity of PO and PPO was maximum. The protein extractwas prepared by homogenizing 1 g of root samples in 2ml of 0.1M sodium phosphate buffer pH 7.0 and centrifuged at 16,000 *g* for 20 min at 4 ◦C. The protein content of the sample was determined. Samples (50 µg protein) were loaded onto8%polyacrylamide gels (Sigma, USA). After electrophoresis, PO isoforms were visualized by soaking the gels in staining solution containing 0.05% benzidine (Sigma, USA) and 0.03% H2O2 in acetate buffer (20 mM, pH 4.2). For assessing PPO isoforms profile, the gels were equilibrated for 30 min in 0.1% p-phenylene diamine followed by addition of 10mMcatechol in the same buffer [3].

Effect of fluorescent pseudomonads on growth of *Alternaria alternate*

Among the ten fluorescent pseudomonads isolates tested for their efficacy in inhibiting the mycelia growth, five isolates showed higher inhibitory effect on mycelial growth of *A. alternata* when compared to other tested isolates. Among these five isolates, *P. fluorescens* isolate AUPF6 exhibited the maximum inhibition of mycelial growth *in vitro* by recording an inhibition zone of 33.0 mm (Table 2).

Table 1: Morphological and Biochemical characterization of *P. fluorescens*

	Pseudomonas fluorescens isolates									
Characteristics	**AUPF1**	**AUPF2**	**AUPF3**	**AUPF4**	**AUPF5**	**AUPF6**	**AUPF7**	**AUPF8**	**AUPF9**	**AUPF2**
Fluorescence test	+	+	+	+	+	+	+	+	+	+
Growth at 41 ^{0}C	-	-	-	-	-	-	-	-	-	-
Growth at 4 ^{0}C	+	+	+	+	+	+	+	+	+	+
Levan formation	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	-	-
Methyl red test	-	-	-	-	-	-	-	-	-	-
Voges Proskauer test	-	-	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-	-	-	-
Gram's staining	-	-	-	-	-	-	-	-	-	-

Table 2: *In vitro* screening of *Pseudomonas fluorescens* against *A. alternate*

S No	*P. fluorescens* Isolates	Mean mycelial growth (mm)*	Inhibition zone (mm)*
1	AUPF1	30.0 ± 2.00^{ab}	28.3 ± 1.53^{bcd}
2	AUPF2	31.0 ± 1.73^{bc}	29.6 ± 1.15^{bc}
3	AUPF3	32.6 ± 1.15^{bcd}	27.0 ± 2.00^{cde}
4	AUPF4	34.6 ± 1.53^{cd}	26.6 ± 1.52^{de}
5	AUPF5	28.6 ± 2.08^{bcd}	30.3 ± 1.52^{ab}
6	AUPF6	26.6 ± 1.52^{a}	33.0 ± 2.00^{a}
7	AUPF7	39.6 ± 2.08^{e}	21.0 ± 2.00^{f}
8	AUPF8	36.0 ± 2.00^{de}	24.3 ± 1.15^{e}
9	AUPF9	45.0 ± 2.00^{f}	18.0 ± 2.00^{g}
10	AUPF10	48.0 ± 1.73^{f}	15.4 ± 0.57^{h}
11	Control	88.0 ± 2.00^{g}	-
		CD (P<0.05) =4.06	CD (P<0.05)= 2.57

*Values are mean of three replications.

In a column, means followed by a common letter(s) are not significantly different at 5% level by DMRT

Efficacy of fluorescent pseudomonads against leaf spot disease under greenhouse conditions

Of the four fluorescent pseudomonads tested under greenhouse conditions, all were effective in reducing the leaf spot disease of stevia. (Table3). Among the isolates tested, *P. fluorescens* isolate AUPF6 showed the lowest disease incidence of 42.0%. This was followed by AUPF5 (46.0%). The other isolates were comparatively less effective. The maximum disease reduction was observed in chemical treatment propiconazole (8.0%) (Table 3).

Table 3. Efficacy of fluorescent pseudomonads isolates against leaf spot disease of stevia under green house conditions

S. No.	Treatment	Leaf spot incidence* (PDI)
1.	AUPF1	49.3[d]
2.	AUPF2	51.5[d]
3.	AUPF5	46.0[c]
4.	AUPF6	42.0[b]
5.	Propiconazole	8.0[a]
6.	Control (Inoculated)	73.0[e]

Induction of defense-related enzymes and phenolic compounds

In Stevia, foliarspray with *P. fluorescens* isolate AUPF6 induced the plants to synthesize PAL, whereas an additional increase in the synthesis was observed in *P. fluorescens* isolate AUPF6 pretreated plants challenge inoculated with *Alternaria alternata*. The activity reached the maximum level on the fifth day after pathogen challenge and thereafter the activity remained at higher levels throughout the experimental period of 7 days. In plants treated with the pathogen alone, increased activity of PAL was observed for a period of 3 days and thereafter declined drastically in both tomato and hot pepper (Figure 1A).

Fig 1A: Phenylalanine ammonia lyase activity in Stevia against A .*alternata*

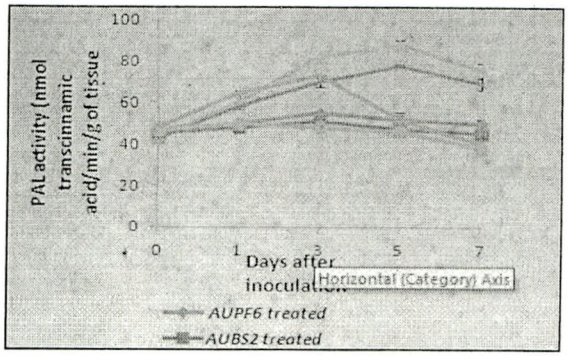


Figure 1B Peroxidase activity in Stevia against *A.alternata*

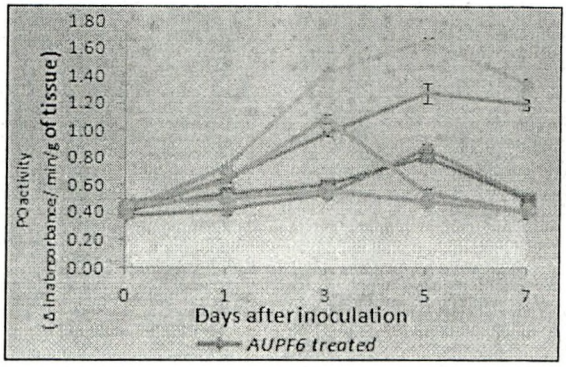
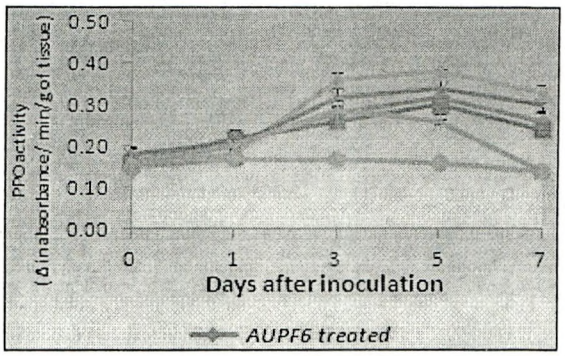
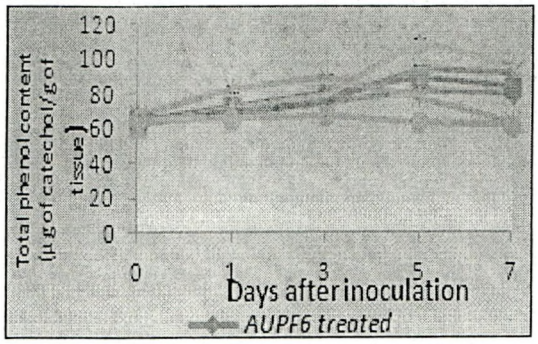


Fig 1C: Polyphenol oxidase activity in Stevia against *A.alternata*



Earlier an increased activities of PO (Figure 1B) and PPO (Figure 1C) were observed in *Pseudomonas*-pretreated stevia plants challenge inoculated with the pathogen and remained at higher levels throughout the experimental period. The activity reached maximum levels on the fifth day after challenge inoculation. The maximum phenolic content was observed in *P. fluorescens*-pretreated plants challenge inoculated with the pathogen and the higher amounts of phenolics were noticed 5th day after the pathogen challenge and remained at higher level. In plants inoculated with the pathogen alone the phenolic content declined to the initial level on the 7th day after inoculation. Plants treated with *P. fluorescens* alone also had increased content of phenolics compared to untreated plants (Figure 1D).

Fig 1D: Total phenol content in Stevia against *A.alternata*



In stevia plants, the expression of PO isoforms, PO1and PO2 was greater in *P. fluorescens* pretreated plants challenged with the pathogen and also plants inoculated with the pathogen alone than in untreated plants or plants treated with *P. fluorescens* alone (Figure 2A). Similarly, PPO isoforms PPO1and PPO2 were prominent instevia plants treated with *P. fluorescens* and challenge inoculated with the pathogen (Figure 2B).

Discussion:

Many of the fluorescent pseudomonads, predominantly *P. fluorescens*, were isolated from suppressive soil for the management of soil-borne diseases. The present study indicates that the all the strains isolated from rhizhosphere of different crops belonged to *P. fluorescens*. The majority of the fluorescent pseudomonads were found to be *P. fluorescens* in rhizosphere soils in Australia [18]. The present study also indicates all the fluorescent pseudomonads isolated from different crops cultivated in India belonged to *P. fluorescens*. Rosales et al. (1993) reported that some of the isolates within each genus of bacteria isolated from rice rhizosphere could be differentiated phenotypically and through protein profile studies.

After isolating and identifying the fluorescent pseudomonads, selecting an effective isolate is the first and foremost important step in biological control. *P. fluorescens* isolate AUPF6 showed the maximum inhibitory effect on mycelial growth. Fluorescent pseudomonads having antagonistic activity and increasing the plant growth would certainly be promising in evaluating suitable isolates in biological control.

In addition to direct antagonism, *P. fluorescens* AUPF6 increased the activities of various defense-related enzymes and chemicals in response to infection by the pathogen. It is well known that all plants are endowed with defense genes which are quiescent in nature and appropriate stimuli or signals are needed to activate them. It has been reported that application of *P. fluorescens* triggers/activates plants' latent defense mechanisms in response to infection by pathogen. Inducing a plant's own defense mechanism by prior application of a biological agent is a novel strategy in plant disease management. In the present study, it has been observed that seeds treated with *P. fluorescens* isolate AUPF6 increased the activities of various defense-related enzymes which lead to the synthesis of defense chemicals in the plants. PAL plays an important role in the biosynthesis of phenolic phytoalexins. When groundnut plants were sprayed with *P. fluorescens*, increase in activity of PAL was observed. Cucumber plants treated with *Pseudomonas corrugata* had initially higher levels of PAL and levels were lower after challenging the plant with *Pythium aphanidermatum*. Increase in mRNAs encoding PAL and chalcone synthase were recorded in the early stages of the interaction between bean roots and various rhizobacteria. De Meyer et al. (1999) reported that rhizosphere colonization by *P. aeruginosa* 7NSK2 activated PAL in bean. roots and increased the salicylic acid levels in leaves. Increased activity of PAL was observed in *P. fluorescens* treated tomato and pepper plants (during flowering stage) in response to infection by

F. oxysporum f. sp. *lycopersici* and *C. capsici* [18]. In the present study, increased activity of PAL was recorded in *P. fluorescens* isolate AUPF6 treated stevia plants challenged with the pathogen, reached maximum on the fifth day after challenge inoculation and was maintained at higher levels throughout the experimental period. In plants inoculated with the pathogen alone the activity declined greatly on the third day after challenge inoculation. Invasion of root tissues by the pathogen might have resulted in decreased activity of PAL whereas earlier and increased activity of PAL due to *P. fluorescens* isolate AUPF6 treatment might have prevented fungal invasion and thus the activity was maintained at the higher levels. PO and PPO catalyze the last step in the biosynthesis of lignin and other oxidative phenols. In the present study, foliar treatment with *P. fluorescens* induced the activities of PO and PPO. In bean, rhizosphere colonization of various bacteria induced PO activity. The higher PO activity was noticed in cucumber roots treated with *P. corrugate* challenged with *Pythium aphanidermatum*. The native-PAGE study indicates that PO and PPO isoforms were prominently expressed in *P. fluorescens* isolate AUPF6-treated leaves in response to infection by the pathogen. PPO transcript levels increased in young leaves of tomato when mature leaflets were injured.

Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall. In the present study, seed treatment with *P. fluorescens* isolate AUPF6 resulted in increased accumulation of phenolic substances in response to infection by the pathogen. M'Piga et al. (1997) reported that *P. fluorescens* isolate 63–28 induced the accumulation of phenolics in tomato root tissues. The hyphae of the pathogen surrounded by phenolic substances exhibited considerable morphological changes including cytoplasmic disorganization and loss of protoplasmic content. Accumulation of phenolics by prior application of *P. fluorescens* in pea has been reported against *P. ultimum* and *F. oxysporum* f. sp. *pisi* (Benhamou et al., 1996). Benhamou et al. (2000) reported that an endophytic bacterium, *Serratia plymuthica* induced the accumulation of phenolics in cucumber roots following infection by *P. ultimum*. *P. fluorescens* Pf1 isolate induced the accumulation of phenolic substances and PR-proteins in response to infection by *C. capsici* in pepper [18]. Since several defense-related genes encoding proteins are synthesized in ISR by fluorescent pseudomonads, it has been hypothesized that induced resistance by *P. fluorescens* isolate AUPF6 is related to multigenic/polygenic (horizontal) resistance in plants which is effective against multiple pathogens/races of pathogens. Tuzun (2001) described that constitutive accumulation of defense-related gene products was an integral part of both multigenic resistance and ISR. In cucumber, rhizobacteria induced resistance against cucumber mosaic virus (CMV) and tomato mottle virus (ToMoV) [12]. Induced resistance by fluorescent pseudomonads has broad spectrum activity against several fungal, bacterial and viral diseases [12].

In conclusion, fluorescent pseudomonads isolated from soils from Tamil Nadu belonged to *P. fluorescens* group. Application of talc-based formulation of *P. fluorescens* consistently reduced the incidence of leaf spot disease in Stevia. Prior treatment of stevia with *P. fluorescens* triggered the plant-mediated defense mechanism in response to infection by *Alternaria alternata*. Thus, it has been found that *P. fluorescens* isolate AUPF6 shows broad-spectrum protection against stevia leaf spot pathogen *Alternaria alternata*.

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