
RESULTS AND DISCUSSION

Stevia rebaudiana Bertoni is one of the herbaceous perennial plant native to subtropical and tropical rainforest areas of South America (Brazil, Venezuela, Colombia and Paraguay). The leaves are used traditionally in various regions of the world including China, Japan, Korea, Taiwan, Thailand, Malaysia and Paraguay. The leaves have been known to contain 100 useful alkaloids among other pharmacologically active compounds (Sumon *et al.*, 2008).

Stevia plants may get affected by soil-borne diseases such as Southern blight caused by *Sclerotium rolfsii*, Charcoal rot, Wilt, Root rot as well as air-borne diseases such as Black spot caused by *Alternaria steviae* and Grey mould caused by *Botrytis cinerea*.

Sclerotium rolfsii root rot of *Stevia* was first observed in India by Kamalakannan *et al.* (2006). The symptoms of this root rot disease observed on 2-month old *Stevia* plants appear as yellowing and drooping of leaves with wilting of plants and white cottony mycelial growth at the collar region. The mycelial growth spread to the stem and roots with associated tissue rotting and mustard like brown sclerotia.

The leaf spot caused by *Alternaria alternata* is very common in medicinal plants cultivated in various districts of West Bengal, India (Maiti *et al.*, 2007b). Symptoms of leaf spot disease initially appear as small circular spots and light brown in colour. Later, many may become irregular and dark brown to grey, while others may remain circular with concentric rings or zones. On severely infected leaves, several spots may coalesce to form large necrotic areas. On older leaves, concentric spots are more common at the tips. Leaf spots vary from 2-18 mm in diameter.

The use of fungicides to control the above mentioned diseases causes environmental pollution and decreased diversity of non- target organisms. The disease protection measures of medicinal plants are still restricted to the application of various chemical fungicides which strictly do not fit with the basic theory of usefulness of herbal drugs. Moreover, the residual effects of different chemicals eventually contaminate the plant drugs (Sharma *et al.*, 2004).

Microorganisms as biological control agents have high potential to control plant pathogens and have no effect on the environment or other non-target organisms (Khamna *et al.*, 2009).

Biological control of plant diseases with bacterial antagonists is a potential alternative to chemical control, as it is safe, effective, economical and eco-friendly. Plant Growth Promoting Rhizobacteria (PGPR) especially *Pseudomonas fluorescens* and *Bacillus subtilis*, are promising candidates for biological control (Latha *et al.*, 2009). Induced systemic resistance (ISR) has been reported as one of the mechanisms by which PGPR could control plant diseases (Saravanakumar *et al.*, 2007).

Therefore, the study was framed to evaluate the efficacy of talc-based powder formulation of bacterial antagonists (*Pseudomonas fluorescens* and *Bacillus subtilis*) in the management of root rot and leaf spot diseases of *Stevia* caused by *Sclerotium rolfsii* and *Alternaria alternata*, respectively. The bacterial antagonists were isolated from rhizosphere soils of Tamil Nadu and their efficacy was studied under *in vitro* conditions using dual plate technique. Then the effective isolates were formulated using talc as carrier and the formulations were tested under pot culture conditions for their efficacy in reducing root rot and leaf spot diseases of *Stevia* and inducing defense mechanism.

The results obtained for the study entitled “**Bio-management of Root rot and Leaf spot diseases in *Stevia rebaudiana* using Plant Growth Promoting Rhizobacteria**” are presented and discussed as follows:

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PHASE I

4.1. Isolation, Characterization and Screening of Plant Growth Promoting Rhizobacteria

4.1.1. Isolation of Plant Growth Promoting Rhizobacteria

Plant Growth Promoting Rhizobacteria (PGPR) are a group of bacteria that are found in the rhizosphere of soil in association with roots which can enhance the growth of plants directly or indirectly. A large number of bacteria including species

of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Bacillus*, *Rhizobium* and *Serratia* have been reported to enhance plant growth (Kumar *et al.*, 2012).

4.1.1.1. *Pseudomonas fluorescens* isolates

Pseudomonas fluorescens are gram-negative rod -shaped bacteria that inhabit soil, plants, and water surfaces. They are non-pathogenic and lack virulence factors of other plant pathogens. The fluorescent Pseudomonads associated with plants includes *P. fluorescens*, *P. putida*, *P. aeruginosa* and *P. aureofaciens*.

Table 1 depicts the *Pseudomonas fluorescens* isolates from various crops and places. Ten strains of *P. fluorescens* were isolated from the rhizosphere regions of different crops like Groundnut, Sugarcane, Banana, Cabbage, Turmeric, Stevia, Tomato, Paddy, Green gram and Maize and named as AUPF1 - AUPF10 (Plate 4).

Table 1
***Pseudomonas fluorescens* isolates from rhizosphere soils**

S.No	Isolates	Source	
		Crop Plant	Location / Place in Tamilnadu
1	AUPF1	Groundnut	Bhavanisagar
2	AUPF2	Sugarcane	Sathyamangalam
3	AUPF3	Banana	Dindugal
4	AUPF4	Cabbage	Ooty
5	AUPF5	Turmeric	Erode
6	AUPF6	Stevia	Gobichettipalayam
7	AUPF7	Tomato	Salem
8	AUPF8	Paddy	Dharapuram
9	AUPF9	Greengram	Annur
10	AUPF10	Maize	Palani

Plate 4

Pseudomonas fluorescens isolates



From Table 1, it is clearly understood that *Pseudomonas* species is present in the rhizospheresoils of many crops/ plants in Tamil Nadu and can be isolated.

This finding can be correlated with reports of Kumar *et al.* (2012) who stated that thirty bacterial strains were isolated from a total of six rhizospheric soil samples from French bean plants collected from different locations of Shimla and Solan in H.P (India).

Another report by Saravanakumar and Samiyappan (2007) showed that *Pseudomonas* strains such as *P. fluorescens* Pf 2, *P. fluorescens* TDK1 and *P. fluorescens* RMD1 could be isolated from the rhizosphere soils of coastal and forestry ecosystems of Tamil Nadu.

Thus from Table 1, it can be inferred that *Pseudomonas* species is present in the rhizosphere soils of many crop plants in Tamil Nadu and can therefore be isolated.

4.1.1.2. *Bacillus subtilis* Isolates

Bacillus is frequently isolated from rhizosphere soil and is a common plant endophyte. The gram positive bacterium *Bacillus subtilis* is known to positively influence plant growth, vitality and the ability of the plant to cope with pathogens often resulting in higher yield (Wu *et al.*, 2005).

Table 2 shows the various places and crops from where *B. subtilis* were isolated.

Table 2
***Bacillus subtilis* isolates from rhizosphere soils**

S.No	Isolates	Source	
		Crop Plant	Location / Place in Tamil nadu
1	AUBS1	Groundnut	Bhavanisagar
2	AUBS2	Sugarcane	Sathyamangalam
3	AUBS3	Banana	Dindugal
4	AUBS4	Cabbage	Ooty
5	AUBS5	Turmeric	Erode
6	AUBS6	Stevia	Gobichettipalayam
7	AUBS7	Tomato	Salem
8	AUBS8	Paddy	Dharapuram
9	AUBS9	Green gram	Annur
10	AUBS10	Maize	Palani

Ten strains of *Bacillus* species were isolated from the rhizosphere soils of various crop plants collected from ten different places of Tamil Nadu and named from AUBS1 to AUBS10 (Plate 5).

Plate 5
***Bacillus subtilis* isolates**



It is obvious from the table that *Bacillus* species survive in the rhizosphere soils of crop plants like Groundnut, Sugarcane, Banana, Cabbage, Turmeric, Stevia, Tomato, Paddy, Green gram and Maize.

The possibility of isolating *B. subtilis* was reported by Cazorla *et al.* (2007) who mentioned that a collection of 905 bacterial isolates obtained from the rhizoplane of healthy avocado trees, contained 277 gram-positive isolates. From these gram-positive isolates, four strains, namely, PCL1605, PCL1608, PCL1610 and PCL1612 were identified to be *B. subtilis*. In another research, Toledo *et al.* (2006) isolated fifteen bacterial strains from solid waste oil samples. The majority of which, belonged to the genera *Bacillus*, *Bacillus pumilus* (8 strains) and *B. subtilis* (2 strains).

4.1.2. Characterization of Plant Growth Promoting Rhizobacteria

Various phenotypic and biochemical methods have been developed and used for characterizing plant growth promoting bacteria such as *Pseudomonas* and *Bacillus* isolates. Rapid identification of potentially and economically viable bioagents is possible through various methods of biochemical characterization (Weller *et al.*, 2002).

4.1.2.1. Morphological and Biochemical characterization of *Pseudomonas fluorescens*

The isolated bacterial strain *P. fluorescens* was examined for its morphological and also biochemical characteristics.

The results of morphological and biochemical characterization of *P. fluorescens* is given in Table 3.

The table reveals that all the ten isolates were found to be gram negative, rod shaped, fluoresced in King's B medium and showed a positive response for growth at 4° C, Levan formation, Gelatin liquefaction and Catalase tests. However, there was a negative response for growth at 41°C, Methyl red, Voges Proskaur and Indole tests. Thus, based on morphological and biochemical characteristics, the isolated strains were identified to be *Pseudomonas fluorescens*.

Table 3
Morphological and Biochemical characterization of *P. fluorescens*

Characteristics	<i>Pseudomonas fluorescens</i> isolates									
	AUPF1	AUPF2	AUPF3	AUPF4	AUPF5	AUPF6	AUPF7	AUPF8	AUPF9	AUPF10
Fluorescent	+	+	+	+	+	+	+	+	+	+
Growth at 41 ^o C	-	-	-	-	-	-	-	-	-	-
Growth at 4 ^o C	+	+	+	+	+	+	+	+	+	+
Levan formation	+	+	+	+	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+	+	+	-	-
Methyl red test	-	-	-	-	-	-	-	-	-	-
Voges Proskauer test	-	-	-	-	-	-	-	-	-	-
Catalase test	+	+	+	+	+	+	+	+	+	+
Indole test	-	-	-	-	-	-	-	-	-	-
Gram's staining	-	-	-	-	-	-	-	-	-	-

Symbol (+) denotes a positive result, (-) denotes a negative result.

Similar findings have been reported by Reddy *et al.* (2010) who isolated ten bacterial strains from rhizosphere soil samples collected from rice seedlings grown in Andhra Pradesh, and found that all the strains were gram negative; rod shaped and produced yellowish green pigment in King's B medium. All isolates they revealed, showed positive response to Gelatin liquefaction test, Oxidase test and Arginine dehydrogenase test and were therefore identified as *P. fluorescens*. Biochemical reactions such as Fluorescien production, Levan formation, certain carbohydrate utilizations and morphological features of the isolates obtained in the present study were similar to the results reported by Kuarabachew *et al.* (2007) also. Similarly, Padmaja and Adlene (2007) also characterized *P. fluorescens* isolates based on their biochemical characters. The isolated petroleum degrading bacteria were identified based on morphological, biochemical characterization and carbohydrate fermentation test. The morphologically distinct organisms were further identified to be *Pseudomonas fluorescens* (Kuberan *et al.*, 2011). Based on the antagonistic potential and other characteristics, seven isolates of *P. fluorescens* viz., PF4, PF6, PF8, PF11, PF13, PF15 and PF20 were studied in detail for colony, colour, growth type, fluorescence and cell shape and it was evident from the observations that all seven isolates produced round shaped colonies and rod shaped cells (Meera and Balabaskar, 2012).

In another study, all the seven identified gram-negative *Pseudomonas* isolates were found to have a green fluorescence in King's B medium under ultraviolet light at 365 nm. The biochemical tests like Gelatin liquefaction, Starch hydrolysis, Catalase test, Oxidase test, IAA production, Siderophore production and Hydrogen cyanide production further confirmed the isolates to be *P. fluorescens* as reported by earlier workers (Nathan *et al.*, 2011).

Thus, from the results of the biochemical tests performed, it can be concluded that the isolated *P. fluorescens* was found to be gram negative, produced negative response for Starch hydrolysis, growth at 41°C, Methyl red, Voges Proskauer and Indole tests and positive response for growth at 4° C, Levan formation, Gelatin liquefaction, Catalase tests and Fluorescence test.

Table 4
Morphological and Biochemical characterization of *B.subtilis*

Characteristics	<i>Bacillus subtilis</i> isolates									
	AUPF1	AUPF2	AUPF3	AUPF4	AUPF5	AUPF6	AUPF7	AUPF8	AUPF9	AUPF10
Catalase test	+	+	+	+	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+
Methyl red test	-	-	-	-	-	-	-	-	-	-
Indole test	-	-	-	-	-	-	-	-	-	-
Voges Proskauer test	+	+	+	+	+	+	+	+	+	+
Blood agar haemolysis	+	+	+	+	+	+	+	+	+	+
Gram's staining	+	+	+	+	+	+	+	+	+	+

Symbol (+) denotes a positive result, (-) denotes a negative result.

4.1.2.2. Morphological and Biochemical characterization of *Bacillus subtilis*

Table 4 shows the results of the morphological and biochemical characterization of *Bacillus subtilis*. It is understood from the table that all the ten isolates were found to be gram positive, rod shaped and showed positive response for Catalase test, Starch hydrolysis test, Voges Proskauer test and Blood agar haemolysis test.

These observations are in accordance with the work of Ashiru *et al.* (2012) who confirmed the strains OB6 and OB7 to be *Bacillus megaterium* and *Bacillus subtilis* respectively by various biochemical tests. Similarly, Roostan *et al.* (2012) also confirmed the presence of *Bacillus subtilis* strain BL10 based on biochemical tests.

Thus, the bacterial isolates from the various sources in the present study were confirmed to belong to *Bacillus subtilis*.

4.1.3. Testing for antagonism of PGPR

The *Pseudomonas fluorescens* and *Bacillus subtilis* isolates were assayed for their antifungal activity against the fungal pathogens *Sclerotium rolfsii* and *Alternaria alternata*.

4.1.3.1. *In vitro* screening of *Pseudomonas fluorescens* isolates against *Sclerotium rolfsii*

The results of the *in vitro* screening of *P. fluorescens* isolates against the mycelial growth of *S. rolfsii* are recorded in Table 5 and Plate 6. The results revealed that all the ten isolates except AUPF3 recorded significant ($p < 0.05$) growth reduction when compared to the control. The isolate AUPF6 showed significantly ($p < 0.05$) the maximum reduction (42.3 ± 1.53 mm) in growth followed by AUPF5 (53.7 ± 0.58 mm) when compared to untreated control plates which exhibited a maximum radial growth of 90 ± 0.57 mm.

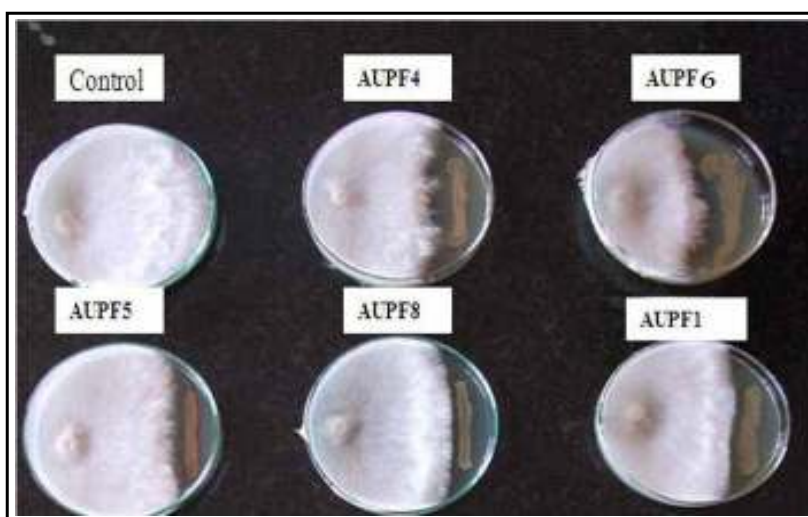
As regards the inhibition zone, maximum inhibition zone was observed by AUPF6 (21.3 ± 0.57 mm) which was significantly ($p < 0.05$) higher than the other samples. This was followed by AUPF5 (11 ± 2.00 mm) isolate.

Table 5***In vitro* screening of *Pseudomonas fluorescens* against *Sclerotium rolfsii***

S.No	<i>P. fluorescens</i> isolates	Mean mycelial growth (mm)*	Inhibition zone (mm)*
1	AUPF1	79.0 ± 1.00 ^f	0.00 ± 0.00 ^e
2	AUPF2	59.0 ± 3.00 ^c	3.66 ± 0.58 ^c
3	AUPF3	89.3 ± 1.15 ^g	0.00 ± 0.00 ^{de}
4	AUPF4	65.3 ± 2.51 ^d	2.00 ± 1.00 ^{cd}
5	AUPF5	53.7 ± 0.58 ^b	11.00 ± 2.00 ^b
6	AUPF6	42.3 ± 1.53 ^a	21.30 ± 0.57 ^a
7	AUPF7	73.3 ± 1.52 ^e	0.00 ± 0.00 ^{de}
8	AUPF8	56.3 ± 2.52 ^{bc}	10.30 ± 0.58 ^b
9	AUPF9	56.6 ± 2.08 ^{bc}	10.66 ± 1.52 ^b
10	AUPF10	62.3 ± 2.51 ^d	2.66 ± 0.57 ^c
11.	Control	90.0 ± 0.57 ^g	-
CD (P<0.05)		3.22	1.67

*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

Plate 6***In vitro* screening of *P. fluorescens* isolates against *S. rolfsii***

The above findings are in accordance with those of Mundhe *et al.* (2009) who reported that *P. fluorescens* was the most effective in reducing the mycelial growth of *S. rolfsii* and recorded the maximum inhibition zone. Rachana and Shalini (2008) also stated that out of five *P. fluorescens* strains (PSB-1, PUR-46, R1, R2 and R3) screened, strain PUR-46 showed almost complete inhibition against *Fusarium* sp. (96.30%), *Curvularia lunata* (96.07%) and *Bipolaris* species (95.08%). Guo *et al.* (2007) observed a maximum inhibition of mycelial growth of pathogenic fungi by *Pseudomonas corrugata* strain P94.

Thus it can be stated that the *P. fluorescens* isolates, namely, AUPF6 and AUPF5 were found to be the most effective in reducing the mycelial growth of *S. rolfsii* since they produced maximum inhibition zones.

4.1.3.2. *In vitro* screening of *Bacillus subtilis* isolates against *Sclerotium rolfsii*

The results of the *in vitro* screening of *Bacillus subtilis* isolates against *Sclerotium rolfsii* are shown in Table 6 and Plate 7.

Table 6

In vitro screening of *Bacillus subtilis* against *Sclerotium rolfsii*

S.No	<i>P. fluorescens</i> isolates	Mean mycelial growth (mm)*	Inhibition zone (mm)*
1	AUBS1	50.0 ± 2.0 ^{bc}	20.0 ± 1.00 ^b
2	AUBS2	42.0 ± 2.64 ^a	23.0 ± 2.00 ^a
3	AUBS3	68.0 ± 2.65 ^e	12.0 ± 2.00 ^d
4	AUBS4	89.7 ± 0.57 ^f	0.00 ^e
5	AUBS5	48.0 ± 1.00 ^b	14.3 ± 1.52 ^d
6	AUBS6	89.7 ± 0.58 ^f	0.00 ^e
7	AUBS7	89.0 ± 2.00 ^f	0.00 ^e
8	AUBS8	54.0 ± 1.00 ^d	17.0 ± 2.00 ^c
9	AUBS9	89.0 ± 1.00 ^f	0.00 ^e
10	AUBS10	51.0 ± 1.73 ^c	19.0 ± 3.00 ^{bc}
11	Control	90.0 ± 1.00 ^f	-
CD (P<0.05)		2.78	2.65

*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

Plate 7

In vitro screening of *Bacillus subtilis* isolates against *Sclerotium rolfsii*



It can be noticed from the table and plate that among the ten *Bacillus subtilis* isolates tested against *Sclerotium rolfsii*, the minimal mycelial growth was recorded in petriplates having the isolate AUBS2 (42.0 ± 2.64 mm), followed by the petriplate having AUBS5 (48 ± 1.00 mm). which were significantly ($p < 0.05$) lower than the other samples.

In the case of inhibition zone, the maximum inhibition zone of 23 ± 2.65 mm was observed in AUBS2 followed by AUBS1 (20.0 ± 1.00 mm) which were significantly ($p < 0.05$) higher than the other samples. On the other hand, there was absolutely no inhibition zone recorded by AUBS4, AUBS6, AUBS7 and AUBS9.

Similar observations have been reported by Araujo *et al.* (2005) who stated that the *Bacillus* strains AP3 and PRBS1 had an inhibition zone of 31.0 and 30.0 mm respectively against *S. rolfsii*. Chung *et al.* (2008) and Lee *et al.* (2007) also studied the antagonistic activity of *B. subtilis* against *Phytophthora* blight in Red Pepper and other fungal pathogens.

It can therefore be deduced from the results of the study that the *Bacillus subtilis* isolates AUBS2 and AUBS5 significantly inhibited the mycelial growth of *S. rolfsii*.

4.1.3.3. *In vitro* screening of *Pseudomonas fluorescens* isolates against *Alternaria alternata*

The results of the *in vitro* screening of *P. fluorescens* isolates against the mycelial growth of *A. alternata* are recorded in Table 7 and Plate 8

It can be inferred from the table that all the ten isolates of *P. fluorescens* significantly ($p < 0.05$) reduced the radial growth of *A. alternata* when compared to the control. Among them, the maximum reduction was observed in AUPF6 (26.6 ± 1.52 mm), followed by AUPF5 (28.6 ± 2.08 mm). As regards the inhibition zone, the highest value was revealed by AUPF 6 (33.0 ± 2.00 mm) followed by AUPF5 (30.3 ± 1.52 mm). The other isolates recorded an inhibition zone between 15.4 ± 0.57 mm and 26.6 ± 1.52 mm.

Table 7

In vitro screening of *Pseudomonas fluorescens* against *A. alternata*

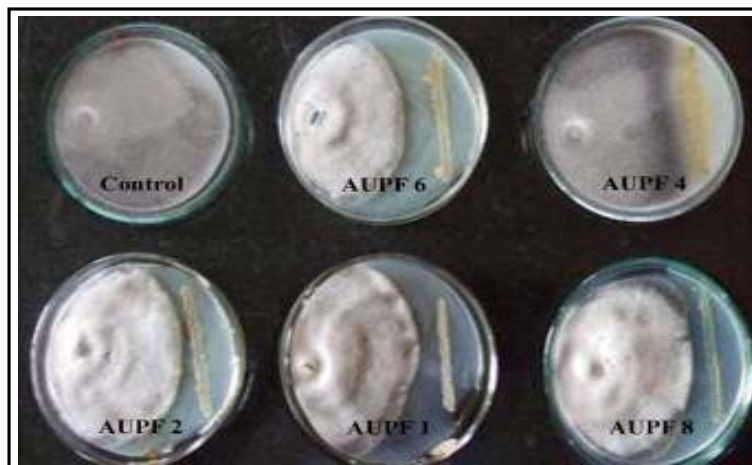
S.No	<i>Pseudomonas fluorescens</i> isolates	Mean mycelial growth (mm)*	Inhibition zone (mm)*
1	AUPF1	30.0 ± 2.00 ^{ab}	28.3 ± 1.53 ^{bcd}
2	AUPF2	31.0 ± 1.73 ^{bc}	29.6 ± 1.15 ^{bc}
3	AUPF3	32.6 ± 1.15 ^{bcd}	27.0 ± 2.00 ^{cde}
4	AUPF4	34.6 ± 1.53 ^{cd}	26.6 ± 1.52 ^{de}
5	AUPF5	28.6 ± 2.08 ^{bcd}	30.3 ± 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)		4.05	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

Plate 8

In vitro screening of *Pseudomonas fluorescens* isolates against *Alternaria alternata*



The above findings are on par with several other similar studies. Ghai *et al.* (2007) stated that thirty seven bacterial cultures isolated from soil samples obtained from different locations when tested for their antagonistic activity against 5 bacterial strains, viz., A1 6 (*Bacillus sphaericus*), K1 24 (*Pseudomonas fluorescens*), M1 42 (*Bacillus circulans*), M1 66 (*Bacillus brevis*) and T1 22 (*Bacillus brevis*) showed positive antagonistic activity against *Sclerotium rolfsii*, *Fusarium oxysporum* and *Rhizoctonia solani*. The *in vitro* antagonistic activity of *P. fluorescens* was also reported by Arunkumar (2008) where *P. fluorescens* was found to be efficient in inhibiting the colony growth of *A. alternata* to 36.4 per cent.

It can therefore be derived from the results of the study that the *Pseudomonas fluorescens*, isolates AUPF5 and AUPF6 significantly inhibited the mycelial growth of *A. alternata*.

4.1.3.4. *In vitro* screening of *Bacillus subtilis* isolates against *Alternaria alternata*

Table 8 and Plate 9 depict the effect of *Bacillus subtilis* isolates on the mycelial growth and inhibition zone of *Alternaria alternata*. It is clear from the above that all the ten isolates of *Bacillus subtilis* showed a significant ($p < 0.05$) inhibitory effect on the mycelial growth of *A. alternata* in varying degrees as compared to the control.

Table 8***In vitro* screening of *Bacillus subtilis* against *Alternaria alternata***

S.No	<i>Bacillus</i> spp. isolates	Mean mycelial growth (mm)*	Inhibition zone (mm)*
1	AUBS1	34.6 ± 1.16 ^b	17.0 ± 1.00 ^{cd}
2	AUBS2	30.0 ± 1.00 ^a	27.0 ± 1.00 ^a
3	AUBS3	38.0 ± 2.00 ^c	20.6 ± 1.53 ^b
4	AUBS4	40.0 ± 1.00 ^{cd}	16.0 ± 2.00 ^{de}
5	AUBS5	32.0 ± 2.00 ^{ab}	25.0 ± 1.73 ^a
6	AUBS6	43.0 ± 2.00 ^e	14.0 ± 2.00 ^{ef}
7	AUBS7	43.3 ± 2.08 ^{ef}	20.3 ± 1.52 ^b
8	AUBS8	41.6 ± 1.53 ^{de}	19.6 ± 1.53 ^{bc}
9	AUBS9	46.0 ± 1.00 ^f	12.5 ± 1.52 ^f
10	AUBS10	39.0 ± 2.64 ^{cd}	15.0 ± 1.00 ^{def}
11	Control	89.0 ± 1.00 ^g	-
CD (P<0.05)		2.84	2.67

*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

The least mycelial growth was observed in petriplates streaked with the isolate AUBS2 (30 ± 1.00 mm), followed by AUBS5 (32.0 ± 2.00 mm) which were significantly lower than all the other samples except AUBS1.

The isolate AUBS2 showed significantly (p<0.05) the highest inhibition zone of 27.0 ± 1.00 mm, followed by AUBS5 (25.0 ± 1.73 mm).

The above results are in accordance with those obtained for *A. alternata* leaf spot of pepper, where the antagonistic activity of a *B. subtilis* strain HS 93 was found to be 54% (Sid *et al.*, 2003). Hou *et al.* (2006) also reported that a *Bacillus* LEV 006 was antagonistic to major fungal pathogens of Canola including *A. brassicae*. The radial mycelial growth of *A. palandui*, a pathogen of onion leaf

blight was drastically inhibited by *Pseudomonas fluorescens* followed by *B. subtilis* (Karthikeyan *et al.*, 2008).

Plate 9

In vitro* screening of *Bacillus subtilis* isolates against *Alternaria alternata



It can therefore be deduced from the above observations, that the *Bacillus subtilis* isolates AUBS2 and AUBS5 significantly inhibited the mycelial growth of *A. alternata*.

4.1.4. Molecular confirmation

The effective isolates of *Pseudomonas fluorescens* viz., AUPF6 and AUPF 5 and *Bacillus subtilis* isolates AUBS2 and AUBS5 were further confirmed by polymerase chain reaction (PCR)

4.1.4.1. Molecular confirmation of *Pseudomonas fluorescens*

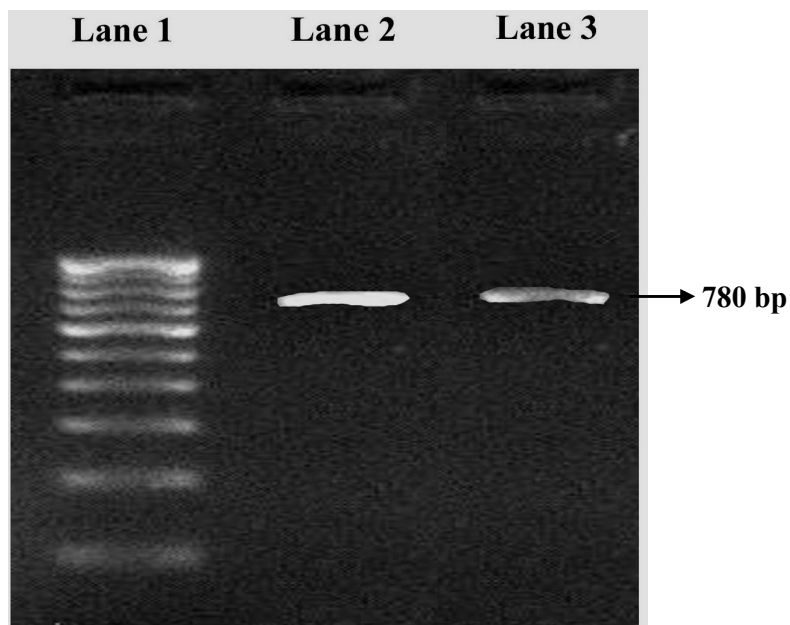
The results of the molecular confirmation of *P. fluorescens* by Polymerase Chain Reaction (PCR) are given in Plate 10.

The effective isolates of *P. fluorescens* AUPF5 and AUPF6 obtained from *in vitro* screening were further confirmed by molecular characterization. The molecular characterization was done by Polymerase Chain Reaction with E334F (5' CCAGACTCCTACGGGAGGCAG 3') as forward primer and E1115R

(5'CAACGAGCGCAACCCT3') as reverse primer. The PCR reaction yielded an amplicon size of ~ 780 bp by 1% gel electrophoresis (Plate 10) for both the samples.

Plate 10

Molecular confirmation of *Pseudomonas fluorescens* isolates by PCR



- Lane 1** - 100 bp marker
Lane 2 - *P. fluorescens* AUPF5
Lane 3 - *P. fluorescens* AUPF6

From plate 10 it is evident that a band of an amplicon size of 780 bp corresponding to *Pseudomonas fluorescens* was obtained.

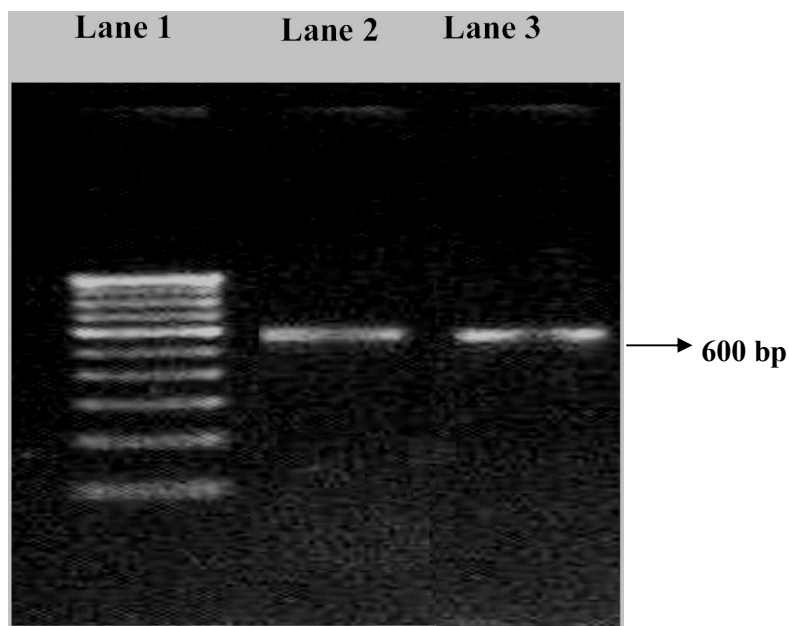
The above study is in concordance with the work of Aysun (2009) who reported that the isolated bacteria in his study to be *Pseudomonas fluorescens* by polymerase chain reaction using the universal bacterial primers such as E334F and E1115R and obtaining an amplicon size of ~ 780 bp by 1% gel electrophoresis. Another study by Ayyadurai *et al.* (2005) confirmed *P. aeruginosa* based on molecular analysis of 16S rRNA using universal bacterial primers yielded an amplicon size of ~ 622-bp by 1% gel electrophoresis.

4.1.4.2. Molecular confirmation of *Bacillus subtilis*

The results of the molecular confirmation of *B. subtilis* by Polymerase Chain Reaction (PCR) are presented in Plate 11.

Plate 11

Molecular confirmation of *Bacillus subtilis* isolates by PCR



Lane 1 - 100 bp marker
Lane 2 - *B.subtilis* AUBS2
Lane 3 - *B.subtilis* AUBS5

It is understood from the plate that the size of the amplicons of the *B.subtilis* strains AUBS2 and AUBS5 corresponds to 600 bp and this confirms the bacterial isolates to be *B.subtilis*.

Similar studies were made by Andretta *et al.* (2004), who confirmed the 4,5,6-trichloroguaiacol degrading bacteria to be *Bacillus subtilis* by PCR reaction using forward and reverse primers. They also obtained an amplicon size of about 600 bp. In another study, Geetha and Manonmani (2008) identified a strain named, VCRC B471 exhibiting mosquito larvicidal and pupicidal activity to be *B. subtilis* by Polymerase chain reaction with an amplicon of the size 600 bp.

Thus, from the results obtained, it can be concluded that plant growth promoting rhizobacteria such as *Pseudomonas fluorescens* and *Bacillus subtilis* can be isolated from the rhizosphere soils of different crop plants and their presence confirmed through morphological, biochemical and molecular characterization. Of the *Pseudomonas fluorescens* isolates, AUPF5 and AUPF6 and of the *Bacillus subtilis* isolates AUBS2 and AUBS5 were found to be the most effective in reducing the mycelial growth of *Sclerotium rolfsii* and *Alternaria alternata* and were thus selected for further study in Phase II.

Phase I - Highlights of the findings

- Ten bacterial strains of *Pseudomonas fluorescens* and *Bacillus subtilis* isolated from rhizosphere regions of different crops were identified by morphological and biochemical tests
- The *P. fluorescens* isolates, AUPF6 and AUPF5 and the *Bacillus subtilis* isolates AUBS2 and AUBS5 were found to be the most effective in reducing the mycelial growth of *S. rolfsii* and *A. alternata*
- The *Pseudomonas fluorescens* strains AUPF5, AUPF6 and the *Bacillus subtilis* strains AUBS2 and AUBS5 were confirmed by Polymerase Chain Reaction.

Phase II

4.2. Anti microbial compounds of biocontrol agents

Plant Growth Promoting Rhizobacteria (PGPR) have been identified to influence the growth and yield of many plants. The effect of PGPR on plant growth can be mediated by direct and indirect mechanisms (Beattie, 2006). This antagonistic activity has been associated with the production of secondary metabolites. Species belonging to *Bacillus* and *Pseudomonas* are frequently used as biocontrol agents, since they excrete hydrolytic enzymes which are able to degrade cell walls, iron-chelating siderophores and several cyclic lipodepsipeptides (LDP). *Pseudomonas* excretes a great variety of antibiotics such as 2, 4-

diacetylphloroglucinol (2, 4-DAPG), pyoluteorin and pyrrolnitrin. *Bacillus* strains also produce important antibiotics such as iturin and surfactin that are useful for plant disease control (Bhattacharyya and Jha, 2012).

Pseudomonas fluorescens produces a variety of secondary metabolites including siderophores, Hydrogen cyanide, salicylic acid, lytic enzymes and antibiotics which showed antimicrobial activity against pathogenic bacteria and fungi (Rachana and Shalini, 2008).

Bacillus species is known to protect plants against pathogens by direct antagonistic interactions between the biocontrol agent and the pathogen, as well as, by induction of host resistance. It depends on a wide variety of traits, such as the production of structurally diverse antibiotics (Liu *et al.*, 2006), production of iron chelators and exo-enzymes such as proteases, lipases, chitinases and β 1,3 - glucanases (Hayat *et al.*, 2010; Deepa *et al.*, 2010), formation of viable spores (Cenci *et al.*, 2006), promotion of plant growth (Ryu *et al.*, 2004), production of bacterial phytohormones and/or the solubilization of mineral phosphates (Calvo *et al.*, 2010, Viruel *et al.*, 2011) and an ubiquitous presence in soil (Gajbhiye *et al.*, 2010).

The most effective PGPR isolates from Phase I of the study, namely, *Pseudomonas fluorescens* - AUPF6 and AUPF5 and *Bacillus subtilis* - AUBS2 and AUBS5 were screened for the production of antimicrobial compounds like hydrogen cyanide, siderophores, salicylic acid, indole acetic acid and chitinase.

4.2.1. Hydrogen cyanide (HCN) production

Cyanide is produced by *Pseudomonas* strains by oxidative decarboxylation of glycine by the three-subunit membrane-bound flavoenzyme (Blumer and Haas, 2000). HCN does not take part in growth, energy storage, or primary metabolism, but is generally considered as a secondary metabolite that has an ecological role and confers a selective advantage to the producer strains. HCN, a volatile metabolite is thought to play a major role in biological control of some soil-borne diseases. Exposing plants to the volatile metabolites of an antagonist causes a

significant increase in peroxide activity, which may contribute to induction of disease resistance.

The results of the HCN production by the selected PGPR isolates are recorded in Table 9, Plates 12 and 13.

Table 9
HCN production by antagonistic bacteria

S. No	Antagonistic bacteria	HCN Production	
		Qualitative analysis	Quantitative analysis (1 unit = 0.001 Absorbance at 625 nm)
1	<i>P. fluorescens</i> AUPF5	Moderate	0.063 ± 0.0018 ^a
2	<i>P. fluorescens</i> AUPF6	Moderate	0.067 ± 0.0015 ^a
3	<i>B. subtilis</i> AUBS2	Weak	0.050 ± 0.0078 ^b
4	<i>B. subtilis</i> AUBS5	Weak	0.042 ± 0.0015 ^c
CD (P<0.05)			0.0056

*Values are mean of five replications.

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

Plate 12
Hydrogen cyanide production by *P. fluorescens* isolates

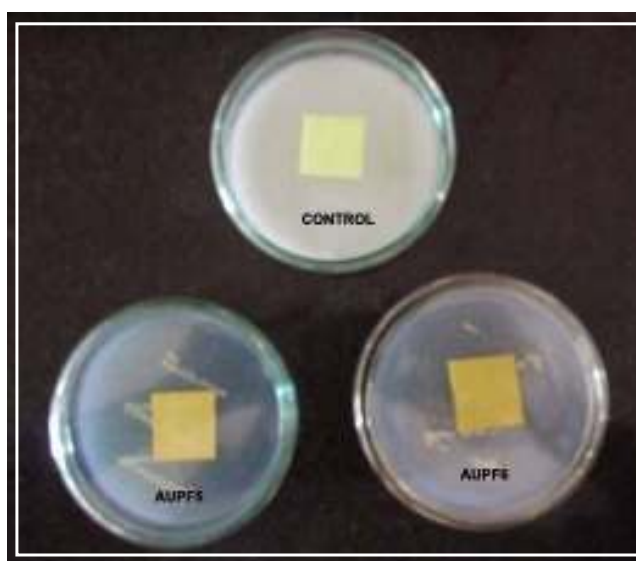


Plate 13

Hydrogen cyanide production by *Bacillus subtilis* isolates



It is obvious from the table that both the isolates of *P. fluorescens* namely AUPF5 and AUPF6 showed moderate HCN production of 0.063 ± 0.0018 and 0.067 ± 0.0015 respectively, whereas in the case of the *B. subtilis* isolates HCN production by AUBS2 (0.050 ± 0.0078 OD) was significantly higher ($P < 0.05$) than that of AUBS 5 (0.042 ± 0.0015 OD). The production of HCN by both the *Pseudomonas fluorescens* strains were significantly higher ($P < 0.05$) than those of the *B. subtilis* strains.

The above results are on par with the reports of Senthilkumar *et al.* (2009), who screened different bacterial isolates for HCN production of which the isolates HKA-72, HKA-107 and HKA-109 belonging to the genus *Bacillus* produced HCN. Similarly, Umamaheshwari *et al.* (2008) reported strong production of HCN in the *Pseudomonas fluorescens* CIAH-196 followed by moderate level in PfC6. They also stated that in the quantitative estimation, the *Pseudomonas fluorescens* strain PfCIAh-196 recorded maximum HCN production (O.D 0.093), while *B. subtilis* isolates did not produce any HCN. Studies have shown that HCN is the sole or primary toxic factor produced by a *P. aeruginosa* strain which is responsible for killing *Caenorhabditis elegans*, a common soil-inhabiting nematode (Gallagher and Manoil, 2001).

From the above study, it can be concluded that *P. fluorescens* isolates AUPF6 and AUPF5 produced more HCN than *B. subtilis* isolates

4.2.2. Salicylic acid production

Salicylic acid is known to play a critical role in the activation of plant defense response after pathogen attack and induces the expression and accumulation of pathogenesis - related proteins in leaves (Loake and Grant, 2007). Salicylic acid is a natural phenolic compound present in many plants and is an important component in the signal transduction pathway. It is also involved in local and systemic resistance to pathogens. These induced defense responses are probably involved in the expression of a range of defense genes, especially those encoding the pathogenesis related (PR) proteins such as chitinase, β -1, 3-glucanase and peroxidase.

The selected isolates were screened for salicylic acid production. Table 10 presents the production of salicylic acid.

Table 10
Salicylic acid production by antagonistic bacteria

S.No	Antagonistic bacteria	Salicylic acid production ($\mu\text{g/ml}$)*
1	<i>P. fluorescens</i> AUPF5	26.64 \pm 0.007 ^b
2	<i>P. fluorescens</i> AUPF6	38.17 \pm 0.015 ^a
3	<i>B. subtilis</i> AUBS2	25.53 \pm 0.012 ^c
4	<i>B. subtilis</i> AUBS5	15.91 \pm 0.007 ^d
CD (P<0.05)		0.015

*Values are mean of five replications.

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

The table clearly indicates that significantly ($P < 0.05$) higher production of salicylic acid was recorded in the *P. fluorescens* isolate AUPF6 (38.17 \pm 0.015 $\mu\text{g ml}^{-1}$) when compared to AUPF5 (26.6 \pm 0.007 $\mu\text{g ml}^{-1}$). In the case of

Bacillus subtilis isolate AUBS2 recorded significantly ($p < 0.05$) more salicylic acid production ($25.53 \pm 0.012 \mu\text{g ml}^{-1}$) than isolate AUBS5 ($15.91 \pm 0.007 \mu\text{g ml}^{-1}$). On comparison of *P. fluorescens* isolates and *Bacillus subtilis* isolates, the *P. fluorescens* strains produced significantly ($P < 0.05$) more salicylic acid than the *Bacillus subtilis* isolates.

These results correlate with the reports of Devi (2009) who reported that among ten *P. fluorescens* isolates, maximum salicylic acid production was recorded in *P. fluorescens* AUPF2 ($28.18 \mu\text{g/ml}$). Among ten *Bacillus subtilis* isolates, AUB2 showed maximum salicylic acid production ($25.50 \mu\text{g/ml}$).

Nandini (2007) also reported that among six isolates of *B. subtilis*, the isolate AUBS2 showed maximum production of salicylic acid ($17.89 \mu\text{g/ml}$). Anand and Kulothungan (2010) who screened five *P. fluorescens* strains 01,02,03,04 and 05 for their ability to produce salicylic acid. Among the five isolates, *P. fluorescens* 04 recorded maximum production of 6.14 mg/ml .

Thus, in our investigation, it was observed that isolates of *P. fluorescens* AUPF6 and AUPF5 produced more salicylic acid than *B. subtilis* isolates.

4.2.3. Siderophore production

Siderophores are low molecular weight (0.5 to 1.5KDa), high specificity Fe^{3+} chelating agents secreted by bacteria on or around the roots that affect the growth of the plant pathogens. The PGPR, being more potent chelators, starve the deleterious rhizobacteria of their iron nutrient, thus protecting the plants from the harmful effects of the deleterious rhizobacteria, resulting in better growth and yield (Sakthivel *et al.*, 2009). Free-living nitrogen-fixing bacteria in soils need to tightly regulate their uptake of metals in order to acquire essential metals such as the nitrogenase metal cofactors iron and molybdenum. They excrete compounds previously identified as siderophores that bind a variety of metals in addition to iron. At low concentrations, complexes of essential metals with siderophores are taken up by the bacteria through specialized transport systems (Kraepiel *et al.*, 2009). Competition for iron is also a possible mechanism to control the phytopathogens.

Table 11 represents the production of siderophores by PGPR.

Table 11
Siderophore production by antagonistic bacteria

S.No	Antagonistic bacteria	Siderophore production (µg/ml)*
1	<i>P. fluorescens</i> AUPF5	45.17 ± 0.007 ^a
2	<i>P. fluorescens</i> AUPF6	51.43 ± 0.018 ^a
3	<i>B. subtilis</i> AUBS2	29.05 ± 0.03 ^b
4	<i>B. subtilis</i> AUBS5	26.00 ± 0.97 ^c
CD (P<0.05)		9.02

*Values are mean of five replications.

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

From the table, it is understood that the *Pseudomonas fluorescens* isolate AUPF6 produced 51.43± 0.018 µg ml⁻¹ of siderophore which is more than that produced by AUPF5 (45.17± 0.007 µg ml⁻¹). Similarly *B. subtilis* isolates produced similar amount of siderophore (29.05± 0.03 µg ml⁻¹ by AUBS2 and 26.00 ± 0.97 by AUBS5). However the production of siderophores by the *P.flourescens* isolates were significantly (P < 0.05) higher than those of the *Bacillus subtilis* isolates.

The above observed results are supported by Devi (2009) who reported that, *P. flourescens* isolate AUPF2 significantly (p<0.05) produced more amount of siderophore (45 µg ml⁻¹) than isolate AUPF3 (43.6 µg ml⁻¹). Similarly maximum siderophore production was observed in the *B.subtilis* isolate AUB2 (19.43 µg ml⁻¹) than isolate AUB4 (15.63 µg ml⁻¹). Similarly, Angayarkanni *et al.* (2005) estimated the siderophore produced by the different isolates of *P.flourescens* of which isolate PFUA1 recorded maximum amount of siderophores (70 µg ml⁻¹).

Tamilselvi (2009) also observed the siderophore production of *P.flourescens* and *B.subtilis* isolates. The *P. flourescens*, isolates AUPF3 and AUPF2 recorded 43.66 and 42.74 µg ml⁻¹ of siderophore, respectively. Among two *B.subtilis* isolates

tested, higher siderophore production was observed in the isolate AUB2 (19.47 $\mu\text{g ml}^{-1}$) than AUB8 (16.33 $\mu\text{g ml}^{-1}$).

It can thus be inferred that all the isolates showed the production of siderophores, with the *P. fluorescens* isolate AUPF6 showing maximum production compared to the other isolates

4.2.4. Indole acetic acid (IAA) production

Indole acetic acid or auxin is the most effective plant growth promoting hormone. Among different auxins, IAA is the commonest one, which is mainly produced by the tryptophan dependent pathway. Rhizobacterial IAA production plays a significant role in the host plant's growth. Indole acetic acid production in microbes has been investigated by several researchers (Ahmad *et al.*, 2008). Indole acetic acid biosynthesis is also widespread in plant associated bacilli and is considered to be directly involved in plant growth promotion (Tsavkelova *et al.*, 2007 and Idris *et al.*, 2007). In addition to siderophores and salicylic acid production, production of IAA by rhizobacterium was also implicated in Induced Systemic Resistance (ISR). The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and the uptake of microbial IAA by plants.

Table 12 shows the production of indole acetic acid by PGPR.

Table 12
Indole acetic acid production by antagonistic bacteria

S.No	Antagonistic bacteria	Indole acetic acid production ($\mu\text{g/ml}$)*
1	<i>P. fluorescens</i> AUPF5	32.4 \pm 0.635 ^b
2	<i>P. fluorescens</i> AUPF6	43.5 \pm 0.731 ^a
3	<i>B. subtilis</i> AUBS2	21.8 \pm 0.768 ^c
4	<i>B. subtilis</i> AUBS5	20.2 \pm 0.707 ^d
CD (P<0.05)		0.96

*Values are mean of five replications.

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

As shown in the table, among the tested isolates, AUPF6 showed significantly ($P < 0.05$) maximum IAA production of $43.5 \pm 0.731 \mu\text{g ml}^{-1}$, followed by AUPF5 (32.4 ± 0.635) in the case of *Pseudomonas fluorescens*. However, in the case of *B. subtilis* isolates, the IAA content of AUBS2 was significantly ($P < 0.05$) higher (21.8 ± 0.768) than AUBS5 (20.2 ± 0.707). In general, it can be said that the IAA production by the *Pseudomonas fluorescens* isolates were significantly ($P < 0.05$) higher than that of *B. subtilis* isolates.

Similar results have been obtained by other researchers also. Singh *et al.* (2008) screened ten isolates of *B. subtilis* for IAA production and found eight isolate to be positive. Karnwal (2009) reported that *Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2 produced more IAA among 30 isolates tested. In another study, Ramesh *et al.* (2002) reported the production IAA by rhizobacterial isolates and the values ranged from 0.02 - 49.51 $\mu\text{g ml}^{-1}$.

Thus, it can thus be inferred that though all the isolates showed production of IAA, the isolate AUPF6 showed maximum production.

4.4.1.5. Chitinase production

Chitinases are ubiquitous enzymes of bacteria, fungi, animals and plants. They are one of the major pathogenesis-related proteins that accumulate extracellularly in infected plant tissues and exhibits high resistance to proteolytic degradation. Chitinases have been implicated in the defense reactions of plants against potential pathogens. In many plants, chitinases rapidly accumulate following pathogen attack, after elicitor treatment and in response to the plant stress hormone ethylene. Microbial chitinases are widely distributed and produced in bacteria such as *Serratia*, *Chromobacterium*, *Klebsiella*, *Pseudomonas*, *Vibrio*, *Arthrobacter*, *Beneckea*, *Aeromonas* and *Streptomyces*.

Table 13 depicts the production of chitinase by PGPR.

It is clearly understood from the table that AUPF6 showed significantly ($P < 0.05$) higher chitinase activity (7.52 ± 0.47 nmol of GlcNAc/min/ml) compared to other isolate AUPF5 (6.30 ± 0.64 nmol of GlcNAc/min/ml). In the case of *B. subtilis*

isolates, AUBS2 and AUBS5 showed chitinase production of 4.65 ± 0.78 , and 4.18 ± 0.70 nmol of GlcNAc/min/ml), respectively and there was no significant difference in chitinase production between the two isolates. On comparison of *P. fluorescens* and *B. subtilis* isolates, the chitinase production was significantly ($P < 0.05$) more by *P. fluorescens* than *B. subtilis* isolates.

Table 13
Chitinase production by antagonistic bacteria

S.No	Antagonistic bacteria	Chitinase activity (nmol of GlcNAc/min/ml)*
1	<i>P. fluorescens</i> AUPF5	6.30 ± 0.64^b
2	<i>P. fluorescens</i> AUPF6	7.52 ± 0.47^a
3	<i>B. subtilis</i> AUBS2	4.65 ± 0.78^c
4	<i>B. subtilis</i> AUBS5	4.18 ± 0.70^c
CD ($P < 0.05$)		0.89

*Values are mean of five replications.

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

Production of chitinase by *Pseudomonas fluorescens* in the above study agree with the findings by Nandakumar *et al.*, (2001), who stated that *Pseudomonas fluorescens* strains viz., PF1, PB2 and FP7 produced chitinase in the culture media. The maximum chitinase activity was observed in the *Pseudomonas fluorescens* strain FP7 (5.5 nmol of GlcNAc /min/ml) whereas isolate PF1 recorded an activity of 4.2 nmol of GlcNAc /min/ml. The isolate PB2 has an activity of 3.8 nmol of GlcNAc /min/ml.

From the above results, it can be inferred that the *Pseudomonas fluorescens* isolate AUPF6 produced more chitinase than AUPF5 and the two *B. subtilis* isolates.

Thus, from the results of Phase II, it can be concluded that among the *P. fluorescens* isolate AUPF6 and among the *B. subtilis* isolate AUBS2 produced more amounts of antimicrobial compounds like HCN, siderophores, salicylic acid, indole acetic acid and chitinase than their counterparts.

Phase II – Highlights of the findings

- *P. fluorescens* isolates AUPF5 and AUPF6 produced more HCN than *Bacillus subtilis* isolates AUBS2 and AUBS5
- Higher production of salicylic acid was recorded in *P. fluorescens* isolate AUPF6 than AUPF5. Among the *Bacillus subtilis* isolates, AUBS2 recorded maximum salicylic acid production than the AUBS5
- Higher siderophore production was recorded in the *P. fluorescens* isolates than the *Bacillus subtilis* isolates.
- *Pseudomonas fluorescens* isolates produced more IAA than that of *B.subtilis*. Among *P. fluorescens* isolates, AUPF6 recorded maximum IAA production.
- *Pseudomonas fluorescens* isolates showed higher activity of chitinase than that of *B.subtilis* isolates

PHASE III

4.3. Efficacy of talc-based formulations on the management of Stevia diseases

The success of biological control of plant diseases depends on the availability of effective formulations of biocontrol agents, their survival during storage and rapid multiplication and colonization after inoculation. Talc-based bioformulations have been reported for the management of plant diseases (Jayaraj *et al.*, 2005).

The results of Phase II clearly indicate the effectiveness of the *P. fluorescens* isolate AUPF6 and *B. subtilis* isolate AUBS2 for their antimicrobial compound production. Hence, talc-based formulations were prepared using these isolates and their efficacy on the management of Stevia diseases was studied on comparison with the fungicide propiconazole and the control (only pathogen).

4.3.1. Management of root rot of *Stevia rebaudiana* by antagonistic bacteria

The results of the efficacy of talc-based formulations of *P. fluorescens* and *B. subtilis* isolates in controlling root rot disease in *Stevia rebaudiana* are recorded in Table 14 and Plate 14.

Table 14
Efficacy of antagonistic bacteria on *Stevia* root rot disease

S.No	Antagonistic bacteria	Root rot incidence (%)*
1	<i>P. fluorescens</i> AUPF6	32.0 ± 2.64 ^b
2	<i>B. subtilis</i> AUBS2	37.0 ± 1.24 ^c
3	Propiconazole	12.0 ± 1.00 ^a
4	Control	83.0 ± 2.33 ^d
CD (P<0.05)		2.55

* Values are mean of five replications.

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

From the table, it is understood that soil application of talc-based formulation of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 isolates, at the rate of 10 g/ kg of soil effectively reduced the root rot incidence significantly ($p < 0.05$) to 32.0 ± 2.64 % and 37.0 ± 1.24 %, respectively in *Stevia rebaudiana* when compared to the control (83 ± 2.33 %). The maximum reduction of disease incidence was observed in propiconazole (12 ± 1.00 %) treated plants, because, it is a chemical fungicide.

Among the bacterial isolates, *P. fluorescens* AUPF6 recorded a significantly ($p < 0.05$) lesser incidence of the disease than *B. subtilis* isolate AUBS2. The maximum root rot incidence of 83 percent was recorded in the pathogen inoculated plants.

The above results are in agreement with the study of Sangita *et al.* (2008), who tested *P. syringae* strain PUR 46 as seed treatment for its ability to suppress collar rot of chickpea caused by *S. rolfsii*. The results indicated that seed

bacterization with PUR 46 showed 46% reduction in plant mortality. Kishore *et al.* (2005) also evaluated 393 bacterial strains against the fungal pathogen of groundnut *S.rolfsii*, out of which the *Pseudomonas aeruginosa* strains GSE18 and GSE 19 were found to be effective in controlling seedling mortality and inhibition of the cell wall degrading enzymes of *S.rolfsii*. Another study by Baysal *et al.* (2008) reported that the *Bacillus subtilis* isolate EU07 reduced crown and root rot disease caused by *S. rolfsii* in tomato by 75 per cent.

Plate 14

Management of root rot of Stevia by antagonistic bacteria



1. Control

3. *P. fluorescens* AUPF6 treated plants

2. Propiconazole treated plants

4. *B. subtilis* AUBS2 treated plants

From the present study, it can be concluded that *P. fluorescens* isolate AUPF6 was more effective in reducing root rot disease of Stevia than *B. subtilis* isolate AUBS2.

4.3.2. Management of leaf spot of *Stevia rebaudiana* by antagonistic bacteria

Table 15 and Plate 15 display the results of the efficacy of talc-based formulation of *Pseudomonas fluorescens* and *Bacillus subtilis* isolates in controlling leaf spot disease in Stevia.

Table 15
Efficacy of antagonistic bacteria on Stevia leaf spot disease

S.No	Antagonistic bacteria	Leaf spot incidence (Percent Disease Index)*
1	<i>P. fluorescens</i> AUPF6	42.0 ± 1.73 ^b
2	<i>B. subtilis</i> AUBS2	48.0 ± 1.00 ^c
3	Propiconazole	8.0 ± 1.58 ^a
4	Control	73.0 ± 2.91 ^d
CD (P<0.05)		2.60

* Values are mean of five replications.

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

It is obvious from the table and plate, that the foliar application of bacterial antagonists, namely *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 reduced the leaf spot disease of Stevia significantly ($p < 0.05$) to 42.0 ± 1.73 % and 48.0 ± 1.00 %, respectively under greenhouse conditions when compared to the control (73.0 ± 2.91 %). The disease incidence was significantly ($p < 0.05$) lower in *P. fluorescens* AUPF6 treated plants than in *B. subtilis* AUBS2 treated plants. The maximum reduction of disease incidence was observed in plants treated with propiconazole (8.0 ± 1.58 %) since it is a chemical fungicide.

The above findings are supported by the observations of Surjith *et al.* (2012) who stated that foliar application of talc-based formulation of a *Pseudomonas fluorescens* strain BRL-1 reduced Stevia leaf spot disease caused by *A. alternata* to the extent of 87 per cent. Lin (2008) also observed a 50 per cent reduction in leaf spot disease of tobacco caused by *Alternaria alternata* when the plants were treated with *P. fluorescens* strain isolate PF7-5 as foliar spray.

Thus, it can be derived from the results that among the PGPR, the *P. fluorescens* isolate AUPF6 was more effective in controlling leaf spot disease than the *B. subtilis* isolate AUBS2.

Plate 15

Management of leaf spot of *Stevia* by antagonistic bacteria



1. *B. subtilis* AUBS2 treated plants

3. Propiconazole treated plants

2. *P. fluorescens* AUPF6 treated plants

4. Control

4.3.3. Study of Induction of plant defense mechanisms

The widely recognized mechanisms of biocontrol mediated by PGPR are competition for an ecological niche or a substrate, production of inhibitory allelochemicals including iron-chelating siderophores, antibiotics, biocidal volatiles, lytic and detoxification enzymes and induction of systemic resistance in host plants to a broad spectrum of pathogens and/or abiotic stresses (Compant *et al.*, 2005).

Induced Systemic Resistance (ISR) is potentiated by plant growth promoting rhizobacteria (PGPR), of which the best characterized are strains that belong to the genus *Pseudomonas* that causes no visible damage to the plant's root system (van Loon and Glick, 2004). Biocontrol strains stimulate the activities of defense enzymes (phenylalanine ammonia lyase, peroxidase and polyphenol oxidase) in plants that could be involved in the synthesis of phytoalexins (Van Loon and Bakker, 2005). Rajendran *et al.* (2007) stated that the *Bacillus* and *Pseudomonas* strains mediate disease resistance in crop plants against plant pathogens.

4.3.3.1. Defense-related compounds in *Stevia rebaudiana* plants against *S.rolfsii*

The decrease in the incidence of disease in plants that occurs on treatment with the bacterial antagonists as compared to untreated control under greenhouse conditions might be partially due to the enhanced induction of pathogenesis-related proteins and other defense enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, phytoalexins and/or chalcone synthase. Induction of defense enzymes and disease suppression in plants by talc-based formulations of PGPR has already been reported (Sendhilvel *et al.*, 2007).

P. fluorescens and *B. subtilis* isolates are known to induce defense mechanisms in plant systems. The level of defense-related enzymes such as peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL) and phenolics were induced to an elevated level in order to resist the pathogens. The acquired resistance in plants in this study was mainly focused on the induction of defense-related enzymes such as PAL, PO, PPO and phenols.

4.3.3.1.1. Phenylalanine ammonia lyase activity against *S.rolfsii*

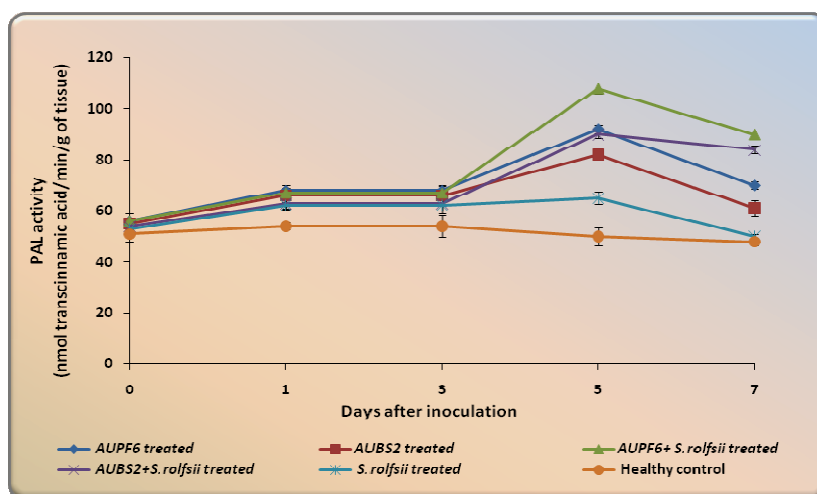
Phenylalanine ammonia-lyase plays an important role in the biosynthesis of various defense chemicals in phenylpropanoid metabolism. Activity of PAL could be induced in plant-pathogen interactions and fungal elicitor treatment (Karthikeyan *et al.*, 2006a).

Figure 1 expresses the induction of phenylalanine ammonia lyase activity in *Stevia* plants against *S. rolfsii*.

From the figure, it is clearly understood that the PAL activity increased from the 0th to 3rd day of inoculation and reached a maximum on the 5th day in all the antagonistic bacteria treated plants and antagonistic bacteria treated and *S.rolfsii* challenge inoculated *Stevia* plants. However, in the plants treated with pathogen alone, PAL activity reached the maximum level (70 nmol of transcinnamic acid/min/g of tissue) 3 days after inoculation and thereafter decreased with increase in the number of days.

Figure 1

Phenyl alanine ammonia lyase activity in Stevia against *S.rolfsii*



Among the two bacterial isolates, *P. fluorescens* AUPF6 induced significantly ($p < 0.05$) more activity of PAL (92 nmol of transcinamic acid/ min/g of tissue) than the *B. subtilis* isolate AUBS2 which induced a lesser activity (82 nmol of transcinamic acid/ min/g of tissue).

However, the plants pre-treated with *P. fluorescens* AUPF6 and challenge inoculated with *S.rolfsii* recorded significantly ($p < 0.05$) more amount of PAL (108 nmol of transcinamic acid/ min/ min/g of tissue) on the 5th day after inoculation, whereas, the plants pre-treated with *B. subtilis* isolate AUBS2 and challenge inoculated with *S.rolfsii* recorded a less PAL activity of 90 nmol of transcinamic acid/ min/g of tissue on the 5th day of inoculation.

After 5 days of challenge inoculation, the activity of PAL has decreased in all the treatments. However, maximum PAL activity on the 7th day after inoculation was observed in the plants pretreated with *P. fluorescens* AUPF6 and challenge inoculated with *S.rolfsii* (90 nmol of transcinamic acid/min/g of tissue) compared to other treatments.

The maximum PAL activity observed in plants treated with AUPF6 in the above study is supported by the findings of Rajendran and Samiyappan (2008), who emphasized that the PAL activity increased in 24 hours after pathogen

inoculation in cotton plants, which were pretreated with bioformulations of *Bacillus* and *Pseudomonas* species. In the roots of bacterized cotton plants inoculated with *R. solani*, the PAL activity was found to increase two fold higher than the control plant. In another study, Lavanya *et al.* (2006) observed the induction of defense enzymes by PGPR *Serratia marcescens* in betel vine against foot and root rot disease caused by *Phytophthora nicotianae*. The PAL activity was found to be 40 nmol of transcinnamic acid/ min/mg of protein in the plants treated with *Serratia marcescens* alone and 60 nmol of transcinnamic acid/ min/g of tissue in plants treated with *S. marcescens* and challenge inoculated with *Phytophthora nicotianae*.

From the above findings, it can be concluded that the activity of PAL in Stevia plants was induced by soil application of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 with the maximum induction observed in plants pretreated with *P. fluorescens* AUPF6 and challenge inoculated with *S. rolfsii*

4.3.3.1.2. Peroxidase activity against *S.rolfsii*

Peroxidases are defense-related enzymes which play key roles in plant-pathogen interactions. PO is believed to be one of the most important factors of the plant's biochemical defense against pathogenic microorganisms and is actively involved in the self-regulation of plant metabolism after infection. It is involved in substrate oxidation, cell wall lignification, photosynthesis, respiration and growth regulation. PO activity has been also associated with pathogenesis which in turn leads to reinforcement of cell walls with phenol compounds. PO and PPO catalyze the last step in the biosynthesis of lignin and other oxidative phenols (Ramamoorthy *et al.*, 2002b).

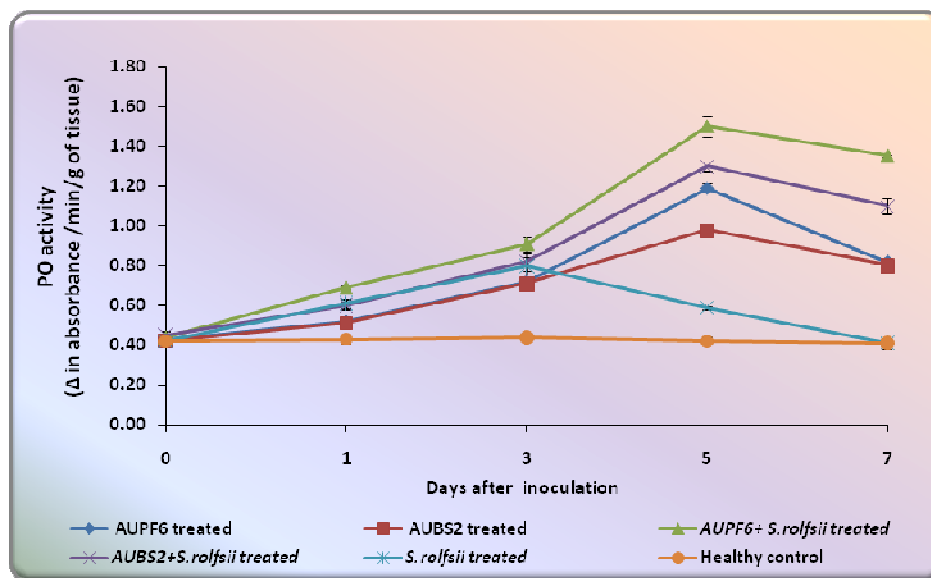
Figure 2 shows the induction of peroxidase activity in Stevia plants against *S. rolfsii*.

From the figure, it can be said that the induction of PO in Stevia plants was observed in those treated with the bacterial antagonists *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 and pathogen *S. rolfsii*. In all the bacterial isolate treated plants and pathogen challenge inoculated plants, PO activity increased from the 0th day after inoculation and reached the maximum on the 5th day of inoculation. However,

in the plants treated with the pathogen alone, PO activity reached the maximum level (0.8 absorbance / min / g of tissue) on the 3rd day of inoculation itself and thereafter decreased with increasing number of days.

Figure 2

Peroxidase activity in Stevia against *S. rolfsii*



Among the two bacterial isolates, *P. fluorescens* AUPF6 induced significantly ($p < 0.05$) more activity of PO (1.19 absorbance / min / g of tissue) than the *B. subtilis* isolate AUBS2 (0.98 absorbance / min / g of tissue) after 5 days of inoculation. However, the plants treated with *P. fluorescens* AUPF6 and challenge inoculated with *S.rolfsii* recorded significantly ($p < 0.05$) higher induction of PO (1.50 absorbance / min / g of tissue) on the 5th day of inoculation than the plants pretreated with *B. subtilis* isolate AUBS2 and challenge inoculated with *S. rolfsii* recorded a PO activity of 1.30 absorbance / min / g of tissue.

After 5 days of challenge inoculation, the activity of PO decreased in all the treatments, while, in the plants pre-treated with *P. fluorescens* AUPF6 and challenge inoculated with *S.rolfsii*, PO activity remained at a higher level of 1.35 absorbance / min / g of tissue after 7 day of inoculation.

The above observations coincide with the work of Kavino *et al.*, (2008) who reported that the peroxidase assay in banana plants revealed that induction of PO enzyme was significantly higher (2.15 change in absorbance/ min/g of tissue) almost twice as high in plants treated with *P. fluorescens* CHA0 + chitin and challenged with Banana bunchy top virus than untreated control (0.793 change in absorbance/ min/g of tissue).

The study of expression of defense-related proteins in rice plants treated with fluorescent *Pseudomonas* bioformulation revealed that PO activity was increased in Pf1+TDK1+ Py 15 treated plants inoculated with *Sarocladium oryzae* compared to untreated control and the activity was found to increase on the 5th day after inoculation and thereafter, a decline was noticed (Sarvanakumar *et al.*, 2009).

From the findings of the present study, it can be concluded that the activity of PO was increased in Stevia plants by soil application of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 and the maximum induction was observed in plants pretreated with *P. fluorescens* AUPF6 and challenge inoculated with *S. rolfsii*.

4.3.3.1.3. Native PAGE analysis of peroxidase

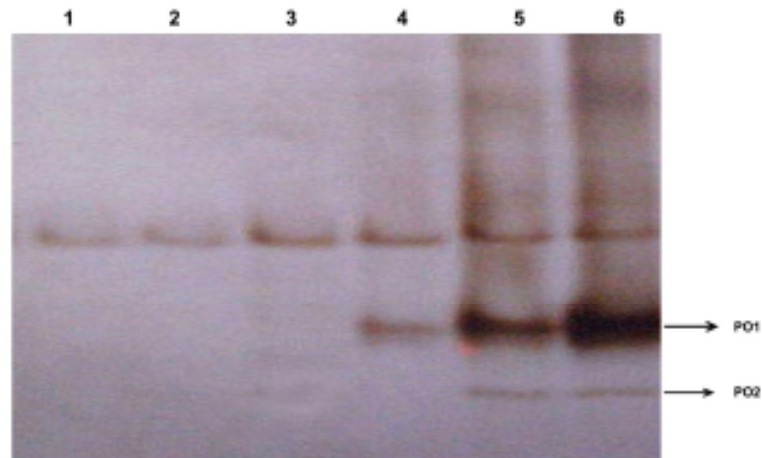
Plate 16 shows the native PAGE pattern of peroxidase in Stevia plants against *S. rolfsii*

It is obvious from the plate that the banding pattern of peroxidase by native PAGE revealed the presence of two additional bands indicating two isoforms PO1 and PO2 in the plants treated with *P. fluorescens* and *B. subtilis* strains and challenge inoculated with *S. rolfsii*. The induction of isoforms was more prominent in the treatment involving AUPF6 and AUBS2 strains when compared to pathogen - inoculated plants and uninoculated healthy plants.

The above findings are in accordance with the work of Sarvanakumar *et al.* (2009) who reported that four isoforms PO1, PO2, PO3 and PO4 of the enzyme PO were induced in *Pseudomonas fluorescens* Pf1+TDK1+ Py 15 treated plants inoculated with *Sarocladium oryzae* causing sheath rot of rice and only two isoforms PO1 and PO2 were observed in non-bacterized plant.

Plate 16

Native PAGE profile of peroxidase isoforms induced by antagonistic bacteria against *S. rolfsii* in Stevia



- | | | |
|--------------------|-------------------------------------|-------------------------------------|
| 1. Healthy control | 2. <i>S.rolfsii</i> treated | 3. AUPF6 treated |
| 4. AUBS2 treated | 5. AUPF6 + <i>S.rolfsii</i> treated | 6. AUBS2 + <i>S.rolfsii</i> treated |

In another study, Kalaiarasan *et al.* (2006) stated the induction of PO by *P. fluorescens* isolates against root-knot nematode in groundnut where the PO activity was higher in bacterized groundnut inoculated with nematode and isoform analysis showed that two isoforms of PO (PO1 and PO2) were induced in all the treatments and the intensity of the PO2 isoform was higher in bacterized groundnut inoculated with nematode than nematode inoculated and uninoculated healthy plants.

Anita and Samiyappan (2012) also reported that native PAGE analysis of Peroxidase from *P.fluorescens* treated root tissues challenged with the root knot nematode (*M.graminicola*) and the expression of isoforms PO2, PO3, PO4 and PO5 were more prominent in bacterized plants challenge inoculated with the nematode compared to other treatments. The expressions of PO4 and PO5 isoforms were more prominent in *P. fluorescens* treated root tissues challenged with the nematode.

From the results of the study, it can be inferred that application of AUPF6 and AUBS2 isolates of antagonistic bacteria and challenge inoculation with *S.rolfsii* induced two additional isoforms- PO1 and PO2 of peroxidase.

4.3.3.1.4. Polyphenol oxidase activity against *S.rolfsii*

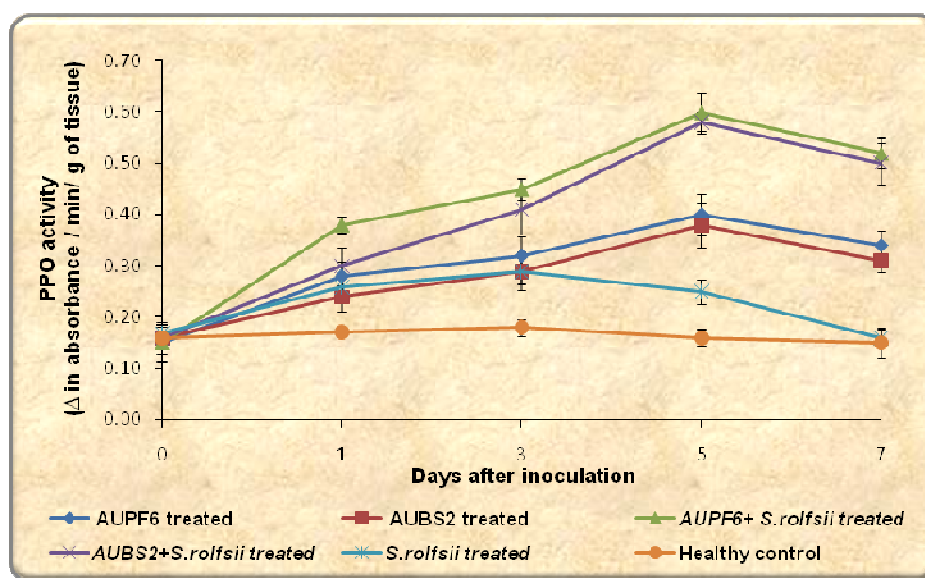
Polyphenol oxidase (PPO) is a copper containing antioxidant enzyme that oxidizes phenolics to highly toxic quinines and contributes to disease resistance. Quinones are presumably toxic to pathogens and also play a significant role in lignin biosynthesis.

PPO usually accumulates upon wounding in plants. Biochemical approaches to understand PPO function and regulation are difficult because the quinone reaction products of PPO covalently modify and cross – link the enzyme (Mayer, 2006).

Figure 3 shows the increase in polyphenol oxidase activity in Stevia plants against *S. rolfsii*

Figure 3

Polyphenol oxidase activity in Stevia against *S.rolfsii*



From the figure, it is evident that the additional increase of PPO in Stevia plants was observed in those treated with the bacterial antagonists *P. fluorescens* AUPF6 + *S. rolfsii*, *B. subtilis* AUBS2 + *S. rolfsii*. In all the bacterial isolate treated and pathogen challenged plants, PPO activity increased from the 0th day after inoculation and reached the maximum on the 5th day of inoculation. However, in the

plants treated with the pathogen alone, PPO activity reached maximum level (0.29 absorbance/min/g of tissue) on the 3rd day of inoculation itself and thereafter decreased with increasing number of days.

The Stevia plants treated with bacterial isolates *P. fluorescens* AUPF6 and *B. subtilis* isolate AUBS2 recorded significantly higher induction of PPO after 5 days of inoculation, (0.4 absorbance / min / g of tissue) and (0.38 absorbance / min / g of tissue), respectively which are on par with each other. However, the plants treated with *P. fluorescens* AUPF6 and challenge inoculated with *S.rolfsii* recorded the highest induction of PPO (0.6 absorbance / min / g of tissue) after 5 days of inoculation, which is on par with *B. subtilis* isolate AUBS2 and challenge inoculated with *S.rolfsii* (0.58 absorbance / min / g of tissue).

It can be noted from the figure that after 5 days of challenge inoculation, the activity of PPO had decreased in all the treatments except in the un-inoculated healthy control plants, whereas, in the plants pre-treated with *P. fluorescens* AUPF6 and challenge inoculated with *S.rolfsii*, PPO activity remained at a higher level of 0.52 absorbance /min / g of tissue even on the 7th day of inoculation.

Similar findings were reported by Ramamoorthy *et al.* (2002a) who stated that an earlier and increased activity of PPO was observed in *P. fluorescens* pre-treated in tomato and pepper plants challenge inoculated with the pathogen and this remained at a higher level throughout the experimental period of 10 days.

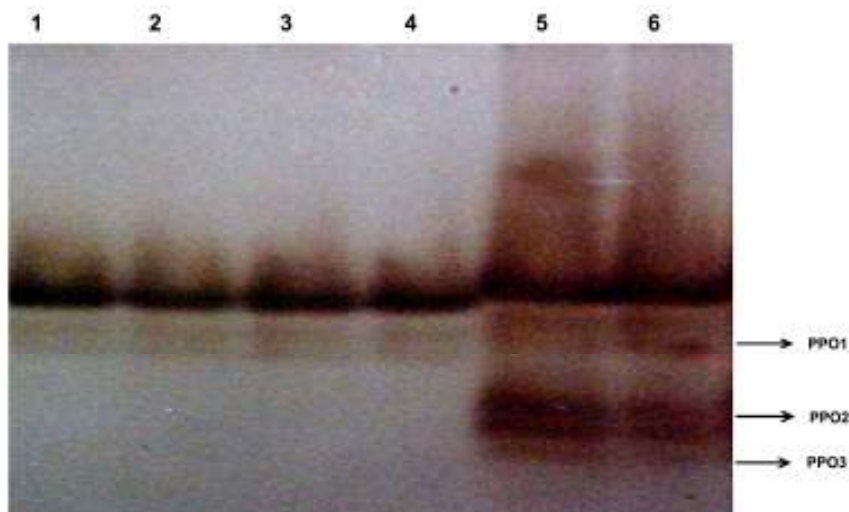
Thus from the findings of the present study, it can be concluded that the activity of PPO was increased in Stevia plants by the soil application of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 and maximum induction was observed in the plants pre-treated with *P. fluorescens* AUPF6 and challenge inoculated with *S. rolfsii*

4.3.3.1.5. Native PAGE analysis of polyphenol oxidase

Plate 17 indicates the native PAGE pattern of polyphenol oxidase activity in Stevia plants against *S. rolfsii*

Plate 17

Native PAGE profile of polyphenol oxidase isoforms induced by antagonistic bacteria against *S. rolf sii* in Stevia



- | | | |
|--------------------|---------------------------------------|---------------------------------------|
| 1. Healthy control | 2. <i>S. rolf sii</i> treated | 3. AUPF6 treated |
| 4. AUBS2 treated | 5. AUPF6 + <i>S. rolf sii</i> treated | 6. AUBS2 + <i>S. rolf sii</i> treated |

It is obvious from the plate, that native PAGE analysis of polyphenol oxidase revealed the presence of three additional isoforms bands representing PPO1, PPO2 and PPO3 which were induced in the plants treated with *P. fluorescens* and *B. subtilis* strains and challenge inoculated with *S. rolf sii*. The induction of isoforms was more prominent in the treatment involving AUPF6 and AUBS2 strains than pathogen inoculated and uninoculated healthy plants.

The above observations are in accordance with the work of Shanmugam *et al.* (2011) who observed the induction of 3 PPO isoforms by the rhizobacterial strains in *Gladiolus*. Another study by Madhaiyan *et al.* (2006) observed that five PPO and PO isozymes could be detected in leaf samples of *Methylobacterium* treated groundnut plants which were challenged with *A. niger* or *S. rolf sii*. Saravanakumar *et al.* (2009) reported that four isoforms of PPO1, PPO2, PPO3 and PPO4 were observed in plants treated with *Pseudomonas* strain after inoculation with *Sarocladium oryzae* causing sheath rot disease in rice, whereas, in the controls, only one isoform was noticed with less intensity.

From that above results, it can thus be inferred that application of AUPF6 and AUBS2 isolates of antagonistic bacteria and challenge inoculation with *S.rolfsii* induced three additional isoforms of PPO, namely, PPO1, PPO2 and PPO3.

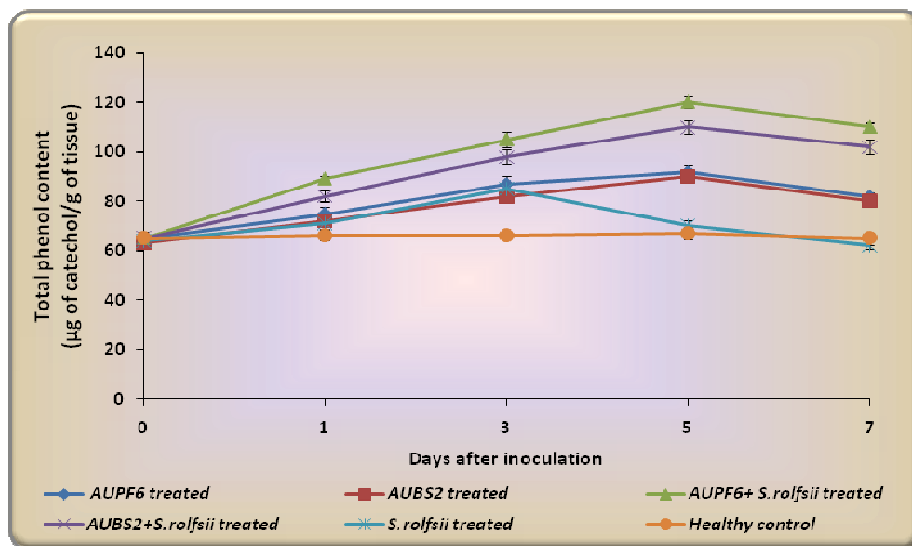
4.3.3.1.6. Total phenol content against *S.rolfsii*

Phenolics are broadly distributed in the plant kingdom and are the most abundant secondary metabolites of plants (Dai and Mumper, 2010). Phenolic compounds may be fungitoxic in nature and may increase the mechanical strength of the host cell wall (Ramamoorthy *et al.*, 2002b).

Figure 4 depicts the induction of total phenols in *Stevia* plants against *S. rolfsii*.

Figure 4

Total phenol content in *Stevia* against *S. rolfsii*



From the figure, it can be deduced that the phenol content had significantly ($p < 0.05$) increased from the 0th day to the 5th day of inoculation and decreased thereafter in all the treatments. The maximum phenol content (120 µg of catechol / g of tissue) was observed on the 5th day in *Stevia* plants pretreated *P. fluorescens* AUPF6 and challenge inoculated with the *S.rolfsii*, which is significantly higher than the other treatments.

Similarly, pre-treatment of *Stevia* plants with *B.subtilis* AUBS2 and challenge inoculated with *S. rolfsii* recorded a phenol content of 110 µg of catechol /g of tissue on the 5th day of challenge inoculation. The other treatments recorded significantly lower phenol content on the same day.

In plants inoculated with the pathogen alone, the phenol content reached maximum level (85 µg of catechol /g of tissue) on the 3rd day of inoculation and thereafter declined to a level below the initial on the 7th day of inoculation.

The above results are in agreement with the reports of Indiragandhi *et al.* (2008) who stated that total phenols were increased on treatment of tomato plant with *Methylobacterium oryzae* CBMB20 and challenge inoculation with *Pseudomonas syringae*. The phenol activity was found to range between 6.32 and 6.76 µg catechol / mg of protein and increased activity was noticed throughout the experimental period. In the inoculated control, the phenol content had gradually declined. Similar results were obtained in rice and peanut also with high accumulation of phenolic compounds when treated with *Methylobacterium* and *S. rolfsii* (Madhaiyan *et al.*, 2006). In another study, Maurya *et al.* (2008) observed that four phenolic acids (gallic, tannic, ferulic and cinnamic acid) were induced in *Trichoderma harizanum* and Plant Growth Promoting Rhizobacteria (Pf4 and Pag) treated and untreated plant leaves. The maximum induction of gallic acid was noted in *Pseudomonas* strain Pf4 (18.86 µg/g) and *T. harizanum* (17.7 µg/g) respectively.

It can therefore be inferred that application of AUPF6 and AUBS2 isolates of antagonistic bacteria and challenge inoculation with *S.rolfsii* in *Stevia* plants induced synthesis of phenols in the plants.

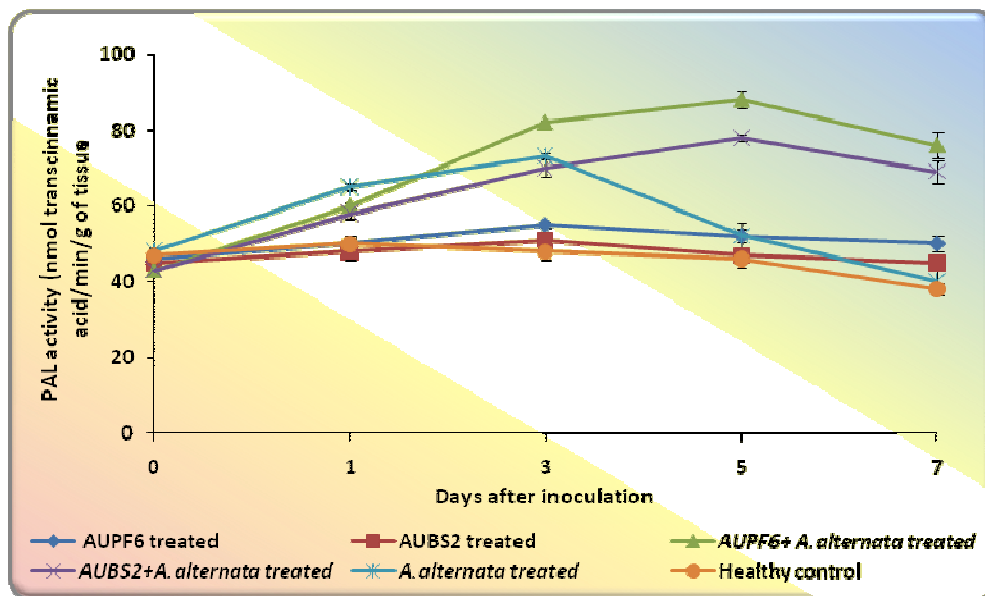
4.3.3.2. Defense-related compounds in *Stevia rebaudiana* plants against *A.alternata*

In the present study, application of bacterial antagonists as foliar spray gave maximum control of *Stevia* leaf spot disease under greenhouse conditions. The efficacy of bacterial antagonists to reduce leaf spot disease might be due to the induction of defense- related enzymes like PAL, PO, PPO and phenols.

4.3.3.2.1. Phenylalanine ammonia lyase activity against *A. alternata*

Figure 5 expresses the induction of phenylalanine ammonia lyase activity in Stevia plants against *A. alternata*

Figure 5
Phenylalanine ammonia lyase activity in Stevia against *A. alternata*



From the figure, it is obvious that the induction of PAL in Stevia plants was observed in those treated with the bacterial antagonists *P. fluorescens* AUPF6 and *B. subtilis* AUBS 2 and pathogen *A.alternata*. In plants treated with the pathogen alone, PAL activity (73 nmol of transscinnamic acid /min / g of tissue) increased from the 0th day of inoculation and reached the maximum on the 3rd day of inoculation and thereafter decreased with increasing number of days.

Among the two bacterial isolates, *P. fluorescens* AUPF6 recorded significantly ($P<0.05$) higher activity of PAL (55 nmol of transscinnamic acid /min / g of tissue) than the *B. subtilis* AUBS2 (51 nmol of transscinnamic acid/ min/ g of tissue) on the 3rd day of inoculation.

However, the plants treated with *P. fluorescens* AUPF6 and challenge inoculated with *A.alternata* recorded significantly ($p<0.05$) higher activity of PAL

(88 nmol of transcinnamic acid/ min/ g of tissue) on the 5th day of inoculation than the plants pre-treated with *B. subtilis* isolate AUBS 2 and challenge inoculated with *A.alternata* (PAL activity of 78 nmol of transcinnamic acid/ min/g of tissue).

After 5 days of challenge inoculation, the activity of PAL had decreased in all the treatments. However, a significantly ($p<0.05$) higher PAL activity (76 nmol of transcinnamic acid/ min/g of tissue) was observed on the 7th day of inoculation in the plants pretreated with *P. fluorescens* AUPF6 and challenge inoculated with *A.alternata* compared to other treatments.

Similar reports have been quoted by several workers. Seedling dip and foliar application of the antagonistic bacteria *B. subtilis* (CBE4) recorded a maximum level of PAL activity in *Phyllanthus amarus* on the 4th day after challenge inoculation with *Corynespora casiiicola* (Mathiyazhagan *et al.*, 2004). Author report stated that the activity of PAL in watermelon plants pretreated with biocontrol agents was induced upon challenge inoculation with *A. alternata* (Umamaheswari *et al.*, 2008). Renuka *et al.* (2007) observed increased activity of PAL in chrysanthemum plants against *Alternaria chlamydospora* pretreated with bacterial antagonist *P. fluorescens* isolate Pf1.

Hence from the above findings, it can be concluded that the activity of PAL in Stevia plants was induced by foliar spraying of *P. fluorescens* AUPF6 and *B. subtilis* AUBS 2 and the maximum induction was observed in the plants pre-treated with *P. fluorescens* AUPF6 and challenge inoculated with *A.alternata*.

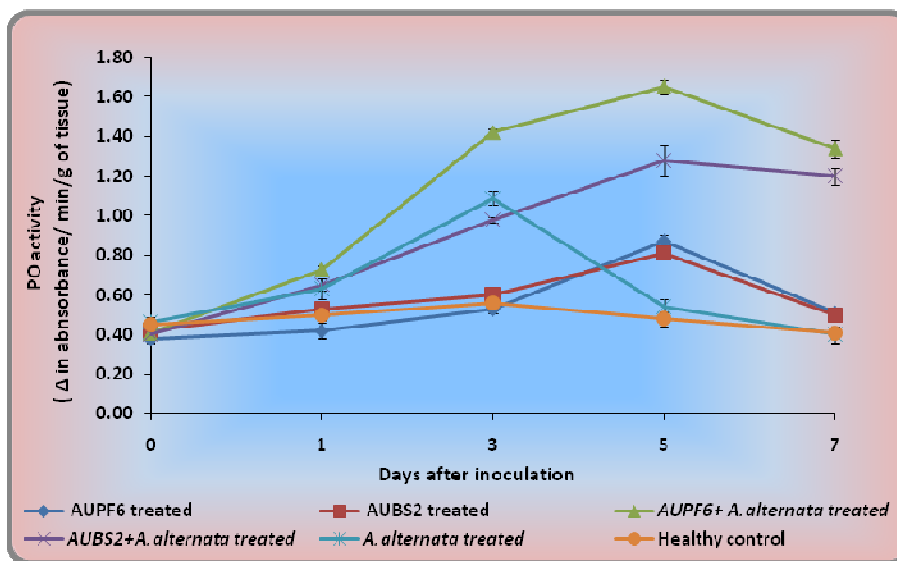
4.3.3.2.2. Peroxidase activity against *A.alternata*

The peroxidase activity in Stevia plants against *A.alternata* is given in Figure 6.

From the figure, it is obvious that the induction of PO in Stevia plants was observed in those treated with the bacterial antagonists *P. fluorescens* AUPF6 and *B. subtilis* AUBS 2 and pathogen *A.alternata*. In all the bacterial isolate-treated and pathogen challenge inoculated plants, PO activity increased from the 0th day of inoculation and reached the maximum on the 5th day of inoculation. However, in the

plants treated with the pathogen alone, PO activity reached the maximum level (1.09 absorbance /min / g of tissue) on the 3rd day of inoculation itself and thereafter decreased with increasing number of days.

Figure 6
Peroxidase activity in Stevia against *A. alternata*



Among the two bacterial isolates, *P. fluorescens* AUPF6 induced significantly ($p < 0.05$) more activity of PO (0.87 absorbance /min / g of tissue) than the *B. subtilis* isolate AUBS 2 (0.81 absorbance /min / g of tissue). However, the plants treated with *P. fluorescens* AUPF6 and challenge inoculated with *A. alternata* recorded a significantly ($p < 0.05$) higher induction of PO (1.65 absorbance / min / g of tissue) on 5th day of inoculation, whereas, the plants pretreated with *B. subtilis* isolate AUBS2 and challenge inoculated with *A. alternata* recorded a PO activity of 1.28 absorbance / min/ g of tissue on the 5th day of inoculation.

After 5 days of challenge inoculation, the activity of PO had decreased in all the treatments, whereas, in the plants pre-treated with *P. fluorescens* AUPF6 and challenge inoculated with *A. alternata*, PO activity remained at a higher level of 1.34 absorbance /min /g of tissue on 7 days of inoculation.

Similar findings were reported by Anand *et al.* (2009) who recorded higher accumulation of PO in Pf1 treated cucumber plants challenged with

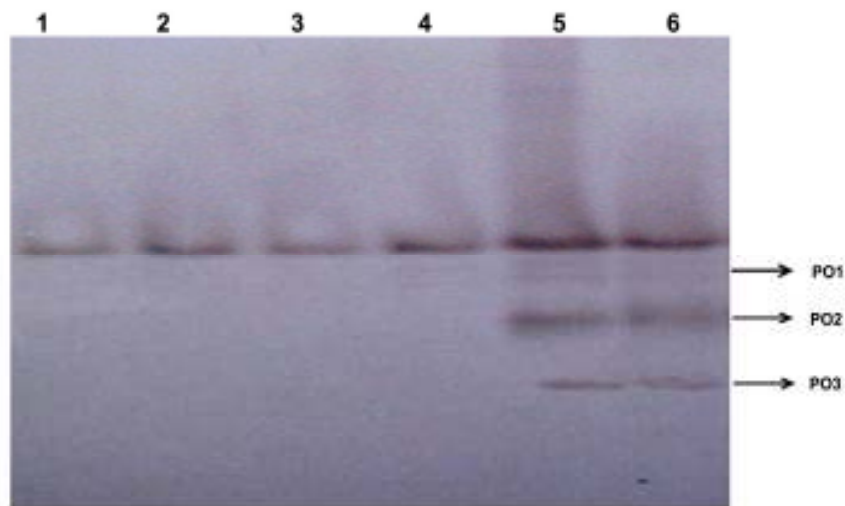
Pseudoperonospora cubensis. In another study, Saravanakumar *et al.* (2007) reported higher PO activity in mungbean plants after challenge inoculation with *M. phaseolina* pretreated with Pf1 bioformulation. High level of expression of PO was reported in *P. fluorescens* (Pf1) treated tomato plants challenged with *Fusarium oxysporum* (Ramamoorthy *et al.*, 2002b). The *P. fluorescens* strains, Pf1 and FP7 were found to be the best inducers of plant chitinase and peroxidases, which are the most important compounds of Induced systemic resistance (Nandakumar *et al.*, 2001).

From the above findings, it can be concluded that foliar application of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 increased the activity of PO in *Stevia* plants against *A. alternata*.

4.3.3.2.3. Native PAGE analysis of Peroxidase

Plate 18 indicates the native PAGE pattern of peroxidase activity in *Stevia* plants against *A. alternata*

Plate 18
Native PAGE profile of peroxidase isoforms induced by antagonistic bacteria against *A. alternata* in *Stevia*



1. Healthy control	2. <i>A. alternata</i> treated	3. AUPF6 treated
4. AUBS2 treated	5. AUPF6 + <i>A. alternata</i> treated	6. AUBS2+ <i>A. alternata</i> treated

From the plate, it is evident that the banding pattern of peroxidase by native PAGE revealed the presence of three additional bands indicating three isoforms PO1, PO2 and PO3 in the plants treated with *P. fluorescens* and *B. subtilis* strains and challenge inoculated with *A. alternata*. The induction of isoforms was more prominent in the treatment involving AUPF6 and AUBS2 strains when compared to pathogen inoculated plants and uninoculated healthy plants.

The results of the present studies are in accordance with the work of Karthikeyan *et al.* (2006a) who reported that the treatment of coconut plants with *P. fluorescens*, *Trichoderma viride* and chitin induced four isoforms PO1, PO2, PO3 and PO4 in coconut palms infected with *Ganoderma lucidum*. Expression of PO1 and PO2 was found in all the groundnut plants treated with Pf1 followed by challenge inoculation with *A. alternata* (Chitra *et al.*, 2006).

In another study, four isoforms PO1, PO2, PO3 and PO4 expressed in pseudomonads Pf1, TDK1 and PY15 treated rice plants inoculated with *Sarocladium oryzae* (Saravanakumar *et al.*, 2009). Latha *et al.* (2009) also confirmed induction of PO isoforms by Pf1 + Py15 + Bs16 + Zimmu treated plants inoculated with *A. solani* and identified expression of three isoforms PO1, PO2 and PO3.

From that above results, it can be inferred that application of AUPF6 and AUBS2 isolates of antagonistic bacteria and challenge inoculation with *A. alternata* induced three additional isoforms of peroxides namely, PO1, PO2 and PO3.

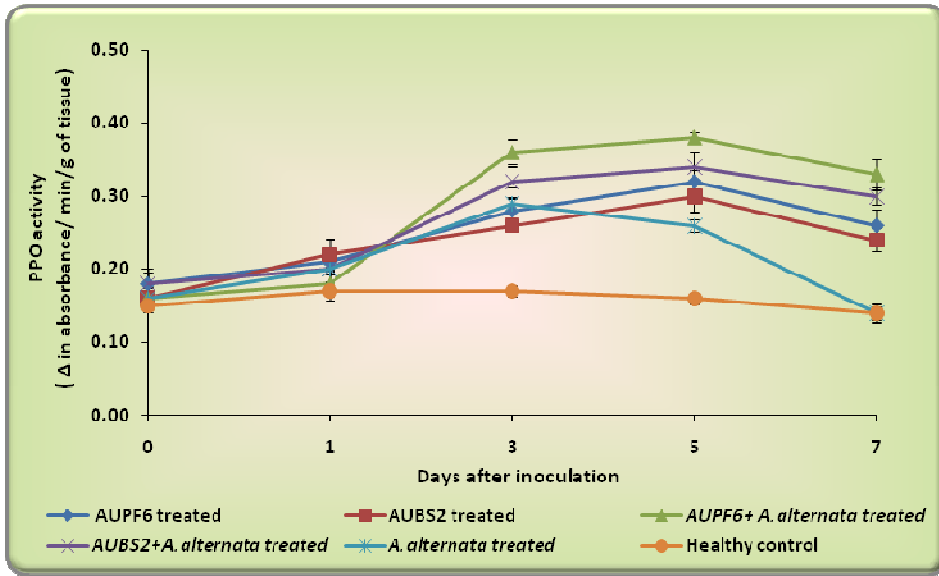
4.3.3.2.4. Polyphenol oxidase activity against *A. alternata*

The polyphenol oxidase activity in Stevia plants is given in Figure 7

From the figure, it can be seen that an additional increase of PPO in Stevia plants was observed in those treated with the bacterial antagonists *P. fluorescens* AUPF6 and *B. subtilis* AUBS 2 and pathogen *A. alternata*. In all the bacterial isolate-treated and pathogen challenge inoculated plants, PPO activity increased from the 0th day of inoculation and reached maximum on the 5th day of inoculation. However, in the plants treated with pathogen alone, PPO activity reached the

maximum level (0.29 absorbance/min/g of tissue) on the 3rd day of inoculation and thereafter decreased with increasing number of days.

Figure 7
Polyphenol oxidase activity in Stevia against *A. alternata*



Among the two bacterial isolates, *P. fluorescens* AUPF6 induced significantly ($p < 0.05$) more activity of PPO (0.32 absorbance /min / g of tissue) than the *B. subtilis* isolate AUBS2 (0.30 absorbance / min/g of tissue). However, the plants treated with *P. fluorescens* AUPF6 and challenge inoculated with *A. alternata* recorded the highest induction of PPO (0.38 absorbance /min /g of tissue) while, the plants pretreated with *B. subtilis* isolate AUBS2 and challenge inoculated with *A. alternata* exerted a PPO activity of 0.34 absorbance /min/ g of tissue on 5th day of challenge inoculation.

After 5 days of challenge inoculation, the activity of PPO had decreased in all the treatments, whereas, in the plants pre-treated with *P. fluorescens* AUPF6 and challenge inoculated with *A. alternata*, PPO activity remained at a significantly ($p < 0.05$) higher level of 0.33 absorbance /min / g of tissue after 7 day of inoculation.

These results are in accordance with those of other researchers. Increased activities of PPO in *P. fluorescens* Pf1 pretreated groundnut plants challenged with *S. rolfisii* was observed by Karthikeyan *et al.* (2006b). Three to four fold increases in the activity of PPO in hot pepper seedlings pretreated with *B. subtilis* strain BSCBE4 and *P. chlororaphis* strain PA23 inoculated with *P. aphanidermatum* was recorded by Nakkeeran *et al.* (2006). The mixture of *Pseudomonas* strains Pf1, TDK1 and PY15 induced a higher activity of PPO in rice plants against *S. oryzae* compared to individual strains and untreated controls has been reported by Saravanakumar *et al.* (2009).

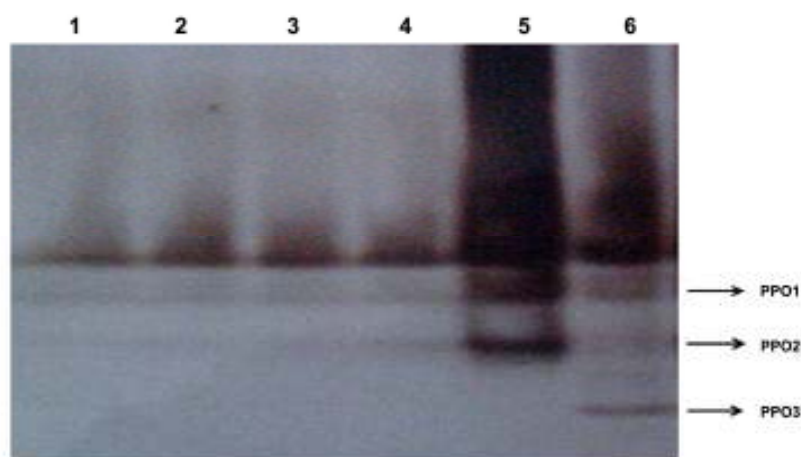
From the above findings, it can be concluded that the activity of PPO induced by foliar application of *P. fluorescens* AUPF6 and *B. subtilis* AUBS 2 in Stevia plants and maximum induction was observed in the plants pre-treated with *P. fluorescens* AUPF6 and challenge inoculated with *A.alternata*.

4.3.3.2.5. Native PAGE analysis of polyphenol oxidase

Plate 19 indicates the Native PAGE analysis of polyphenol oxidase activity in Stevia plants against *A.alternata*

Plate 19

Native PAGE profile of polyphenol oxidase isoforms induced by antagonistic bacteria against *A.alternata* in Stevia



- | | | |
|--------------------|---------------------------------------|--------------------------------------|
| 1. Healthy control | 2. <i>A.alternata</i> treated | 3. AUPF6 treated |
| 4. AUBS2 treated | 5. AUPF6 + <i>A.alternata</i> treated | 6. AUBS2+ <i>A.alternata</i> treated |

It is clearly seen from the plate that Native PAGE analysis of polyphenol oxidase is revealed the presence of two additional isoforms PPO1 and PPO2 in the plants treated with *P. fluorescens* and challenge inoculated with *A.alternata*. On the other hand, the plants treated with *B.subtilis* AUBS2 and challenge inoculated with *A.alternata* recorded a weak induction of three isoforms PPO1, PPO2 and PPO3. The induction of isoforms was more prominent in treatment involving AUPF6 than AUBS2 strains.

The above observations are on par with the reports of Latha *et al.* (2009) who observed three isoforms of PPO1, PPO2 and PPO3 in *Pseudomonas* strains Pf1 + Py15 + Bs16 + Zimmu treated plants after inoculation with *A. solani*, whereas in the controls, only two isoforms were noticed with less intensity. A higher induction of PPO was observed in plants pretreated with mixture of Pf1 + Py15 + Bs16 + Zimmu leaf extract inoculated. Another study by Anita and Samiyappan, (2012) quoted that 5 PPO isoforms, PPO1, PPO2, PPO3, PPO4 and PPO5 were observed in bacterized rice root tissues, inoculated with root-knot nematode. The induction of isoforms PPO1 and PPO2 were observed in all the treatments except in healthy plants. The Isoforms, PPO3 and PPO4 were detected both in bacterized root tissues and root knot infected roots but it was more prominent in root tissues treated with *P. fluorescens* challenge inoculated with the nematode.

From that above results, it can be inferred that foliar application of *P. fluorescens* AUPF6 strongly induced two isoforms of PPO, whereas AUBS2 isolate of *B.subtilis* recorded three weakly induced isoforms.

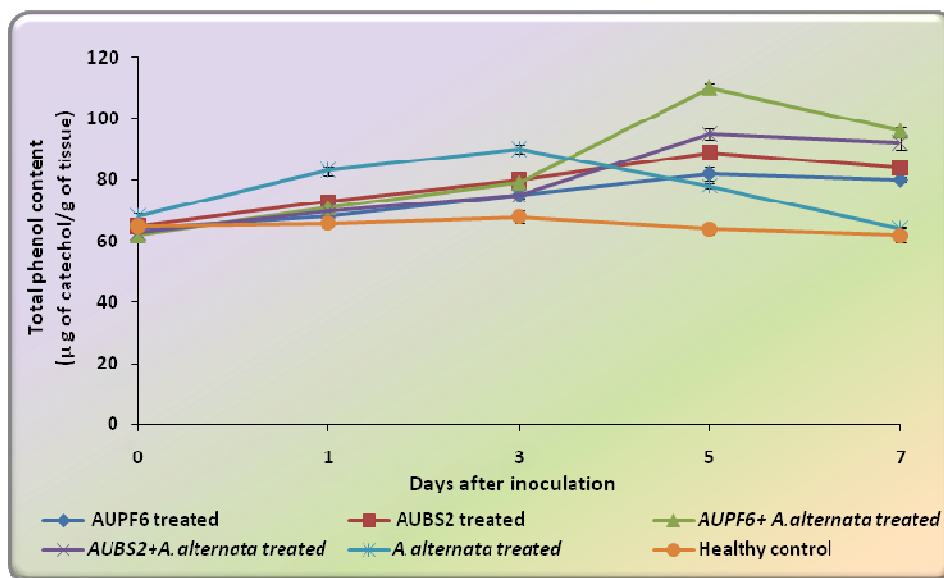
4.3.3.2.6. Total phenol content against *A. alternata*

The results of induction of total phenols in Stevia plants in response to bacterial antagonist *P. fluorescens* AUPF6 and *B.subtilis* AUBS2 and pathogen *A. alternata* are presented in Figure 8.

From the figure, it can be said that the phenol content was increased in Stevia plants by treatment with *P.fluorescens* AUPF6 and *B.subtilis* AUBS2 and pathogen *A. alternata*.

Figure 8

Total phenol content in Stevia against *A. alternata*



Significantly ($p < 0.05$) higher phenol content was observed in *P. fluorescens* AUPF6 pre-treated plants challenge inoculated with the pathogen ($110 \mu\text{g}$ of catechol / g of tissue) on the 5th day of the pathogen challenge. Similarly, pre-treatment of plants with *B. subtilis* AUBS2 recorded the higher phenol content ($95 \mu\text{g}$ of catechol / g of tissue) on the 5th day of inoculation followed by a decline after the 5th day.

In plants inoculated with the pathogen alone, the phenolic content reached a maximum level on the 3rd day of inoculation and thereafter declined below the initial level on the 7th day of inoculation. Plants treated with *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 alone also had a significant increase of phenolics compared to the untreated plants.

The above findings are supported by Girish and Umesha (2005) who stated that the rate of reduction in bacterial canker disease incidence was directly proportional to the increased level of total phenol content. In another study, Dutta *et al.* (2008) recorded increased level of phenol content in the leaves of pigeon pea against *Fusarium* pretreated with PGPR. Anand *et al.* (2009) also reported that

P. fluorescens pre-treated chilli plants challenge inoculated with *Colletotrichum capsici* and *Leveillula taurica* showed higher accumulation of total phenols.

From that above results, it can be inferred that foliar application of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 isolates of antagonistic bacteria and challenge inoculation with *A. alternata* induced synthesis of phenols when compared to un-inoculated control.

Phase III – Highlights of the findings

- Soil application of talc-based formulation of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 isolates, at the rate of 10 g/ kg of soil effectively reduced root rot disease incidence in Stevia
- Foliar spray of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 isolates at 0.2% concentration significantly reduced leaf spot disease incidence in Stevia
- Application of talc-based formulation of *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 isolates induced defense enzymes like PAL, PO, PPO and total phenol content of the Stevia
- Two peroxidase isoforms such as PO1 and PO2 were induced in Stevia plants treated with *P. fluorescens* and *B. subtilis* strains and challenge inoculated with *S. rolfsii* and *A. alternata*
- Three polyphenol oxidase isoforms PPO1, PPO2 and PPO3 were induced in Stevia plants treated with *P. fluorescens* AUPF6 and *B. subtilis* AUBS2 strains and challenge inoculated with *S. rolfsii*
- Two polyphenol oxidase isoforms PPO1 and PPO2 were induced in Stevia plants treated with *P. fluorescens* AUPF6 and challenge inoculated with *A. alternata*
- Three isoforms of PPO like PPO1, PPO2 and PPO3 were induced in Stevia plants pre-treated with *B. subtilis* AUBS2 and challenge inoculated with *A. alternata*

An outline of the findings of the present study and conclusion drawn thereof are presented in the following chapter.