Results and Discussion

4.0 RESULTS AND DISCUSSION

The herb *Stevia rebaudiana* is a potential source of low calorie sweeteners. Leaf spot on Stevia caused by *Alternaria alternata* is a serious foliar disease. In India, research work on this disease is very limited. Keeping in mind the sweetening property and other medicinal uses of *S. rebaudiana,* an attempt has been made to evaluate the efficacy of bacterial antagonists *Pseudomonas fluorescens* and *Bacillus subtilis* against *A. alternata* both under *in vitro* and greenhouse conditions. The results obtained are discussed herein.

4.1 *In vitro* screening of *P.fluorescens* isolates against *A. alternata*

Ten isolates of *P. fluorescens* were screened for their efficacy in inhibiting the mycelial growth of *A. alternata* under *in vitro* condition. Among the ten isolates of *P. fluorescens*, AUPF2 recorded lowest radial mycelial growth of *A. alternata* (59.23 mm). This was followed by AUPF3 (68.10 mm), AUPF5 (70.27 mm), AUPF1 (73.50 mm) and AUPF4 (75.33 mm) compared to control (90 mm).

The isolate AUPF2 was significantly superior followed by AUPF3 by recording maximum higher inhibition zone of 20.80 mm and 11.90 mm, respectively. The isolates AUPF5, AUPF1 and AUPF4 showed inhibition zone of 10.30 mm, 6.76 mm and 5.06 mm, respectively. The other five isolates of *P. fluorescens* (AUPF6, AUPF7, AUPF8, AUPF9 and AUPF10) were lesser in their efficacy to inhibit the mycelial growth of *A. alternata* (Table 1; Plate 6).

These results concur with those of other researchers. The *in vitro* antagonistic activity of *P. fluorescens* was reported by Indira *et al.* (2006) where *P. fluorescens* was found to be more efficient in inhibiting the colony growth of *A. alternata* to 64.9%.

Table 1. Effect of *P. fluorescens* isolates on radial mycelial growth of *A. alternata*

S. No	*P. fluorescens* isolates	Mycelial growth (mm)*	Inhibition zone (mm)*
1	AUPF1	73.50^{d}	6.76^{d}
2	AUPF2	59.23^{a}	20.80^{a}
3	AUPF3	68.10^{b}	11.90^{b}
4	AUPF4	75.33^{e}	5.06^{e}
5	AUPF5	70.27^{c}	10.30^{c}
6	AUPF6	84.33^{g}	-
7	AUPF7	86.50^{h}	-
8	AUPF8	80.50^{f}	-
9	AUPF9	84.33^{g}	-
10	AUPF10	89.0^{i}	-
11	Control	90.0^{i}	-

*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

Siddiqui (2007) studied the effect of 18 isolates of *Bacillus* and *Pseudomonas* on *A. triticina* under *in vitro* condition and reported that the isolates Pa22, Pa23, Pa26, Pf27, Pa28, B25, B27, B28 and B30 showed inhibitory effects. Two isolates of *P. fluorescens* (Pf1 and Py15) and one isolate of *B. subtilis* (Bs16) were effective in reducing the mycelial growth of *A. solani* under *in vitro* condition with 75.5 mm, 78.5 mm and 76.5 mm, respectively (Latha *et al.*, 2009).

4.2 *In vitro* screening of *B. subtilis* isolates against *A. alternata*

Among the ten isolates of *B. subtilis* tested against *A. alternata,* AUB2 recorded minimum radial mycelial growth of *A. alternata* (69.97 mm), followed by AUB4 (71.13 mm) and AUB8 (73.13 mm) compared to control (90 mm). AUB2, AUB4 and AUB8 were significantly superior in recording higher inhibition zone of 10.13 mm, 9.07 mm and 7.10 mm, respectively (Table 2; Plate 7). None of the other isolates of *B. subtilis* (AUB1, AUB3, AUB5, AUB6, AUB7, AUB9 and AUB10) were effective in controlling *A. alternata* which was indicated by the absence of inhibition zone and the pathogen was found to overgrow the antagonist.

The results are similar to those obtained for *A. alternata* leaf spots of pepper, where the antagonistic activity of *B. subtilis* (HS93) against *A. alternata* (54%) was documented by Sid *et al.* (2003). Hou *et al.* (2006) reported that *B. subtilis* strain LEV-006 was antagonistic to four major fungal pathogen of canola including *A. brassicae*.

Table 2. Effect of *B. subtilis* isolates on radial mycelial growth of *A. alternata*

S. No	*B. subtilis* isolates	Mycelial growth (mm)*	Inhibition zone (mm)*
1	AUB1	82.83^{d}	-
2	AUB2	69.97^{a}	10.13^{a}
3	AUB3	84.33^{e}	-
4	AUB4	71.13^{b}	9.07^{b}
5	AUB5	85.83^{gh}	-
6	AUB6	83.50^{d}	-
7	AUB7	85.0^{e}	-
8	AUB8	73.13^{c}	7.10^{c}
9	AUB9	86.33^{h}	-
10	AUB10	85.33^{g}	-
11	Control	90.0^{i}	-

*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 radial mycelial growth of *A. palandui*, a pathogen for onion leaf blight was drastically inhibited by *P. fluorescens* (Pf1) (66.74%) followed by *B. subtilis* (65.59%) (Karthikeyan *et al.*, 2008).

Plate 6

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

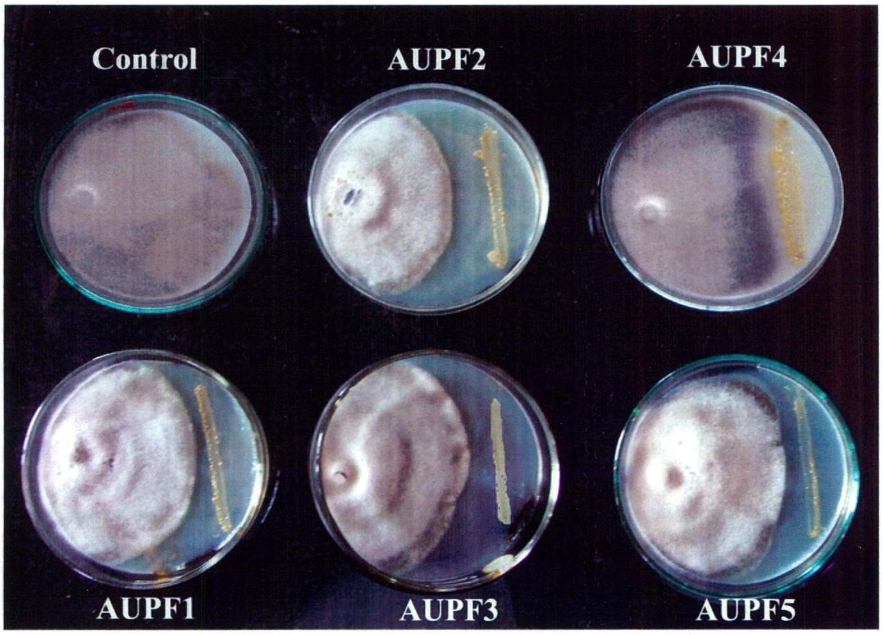
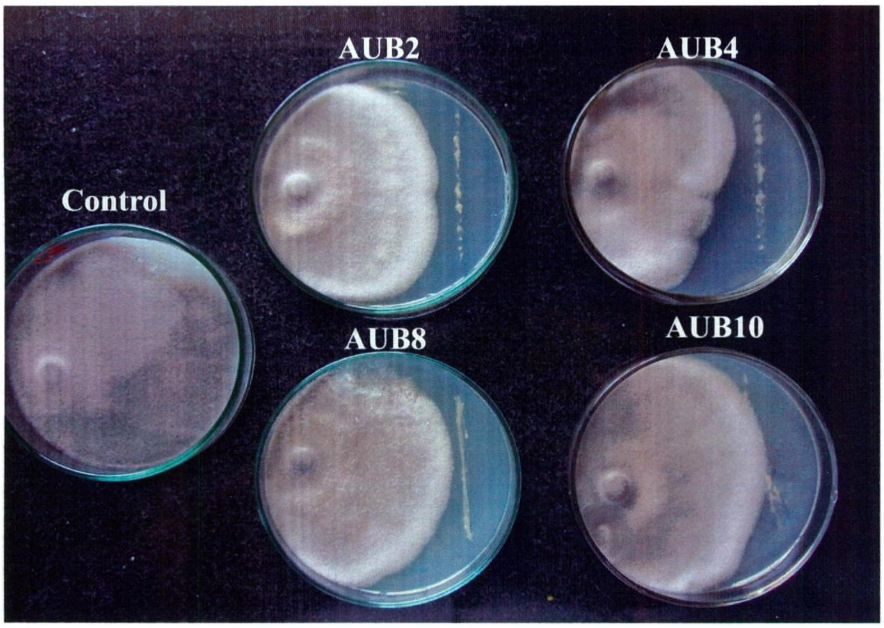


Plate 7

In vitro screening of *B. subtilis* against *A. alternata*



Based on the result, AUPF2, AUPF3, AUB2 and AUB4 were found effective in inhibiting the mycelial growth of *A. alternata* under *in vitro* condition.

4.3 Antimicrobial compounds of *P. fluorescens* and *B. subtilis* isolates

The effective isolates AUPF2, AUPF3, AUB2 and AUB4 were screened for the production of antimicrobial compounds like hydrogen cyanide, salicylic acid, siderophores and indole acetic acid.

4.3.1 Hydrogen cyanide (HCN) production

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 (Vining, 1990). Ellis *et al.* (2000) reported a significant correlation between biological control activity and the accumulation of specific cyclopropane fatty acid and hydrogen cyanide.

Isolate AUPF2 and AUPF3 showed moderate HCN production of 0.063 and 0.066 OD (1 unit = 0.001 Absorbance) whereas isolate AUB2 and AUB4 recorded weak HCN production with 0.041 and 0.050 OD, respectively (Table 3; Plate 8 and Plate 9).

These results concur with those of other researchers. The production of HCN by *Pseudomonas* GRC2 was recorded by Gupta *et al.* (2002). Kamalakannan *et al.* (2004) reported that *P. fluorescens* isolate PfC6 produced HCN in higher amount.

Table 3. Hydrogen cyanide production by antagonistic bacterial isolates

S. No	Antagonist	HCN production*	
		Qualitative analysis	Quantitative analysis (1 unit = 0.001 Absorbance at 625 nm)
1	AUPF2	Moderate	0.063^{b}
2	AUPF3	Moderate	0.066^{a}
3	AUB2	Weak	0.041^{d}
4	AUB4	Weak	0.050^{c}

*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

Senthilkumar *et al.* (2009) screened different bacterial isolates for HCN production of which isolate HKA-72, HKA-107 and HKA-109 belonging to genus *Bacillus* produced HCN. Ahmadzadeh and Tehrani (2009) have documented that *P. fluorescens* isolates UTPF7, UTPF13, UTPF18, UTPF22, UTPF27 and CHA0 produced HCN.

4.3.2 Salicylic acid production

Salicylic acid is a natural phenolic compound present in many plants and is an important component in the signal transduction pathway. Salicylic acid is also involved in local and systemic resistance to pathogens. These induced defense responses by SA are probably involved

Plate 8
Production of HCN by *P. fluorescens* isolates

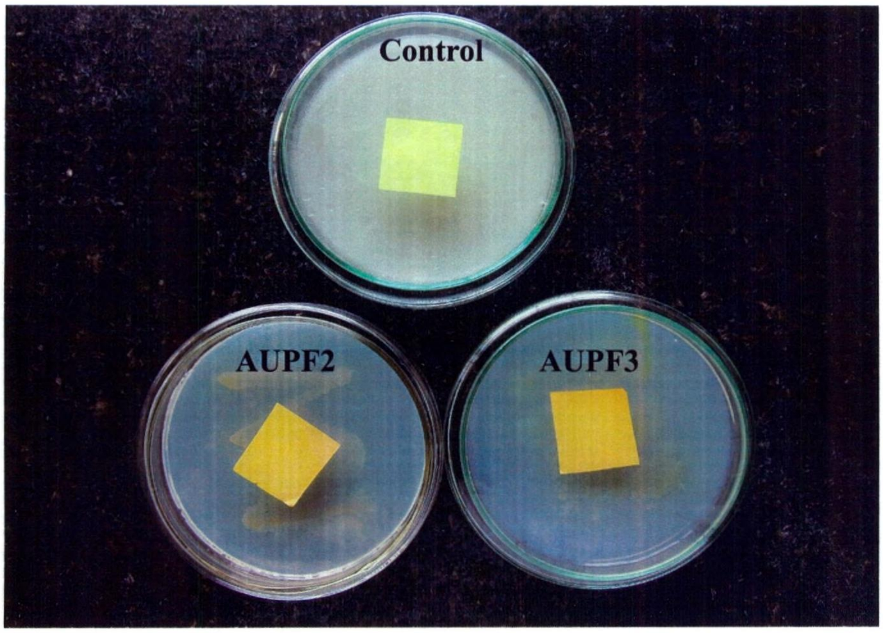
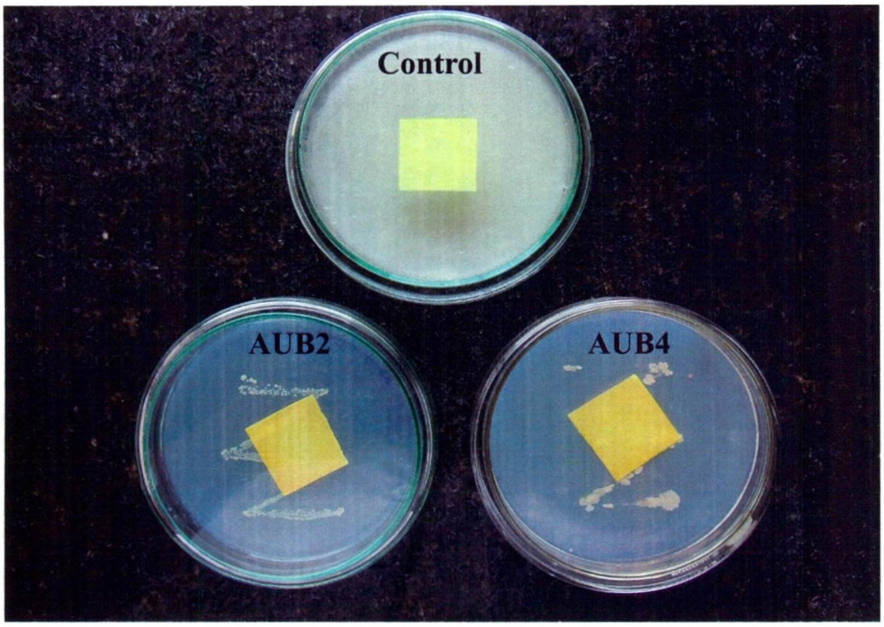


Plate 9
Production of HCN by *B. subtilis* isolates



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 (Meena *et al*., 2001).

The selected isolates were screened for salicylic acid production. Maximum production of salicylic acid was recorded in AUPF2 (28.18 μg ml^{-1}) followed by AUPF3 (26.63 μg ml^{-1}), AUB2 (25.50 μg ml^{-1}) and AUB4 (16.20 μg ml^{-1}) (Table 4).

Karthikadevi (2004) reported that among the five isolates of *P. fluorescens* tested for their ability to produce salicylic acid, Pf KS 3 showed more salicylic acid production (21.8 μg/ml). Similar studies have been documented by Santhini *et al.* (2005), where the isolate PfUA7 showed maximum salicylic acid production (159.70 μg/ml) among the twelve isolates of *P. fluorescens* tested.

Table 4. Salicylic acid production by antagonistic bacterial isolates

S. No	Antagonist	Salicylic acid production (μg/ml)*
1	AUPF2	28.18^{a}
2	AUPF3	26.63^{b}
3	AUB2	25.50^{c}
4	AUB4	16.20^{d}

*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

Kloepper *et al.* (2004) reported that specific strain of *Bacillus* sp. elicits significant reductions in the incidence or severity of various diseases by salicylic acid production. Nandini (2007) tested six isolates of *B. subtilis* for their ability to produce salicylic acid and revealed that all the isolates produced salicylic acid, among which the isolate AUBS2 showed maximum production (17.89 μg/ml).

4.3.3 Siderophore production

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 Fe, Mo and V) while excluding toxic ones (such as W). They excrete compounds previously identified as siderophores that bind a variety of metals in addition to iron. At low concentrations, complexes of essential metals (Fe, Mo,V) 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 (Tokala *et al.,* 2002).

None of the isolates were found to produce carboxylate type of siderophore. All isolates produced hydroxamate type of siderophore. In the quantitative production of siderophore, AUPF2 significantly produced more amount of siderophore (45 μg ml^{-1}) followed by AUPF3 (43.6 μg ml^{-1}), AUB2 (19.43 μg ml^{-1}) and AUB4 (15.63 μg ml^{-1}) (Table 5).

Similar results were achieved by several researchers. Gupta *et al.* (2002) detected hydroxamate type of siderophore production by *Pseudomonas* GRC2 strain. Lee *et al.* (2007) reported that isolates of

B. subtilis R33 and R13 exhibited positivity towards siderophore production.

Table 5. Siderophore production by antagonistic bacterial isolates

S. No	Antagonist	Siderophore production (µg/ml)*
1	AUPF2	45.0^{a}
2	AUPF3	43.6^{b}
3	AUB2	19.43^{c}
4	AUB4	15.63^{d}

*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 majority of bacteria isolated from rhizospheres of groundnut and mung bean predominantly produced catechol type siderophores except for a few fluorescent pseudomonads that produced hydroxamates in addition to catecholates (Joshi *et al*., 2006).

Recently, Sayyed and Chincholkar (2009), demonstrated the antifungal activity of siderophore rich culture broth, siderophore rich supernatant and purified siderophore preparation against *Aspergillus niger* NCIM 1025, *A. flavus* NCIM 650, *Fusarium oxysporum* NCIM 1008 and *Alternaria alternata* IARI 715.

4.3.4 Indole Acetic Acid (IAA) production

Indole acetic acid biosynthesis is also wide spread in plant associated bacilli and considered to be directly involved in plant growth promotion (Tsavkelova *et al*., 2007; Idris *et al*., 2007). In addition to siderophores and salicylic acid production, production of IAA by rhizobacterium was also implicated in ISR (Gupta *et al*., 2000).

Among the tested isolates, AUPF2 showed maximum IAA production of 3.5 μg ml^{-1}, followed by AUPF3, AUB2 and AUB4 with 2.4, 1.8, and 1.2 μg ml^{-1}, respectively (Table 6).

Table 6. Indole acetic acid (IAA) production by antagonistic bacterial isolates

S. No	Antagonist	Indole acetic acid production (μg/ml)*
1	AUPF2	3.5^{a}
2	AUPF3	2.4^{b}
3	AUB2	1.8^{c}
4	AUB4	1.2^{d}

*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

Similar result has also been obtained by other researchers. Singh *et al*. (2008) screened ten isolates of *B. subtilis* for IAA production and found eight isolates positive. Karnwal (2009) tested 30 isolates of fluorescent pseudomonads for their ability to produce indole acetic acid in pure culture in the absence and presence of L-tryptophan at 50, 100, 200 and 500 µg/ml. Among which two strains, *Pseudomonas fluorescens* AK1 and *Pseudomonas aeruginosa* AK2, showed increased indole production with increase in tryptophan concentration (0.5, 1.2, 4.3 and 9.3 µg/ml and 0.2, 0.7, 3.8, and 8.3 µg/ml, respectively). Ali *et al.* (2009) reported that production of IAA by *Bacillus* spp ranges from 0.60 to 3.0 µg/ml in L-broth medium supplemented with 1,000 µg/ml L-tryptophan.

4.4 Induction of defense related enzymes by biocontrol agents

Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, whereby the plant's innate defenses are potentiated against subsequent biotic challenges. This enhanced state of resistance is effective against a broad range of pathogens and parasites (van Loon, 2000). Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are two forms of induced resistance wherein plant defenses are preconditioned by prior infection or treatment that results in resistance against subsequent challenge by a pathogen or parasite (Choudhary *et al.*, 2007).

ISR is potentiated by plant growth promoting rhizobacteria (PGPR), of which best characterized are strains that belong to genus *Pseudomonas* that cause no visible damage to the plant's root system (van Loon and Glick, 2004). Biocontrol strains stimulate the activities of defence enzymes (phenylalanine ammonia lyase, peroxidase and polyphenol oxidase) in plants that could be involved in the synthesis of phytoalexins (Chen *et al.*, 2000; van Loon and Bakker, 2005). Rajendran *et al.* (2007) demonstrated the endophytic behaviour of *Bacillus* and *Pseudomonas* strains that mediates the disease resistance in crop plants against plant pathogens.

In the present study, application of biocontrol agents as foliar spray gave maximum control of Stevia leaf spot under greenhouse condition (Plate 10). The efficacy of biocontrol agents to reduce leaf spot disease might be due to the induction of defense related enzymes like PAL, PO, PPO and phenols.

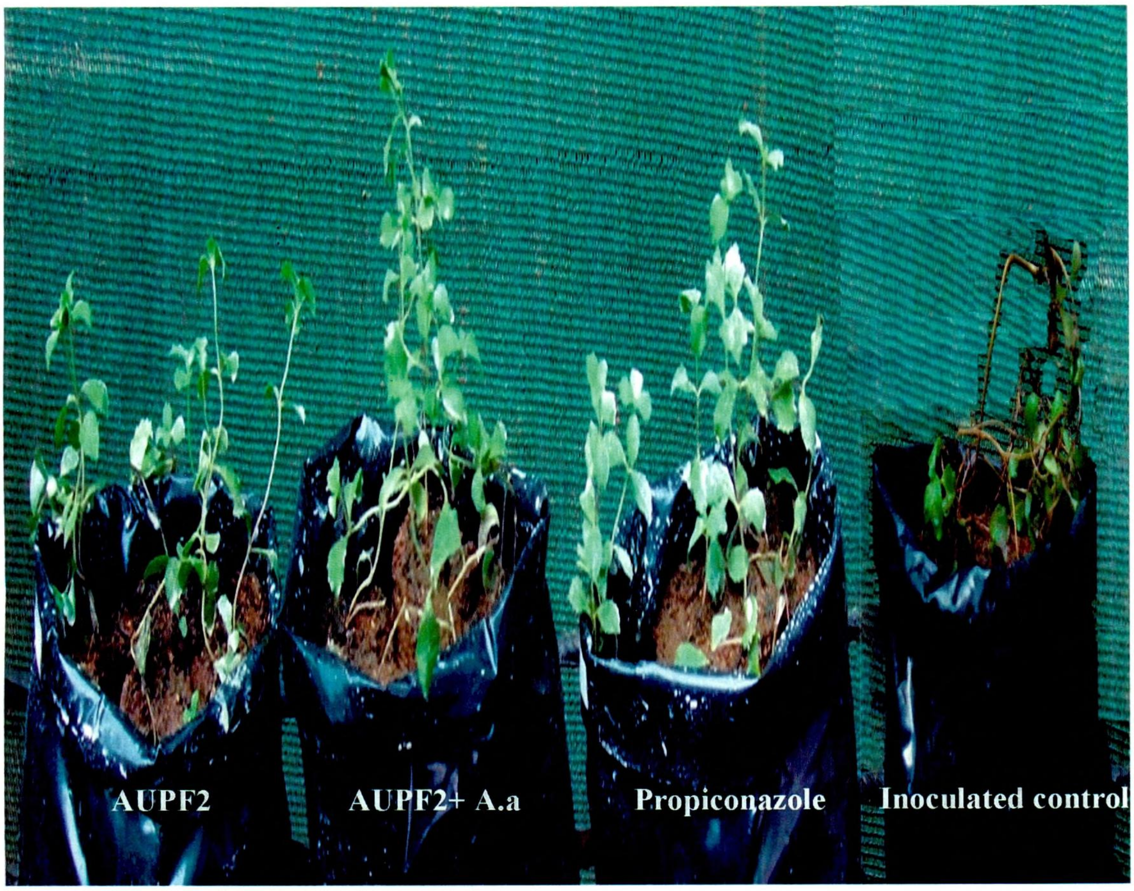
4.4.1. Changes in phenylalanine ammonia lyase (PAL) activity in *Stevia* plants

PAL plays an important role in the biosynthesis of various defense chemicals in phenyl propanoid metabolism (Daayf *et al.*, 1997). Induction of defense enzymes like PAL is one of the responses of the host for treatment with biocontrol agents (Zodor and Anderson, 1992). PAL activity could be induced in plant pathogen interactions and fungal elicitor treatment (Ramanathan *et al.*, 2000).

In Stevia, foliar application of biocontrol agents induced the plants to synthesis PAL, where as an additional increase in the PAL was observed in biocontrol agents challenged with *A. alternata.* Thus there was an increase in the activity of PAL in all the treatments when compared to the uninoculated control. In plants treated with pathogen alone, increased activity of PAL was observed for a period of 1-3 days than uninoculated plants, but lower than the treated plants and thereafter the activity declined to the level less than the uninoculated plants .The

Plate 10

Effect of foliar application of biocontrol agents against leaf spot disease on *S. rebaudiana* under greenhouse condition



A.a - ***Alternaria alternata***

activity of PAL reached a maximum level on 5th day (138 nmols of trans-cinnamic acid/min/g of leaf tissue) after challenge inoculation in AUPF2 treatment and thereafter the activity declined slowly throughout the experimental period up to 7th day. AUB2 treated plants challenged with *A. alternata* recorded 115 nmols of trans-cinnamic acid/min/g of leaf tissue on 5th day (Fig 1).

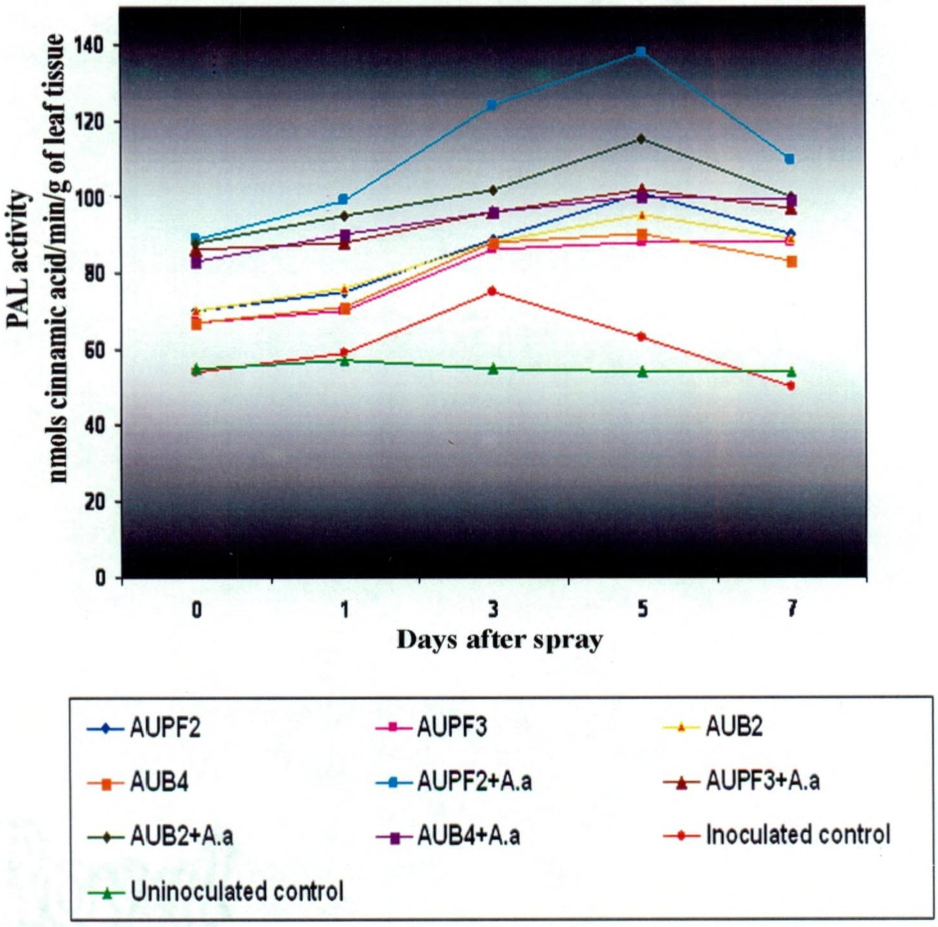
Similar reports have been reported by several workers. Seedling dip and foliar application of the antagonistic bacteria *B. subtilis* (CBE4) recorded a maximum level of PAL activity in *Phyllanthus amarus* on 4th day after challenge inoculation with *Corynespora casiicola* (Mathiyazhagan *et al.*, 2004). Activity of PAL in watermelon plants pretreated with biocontrol agents was induced upon challenge inoculation with *A. alternata* (Uma Maheswari *et al.*, 2007). Renuka *et al.* (2008) observed increased activity of PAL in chrysanthemum plants against *Alternaria chlamydospora* pretreated with bacterial antagonist *P. fluorescens* (isolate Pf1). The present results also revealed that plants treated with *P. fluorescens* increased the PAL activity and *P. fluorescens* pretreated Stevia plants challenged with *A. alternata* showed an additional increase in the PAL activity. The biocontrol agents induced the host defense mechanism by increasing PAL activity.

4.4.2. Changes in the peroxidase (PO) activity in *Stevia* plants

Peroxidases have been implicated in the regulation of plant cell elongation, phenol oxidation, polysaccharide cross-linking, IAA oxidation, cross-linking of extension monomers, oxidation of hydroxy-

Figure 1

Induction of phenylalanine ammonia lyase activity in *S. rebaudiana* leaves treated with biocontrol agents against *Alternaria* leaf spot



(A.a - *Alternaria alternata*)

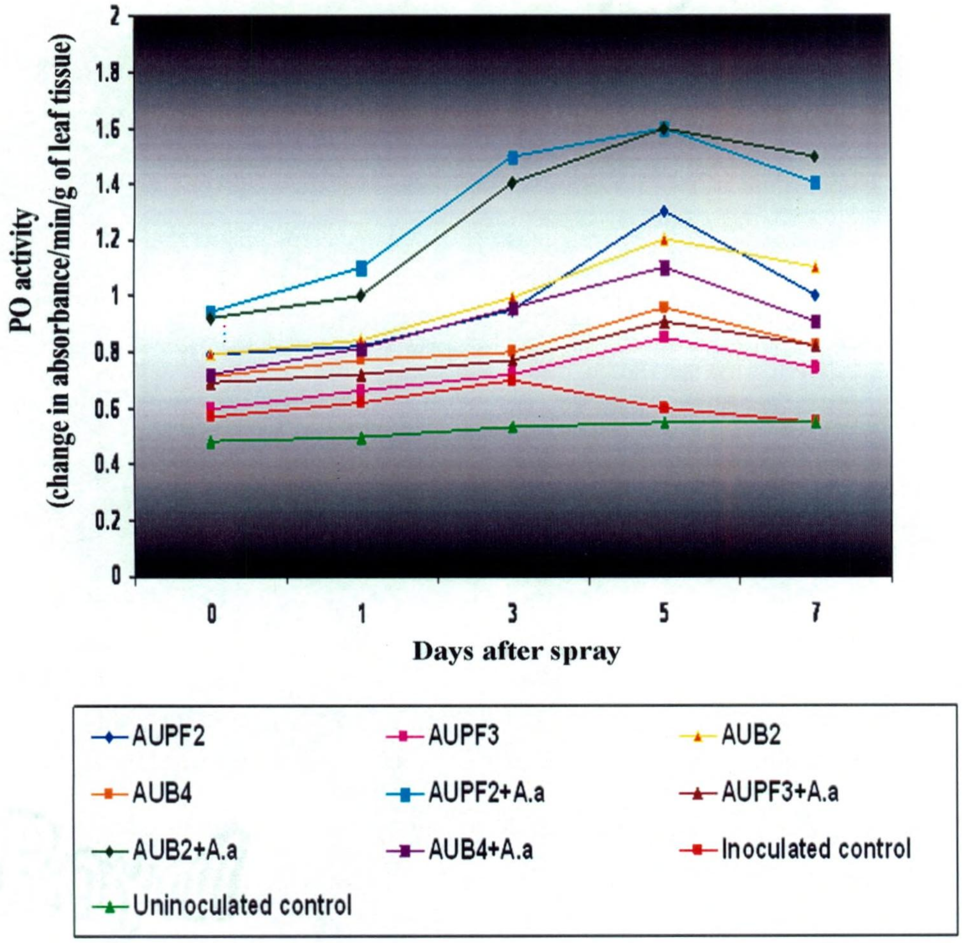
cinnamyl alcohols into free radical intermediates and wound healing (Vidhyasekaran, 1997). Peroxidase, a part of the PR 9-family, catalyses the condensation of phenolic compounds into lignin and the peroxidase activity is associated with disease resistance in plants and increases in host plants following pathogen infection (Scott-craiz *et al.*, 1995). Besides, PO itself was found to inhibit the spore germination and mycelial growth of certain fungi (Joseph *et al.,* 1998).

The activity of peroxidase was induced on plants treated with biocontrol agents when compared to the uninoculated control. But an additional increase in the activity was observed in plants pretreated with biocontrol agent and challenge inoculated with *A. alternata*. The treatment with AUPF2 and AUB2 recorded higher PO activity (1.6 change in absorbance/min/g of leaf tissue) on 5th day after challenge inoculation and thereafter the activity declined slowly. In plants treated with pathogen alone, the activity of PO was lower when compared to biocontrol agent treated plants and were several folds lower than plants challenge inoculated with biocontrol agents (Fig 2).

Anand *et al.* (2009b) recorded higher accumulation of PO in Pf1 treated cucumber plants challenged with *Pseudoperonospora cubensis.* Saravanakumar *et al.* (2007b) 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.*, 2002).The strains, Pf1 and FP7 are the

Figure 2

Induction of peroxidase activity in *S. rebaudiana* leaves treated with biocontrol agents against *Alternaria* leaf spot



(A.a - *Alternaria alternata*)

best inducers of plant chitinase and peroxidases, which are the most important compounds of ISR (Nandakumar *et al.*, 2001).

4.4.3. Changes in polyphenol oxidase (PPO) activity in *Stevia* plants

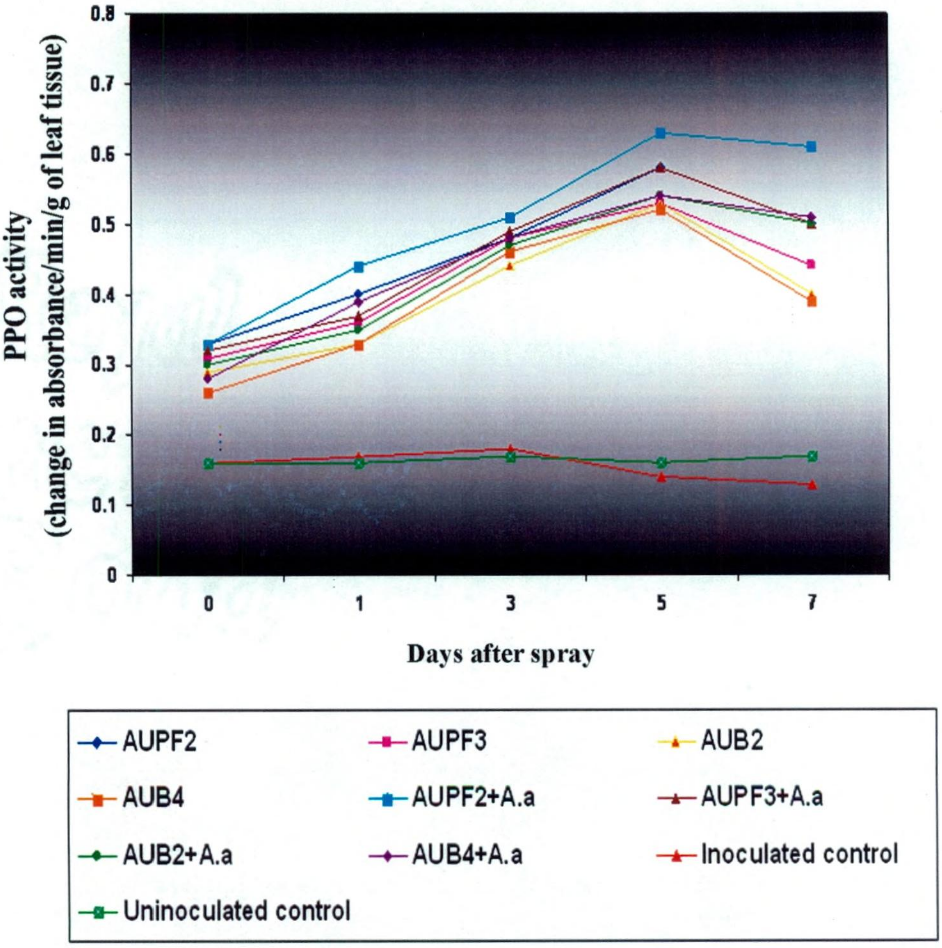
Polyphenol oxidases are a group of copper-proteins, widely distributed phylogenetically from bacteria to mammals, which catalyze the oxidation of phenolics to quinines which produce brown pigments in wounded tissues (Mayer, 1987). PPO has been implicated in the formation of pigments, oxygen scavenging (Trebst and Depka, 1995) and defense mechanism against plant pathogens (Mohammadi and Kazemi, 2002).

The activity of PPO was induced on plants treated with biocontrol agents and challenge inoculated with *A. alternata*. The higher PPO activity of 0.63 (change in absorbance/min/g of leaf tissue) was recorded in plants treated with AUPF2 and challenge inoculated with *A. alternata* followed by plants treated with AUPF3 and challenge inoculated with *A. alternata.* The plants treated with biocontrol agents were less in inducing PPO activity. In inoculated control, the activity was decreased from third day and reached below initial level (Fig. 3).

These results concur with those of other researchers. Increased activities of PPO in *P. fluorescens* Pf1 pretreated groundnut plants challenged with *S. rolfsii* was observed by Karthikeyan *et al.* (2006a). 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*

Figure 3

Induction of polyphenoloxidase activity in *S. rebaudiana* leaves treated with biocontrol agents against *Alternaria* leaf spot



(A.a - *Alternaria alternata*)

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).

4.4.4. Changes in phenolic content in *Stevia* plants

Phenols play an important role in determining resistance or susceptibility of a host to parasite infection. Plant phenolics and their oxidation products such as quinones are highly toxic to invading fungi (Bennett and Wallsgrove, 1994). The phenolic compounds may contribute to enhance the mechanical strength of host cell wall and may also inhibit the fungal growth as phenolics are fungitoxic in nature. Seed treatment with *P. fluorescens* induced the accumulation of phenolics in tomato root tissues (M'piga *et al*., 1997).

The results revealed that the phenolic content increased progressively up to five days in all the treatments and thereafter declined. Increase in total phenol content was significantly very high in AUPF2 treated plants (120 µg of catechol/g of leaf tissue) challenge inoculated with *A. alternata* followed by plants treated with AUB2 (112 µg of catechol/g of leaf tissue) and challenge inoculated with *A. alternata.* Accumulation of total phenols started one day after challenge inoculation and the maximum accumulation was observed five days after challenge inoculation. Plants inoculated with pathogens alone also recorded increased accumulation of total phenols and the content started decreasing after third day of inoculation. Plants treated with biocontrol agents had an increased content of phenols when compared to untreated plants (Fig. 4).

Girish and Umesha (2005) observed that the rate of reduction in the bacterial canker disease incidence was directly proportional to the amount of increased level of total phenol content. Dutta *et al.* (2008) recorded increased level of phenol content in the leaves of pigeon pea against *Fusarium* pretreated with PGPR. Anand *et al.* (2009a) reported that *P. fluorescens* pretreated chilli plants challenge inoculated with *Colletotrichum capsici* and *Leveillula taurica* showed higher accumulation of total phenols. It is well known that phenolic compounds are fungi toxic in nature. Moreover, phenolic compounds increase the physical and mechanical strength of host cell walls resulting in inhibition or fungal invasion.

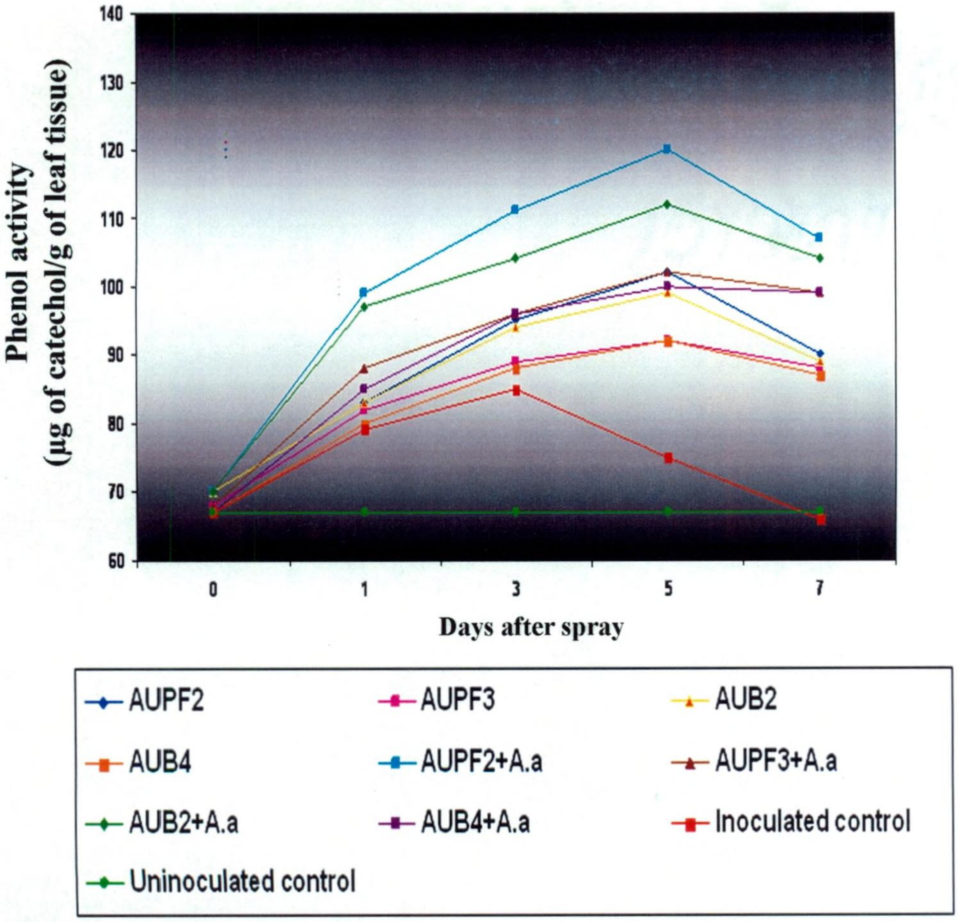
4.5. Native gel electrophoresis

4.5.1. Isoform pattern of peroxidase

Native PAGE analysis revealed that two PO isoforms such as PO1 and PO2 were present in all the treatments. However, the plants treated with biocontrol agents and challenged with *A. alternata* showed prominent induction than the healthy and inoculated control. Increased intensity of the band was observed in biocontrol agents treated plants challenged with and without *A. alternata* when compared to the healthy and inoculated control (Plate 11). The higher level of expression of PO1 and PO2 in the plants treated with biocontrol agents and challenged with *A. alternata* might have induced defense responses. From this observation,

Figure 4

Induction of phenol activity in *S. rebaudiana* leaves treated with biocontrol agents against *Alternaria* leaf spot



(A.a - *Alternaria alternata*)

it is assumed that the biocontrol agents treated plants mediated induction of defense responses could play a significant role in reducing the leaf spot disease under greenhouse conditions.

Radjacommare *et al.* (2004b) studied the induction of PO by Pf1 against blast fungus, *Pyricularia grisea*, in ragi and reported that Native PAGE of PO showed the single isoform in all the treatments including the control and the only difference observed was in the increased intensity of the band in the pathogen inoculated control and Pf1 treatment. Karthikeyan *et al.* (2006b) reported that the treatment with *P. fluorescens*, *T. 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). Four isoforms PO1, PO2, PO3 and PO4 where expressed in pseudomonads Pf1, TDK1 and PY15 treated rice plants inoculated with *Sarocladium oryza*e (Saravanakumar *et al.*, 2009).

4.5.2. Isoform pattern of polyphenol oxidase

Studies on the expression of various isoforms of PPO through native gel electrophoresis showed no induction of PPO isoforms. Hence the results revealed that PPO does not play a major role in the suppression of *Alternaria* leaf spot of Stevia when compared to other enzymes (Plate 12). Menaka (2006) reported that PPO does not play a major role in the suppression of *Alternaria* leaf spot of sugarbeet plants.

Plate 11

NATIVE PAGE profile of peroxidase in response to biocontrol agents in *S. rebaudiana* leaves

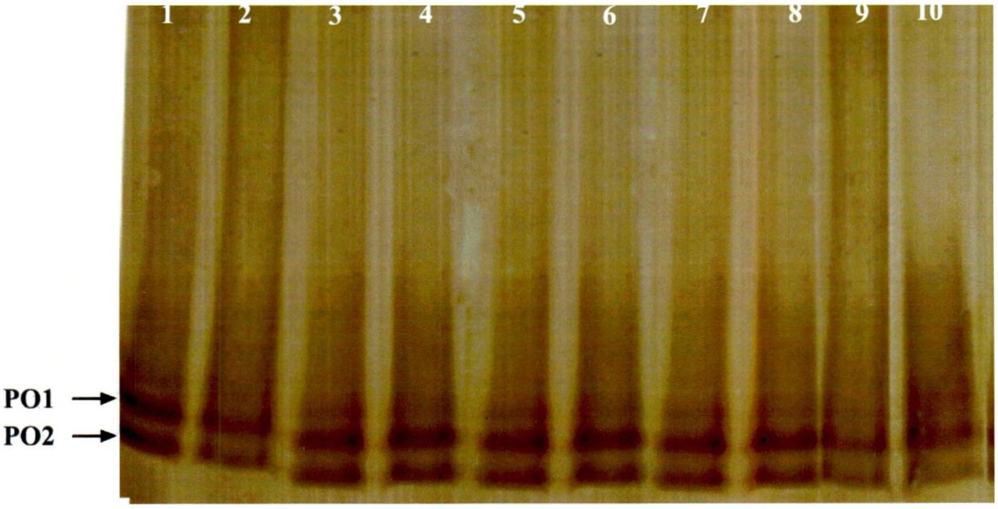
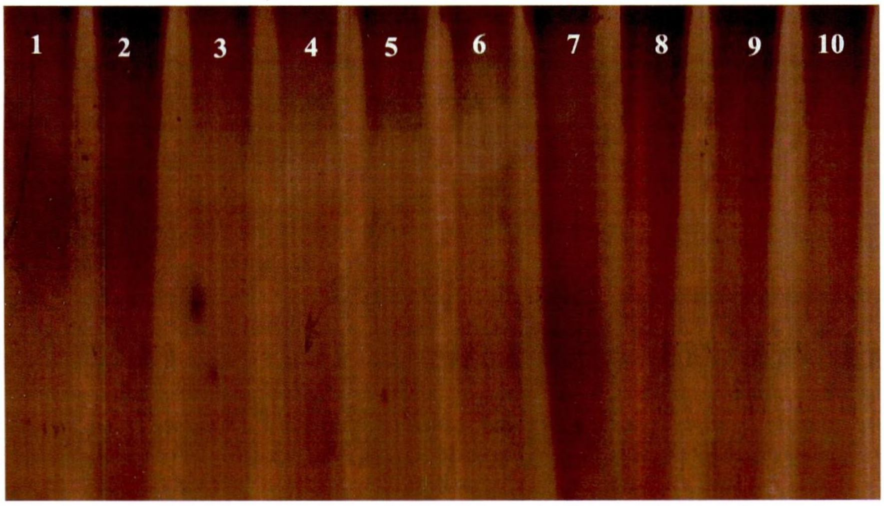


Plate 12

NATIVE PAGE profile of polyphenol oxidase in response to biocontrol agents in *S. rebaudiana* leaves



Lane 1 - AUPF2
Lane 2 - AUPF3
Lane 3 - AUB2
Lane 4 - AUB4
Lane 5 - AUPF2 + *A. alternata*
Lane 6 -AUPF3 + *A. alternata*
Lane 7 - AUB2+ *A. alternata*
Lane 8 - AUB4+ *A. alternata*
Lane 9 - Inoculated control
Lane 10- Uninoculated control