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Materials and Methods

Stevia (*Stevia rebaudiana* Bertoni) is a herbaceous perennial plant native of South America. *Stevia* is composed of several natural, heat-stable *ent*-kaurene glycosides (steviol glycosides) whose intensities of sweetness and flavour profiles differ from each other and vary according to concentration and environment.

The two main alkaloids, namely, stevioside (ST) and rebaudioside A (R-A) are the sweetest compounds tasting about 300 and 450 times sweeter than sucrose, respectively (Geuns, 2003). Stevioside has been used as a dietary supplement by the United States since 1995 (Mizushina *et al.,* 2005). Besides stevioside, Stevia also contains significant quantities of chlorogenic acid, which has hypoglycemic effects (Gregersen *et al*., 2004). In addition, it has already been encouragingly established that the stevioside and benzopyran contents in micropropagated *S. rebaudiana* amount to nearly the same as in the mother plants (Hwang, 2006; Supaibulwattana *et al*., 2011). Some ingredients of Stevia are commercially used as low-caloric sweeteners, i.e. as sugar substitutes.

Like other cultivated plants, medicinal and aromatic plants are also attacked by a number of fungi, bacteria, viruses and nematodes leading to significant quantitative and qualitative losses. Most of the diseases are of fungal origin (Paul and Singh, 2002). Survey for the last five consecutive years confirmed that *Alternaria* leaf disease is very common in medicinal plants cultivated in various districts of West Bengal, India (Maiti *et al*., 2007). Soil-borne diseases such as southern blight caused by *Sclerotium rolfsii* [*Corticium rolfsii*], charcoal rot caused by *Macrophomina phaseolina*, wilt caused by *Fusarium oxysporum*, root rot caused by *F. semitectum* [*F. pallidoroseum*], *F. solani* and *Rhizoctonia solani* as well as air-borne diseases such as black spot caused by *Alternaria steviae* and grey mould caused by *Botrytis cinerea* were recorded for the first time on *Stevia rebaudiana* plants growing in Egypt. Isolation of pathogens from the roots and stems of this

plant revealed that *Sclerotium rolfsii* is the most virulent pathogen, followed by *F. oxysporum, M. phaseolina* and *R. solani*. The susceptibility to fungal diseases increases in excessive levels of water or soils with high organic matter content (Katan, 2010).

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 and are also of serious concern from the environmental point of view (Sharma *et al.,* 2004).

Therefore, biological control agents are gaining importance in the field of disease management of medicinal plants (Mathivanan *et al*., 2005). The diversity of naturally occurring microorganisms of the rhizosphere and phyllosphere and their potential for biological control of plant pathogens have been examined extensively (Jayraj *et al.,* 2007). Pseudomonads are considered to be important rhizosphere organisms, wherein considerable research is underway globally to exploit their potential. Fluorescent pseudomonads help in maintenance of soil health, protect crops from pathogens and are metabolically and functionally more diverse (Choudhury *et al*., 2009). A number of fluorescent pseudomonads have been reported to possess *in vitro* and *in vivo* biocontrol potentiality against a wide range of phytopathogens (Jayraj *et al*., 2007, Sen *et al*., 2006, Maiti *et al*., 2011).

Similarly, *Bacillus* isolates have also been reported to be involved in biocontrol mechanisms against plant pathogens. *Bacillus* species are gram-positive bacteria that are present in substantial numbers in nearly all agricultural soils and other environments. Strains of *Bacillus subtilis* isolated from the rhizosphere region exhibit antagonistic activity against several plant pathogens (Lee *et al*., 2007) Rhizobacterial isolates are able to diminish either root or foliar diseases on field (Wang *et al.,* 2003).

Inducing a plant's own defense mechanisms by pretreatment with a biological inducer is thought to be a novel plant protection strategy (Ramamoorthy

et al., 2002a). Many defense enzymes are involved in defense reactions against plant pathogens. These include oxidative enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and other phenols that contribute to the formation of defense barriers for reinforcing cell structure (Lavania *et al*., 2006). Hence, the present study was undertaken to identify effective isolates as well as to assess the status of secondary metabolite production and to determine defense related compound activity such as phenylalanine ammonia lyase, peroxidase, polyphenol oxidase and total phenols in *Stevia rebaudiana* plants.

The detailed experimental design pertaining to the study "**Bio-management of Root rot and Leaf spot diseases in *Stevia rebaudiana* using Plant growth Promoting Rhizobacteria"** is presented as follows

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3.1.2. Collection and maintenance of fungal pathogens

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3.1.4. Characterization of Plant Growth Promoting Rhizobacteria

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3.4. Statistical analysis

The various steps involved in the materials and methods is given in the following flow chart.

Phase I

3.1. Characterization and Screening of Plant Growth Promoting Rhizobacteria

3.1.1. Collection and maintenance of Stevia plan

The seedlings of Stevia plants were collected from the Forest nursery, Aliyar Nagar and maintained under green house conditions in the Department of Biochemistry, Avinashilingam University, Coimbatore for further studies (Plate 1)

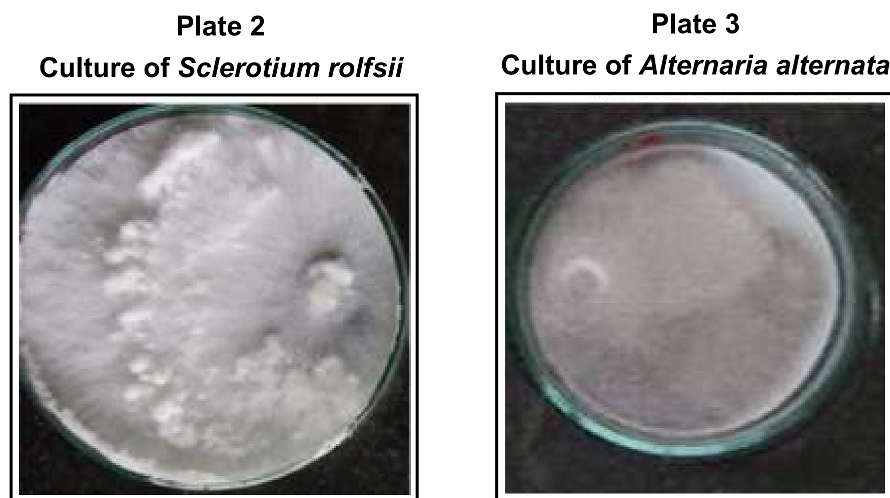
Plate 1
***Stevia rebaudiana* Plant**

3.1.2. Collection and maintenance of fungal pathogens

The soil-borne fungal pathogens, namely, *Sclerotium rolfsii* and *Alterneria alternata* were collected from the Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore and maintained on slants containing Potato Dextrose Agar (PDA) medium at 5° C (Plate 2 and 3).

Plate 2
Culture of *Sclerotium rolfsii*

Plate 3
Culture of *Alternaria alternata*



3.1.3. Isolation and maintenance of Plant Growth Promoting Rhizobacteria

The Plant Growth Promoting Rhizobacteria (PGPR) strains from the rhizosphere of various crops like paddy, groundnut, maize, banana, tomato and Stevia from different locations of Tamil Nadu were isolated by serial dilution method and the pure cultures were maintained on respective agar slants at 4° C.

3.1.4. Characterization of Plant Growth Promoting Rhizobacteria

3.1.4.1. Morphological characterization

Gram staining and motility tests were performed for preliminary identification of the strains (Allegrucci and Sauer, 2007). In order to determine the phenotypic nature, morphological characterization was done on the basis of colony colour, size, shape and margin (Cappucino and Sherman, 2002). The PGPR strains were examined for the colony morphology, growth, pigmentation, cell shape and gram reaction as per the standard procedures given by Barthalomew and Mittewer (1950).

3.1.4.1.1. Gram's staining

Thin smears of all isolates were made on clean glass slides, air dried and then heat fixed. Crystal violet solution was added so that it covers the whole smear. It was allowed to act for 1 minute and washed with distilled water. A few drops of gram's iodine was added to cover the smear and allowed to react for 30 seconds to 1 minute and rinsed with distilled water. The stains were decolourized by adding ethyl alcohol (95%) and counter stained with safranine for 45 minutes and washed with distilled water. The slides were then blotted dry and examined under oil immersion objective. (Sundarajan, 1995)

3.1.4.2. Biochemical characterization

Various phenotypic and biochemical methods have been developed and used for characterizing fluorescent pseudomonads and *Bacillus subtilis* isolates. Rapid identification of potentially and economically viable bioagents is possible through various methods of biochemical characterization (Weller *et al.*, 2002). The biochemical characterization of selected efficient PGPR strains was essentially done as per the procedures outlined by Cappuccino and Sherman (2002).

3.1.4.2.1. Growth at 4ºC (Hildebrand *et al*., 1992)

Each isolate was inoculated on King's B (KB) (Peptone -20.0 g; Dipotassium hydrogen phosphate-1.5 g; Magnesium sulphate-1.5 g; Glycerol -10.0 ml; Agar- 15 g; Distilled water- 1000 ml) slants and incubated at 4ºC for 48 hours and observed for growth.

3.1.4.2.2. Growth at 41ºC (Hildebrand *et al.,* 1992)

Each isolate was inoculated on KB slants and incubated at 41ºC for 48 hours and observed for growth.

3.1.4.2.3. Catalase test (Dubey and Maheswari, 2002)

A smear was prepared using 24 hours old bacterial culture on a clean glass slide and covered with a few drops of 3% Hydrogen peroxide. Effervescence indicates positive result.

3.1.4.2.4. Gelatin liquefaction (Kannan, 2003)

20 ml of a sterile medium containing Nutrient Agar (NA) (15 g, KH_2PO_4 - 0.5 g, K_2PO_4 - 1.5 g, gelatin - 4.0 g, glucose - 0.05 g and distilled water - 1000 ml) was poured into sterile petriplates and allowed to solidify. Then the isolate was inoculated as a single streak. The plates were then incubated in an inverted position at 37ºC for 24 hours. Then the plates were flooded with mercuric chloride solution and the zone of hydrolysis observed.

3.1.4.2.5. Indole test (Dubey and Maheswari, 2002)

Each isolate was inoculated into peptone broth (Potassium dihydrogen phosphate 3.56 g; Di-sodium hydrogen phosphate dihydrate 7.23 g; Sodium chloride 4.3 g; Peptone from pepsin-digested meat 1.0 g and .pH: 7.0 ± 0.2) and incubated at 37ºC for 48 hours. Then 1 ml of Kovac's reagent was added to the tubes and the tubes were shaken gently with intervals of about 10-15 minutes. The tubes were allowed to stand to permit the reagent to rise to the top and then observed for the production of cherry red layer.

3.1.4.2.6. Levan formation (Dubey and Maheswari, 2002)

Sterile medium containing peptone - 0.5 g, beef extract - 0.3 g, NaCl - 0.5 g, agar - 3.0 g, sucrose - 5.0 g and distilled water - 1000 ml was prepared and 20ml of it was poured into sterile petriplates and then each isolate was inoculated by quadrant streak. The plates were then incubated at 37ºC for 24 hours and observed for white mucoid colony formation.

3.1.4.2.7. Methyl-Red test (Dubey and Maheswari, 2002)

Sterile Methyl Red-Voges Proskauer (MR-VP) broth (Peptone from meat 7.0g; D(+)glucose 5.0g; phosphate buffer 5.0 ml) was inoculated with the bacterial culture and incubated at 37ºC for 24 hours. Methyl red indicator was added to each isolate. Change in the colour of the broth from yellow to red indicates positive result.

3.1.4.2.8. Voges Proskauer test (Dubey and Maheswari, 2002)

Sterile Methyl Red-Voges Proskauer (MR-VP) broth was inoculated with each isolates and then incubated at 37ºC for 24 hours. 40 percent KOH solution

and Barrits reagent were added after incubation period. The tubes were shaken gently for 30 seconds and exposed to oxygen. Change in the color of the broth from yellow to pink indicates positive result.

3.1.4.2.9. Starch hydrolysis test

Sterile starch agar (Agar -12.0g; Soluble starch -10.0 g; Beef extract – 3g; sterile water – 1000 ml; pH 7.0 ± 0.2) plates were prepared and the bacterial culture was plated onto the plates. The plates were incubated at 37 °C for 48 hrs and flooded with iodine solution. A clear zone around the organism indicates positive result. Dark blue coloration of medium with no clear zone formation indicates negative result.

3.1.4.2.10. Blood agar hemolysis test (Hsieh *et al.,* 2004)

Sterile blood agar (Meat- 10.0g; Peptone- 10.0 g; NaCl- 5.0 g; Agar –15.0 g; Distilled water 1 liter ; pH 7.3 ±0.2) plates were prepared and the bacterial culture was plated onto the plates. The plates were incubated at 37º C for 48 hrs. A clear zone of hemolysis observed around the organism indicates a positive result for *Bacillus* sp. Absence of hemolysis zone indicates negative result.

3.1.4.3. Molecular characterization

3.1.4.3.1. Polymerase chain reaction (PCR) of *Pseudomonas fluorescens* and *Bacillus subtilis*

3.1.4.3.1.1. 16S rRNA gene of *Pseudomonas fluorescens*

Total genomic DNA of the *Pseudomonas fluorescens* strain was isolated by CTAB/NaCl method (Jaufeerally-Fakim and Dookun 2000) which consists of chemical lysis with detergent, incubation with a non specific protease followed by a CTAB extraction in which CTAB complexes both with polysaccharides and with residual protein. This procedure is very effective in producing high quality chromosomal DNA from a variety of gram-negative bacteria, all of which normally produce large amounts of polysaccharides (Wilson, 1990). 30 ml of 24 hour bacterial culture (OD 600 nm) grown at 25 °C with shaking (200 rpm) was used for the genomic DNA isolation. Isolated genomic DNA concentration was measured

visually after electrophoresis in 1% agarose gel. 16S rRNA gene of the isolated strain was amplified from its genomic DNA using a pair of universal bacterial primer, E334F (5' CCAGACTCCTACGGGAGGCAG 3') as forward primer and E1115R (5'CAACGAGCGCAACCCT3') as reverse primer. PCR was performed as follows: genomic DNA (100 ng/ µl) 1 µl, E334F- E1115R primers (10 µM) 1 µl each, 25 µl 2X PCR master mix (Fermentas) and 22 µl distilled water were mixed in a total volume of 50 µl. The conditions for PCR amplification were as follows : an initial denaturation step at 94 °C for 2 minutes; followed by 25 cycles including denaturation at 94°C for 30 seconds , primer annealing at 62°C for 30 seconds and elongation at 72°C for 1 minute and also final elongation at 72°C for 5 minutes. PCR products were visualized by agarose gel electrophoresis.

3.1.4.3.1.1. 16SrRNA gene of *Bacillus subtilis*

The 16S rRNA gene, corresponding to an internal portion of the *B. subtilis* group, *was* PCR-amplified using the primers, Bsub 5F (5' - AAG TCG AGC GGA CAG ATG G -3') and Bsub 3R (5' - CCA GIT CCA ATG ACC CTC CCC -3'). Total genomic DNA was extracted from *Bacillus subtilis* isolates using Gen Elute Bacterial Genomic DNA kit (Sigma, St. Louis, USA) according to the manufacturers instructions. The PCR mixture comprised 3.5 µM $MgC1_2$, 200 µM of each dNTP's (Promega, Madison, USA), 0.4 µM of each primer (Metabmn, Bangalore, India), 2.5 pl of 10X Taq buffer and 1.25 units of Taq polymerase (AmpliTaq Gold Applied Biosystems, New Jersey (NJ), USA). To this mixture 1 µl of the DNA template was added**.** The control tube was added with 1 µl de-ionized water in place of DNA sample and the reaction mixture in the tubes were made up to 25 µl volume using de-ionized water. The reaction mixture was amplified in a Thermal Cycler (Biorad, USA). The PCR conditions were: denaturation at 94° C for 12 minutes, 30 cycles of denaturation at 95° C for 0.5 min, annealing at 65°C for 2 min, extension at 72°C for 2 min and a final extension step at 72° C for 7 minutes. The amplified product was checked by electrophoresis of 8 µl on 1.5% agarose (Sigma, St. Louis, USA) gel containing ethidium bromide. To verify the size and purity of the product, a 100 bp ladder (Promega, Madison, USA) was run alongwith the sample and size of the amplicon was confirmed (Wattiau *et al*., 2001).

3.1.5. Testing the antagonism of Plant Growth Promoting Rhizobacteria

The Plant Growth Promoting Rhizobacteria are known to antagonize fungal pathogens by direct parasitism and by secreting lytic enzymes, antibiotics and various secondary metabolites. The antagonistic effect of *Pseudomonas fluorescens* and *Bacillus subtilis* was proved under *In vitro* conditions by several workers like Ramamoorthy *et al.* (2002b), Anith *et al.* (2004), Maiti *et al*. (2011) and Kumar *et al.* (2012).

3.1.5.1. Dual culture technique (Vidhyasekaran *et al.,* 1997)

The antagonistic activity of PGPR against the fungal pathogens was tested by dual culture technique. Bacterial isolates were streaked on one side of the petridish (one cm away from the edge) containing PDA and 8-9 mm mycelial disc from four to seven days old PDA culture of fungal pathogen was placed at the opposite side of the petridishes perpendicular to the bacterial streak and incubated at 28 ± 2° C for 4-7 days. Petridishes inoculated with fungal discs alone served as control. Three replications were maintained for each isolate. Observations on width of inhibition zone and mycelia growth of test pathogen were recorded and the per cent inhibition of pathogen growth was calculated using the formula proposed by Vincent (1947).

Per cent inhibition (I) = C-T/C ×100

Where, C- mycelial growth of pathogen in control

T- mycelial growth of pathogen in dual plate.

3.1.5.1.1. *In vitro* screening of *Pseudomonas fluorescens* and *Bacillus subtilis* isolates against *Sclerotium rolfsii*

The effective isolates of *P. fluorescens* and *B. subtilis* were identified by dual plate technique. The bacterial strains were streaked on one side of the petridish (1 cm away from the edge of the plate) with PDA medium and a mycelial disc (8 mm diameter) of three day old culture of *Sclerotium rolfsii* was placed on the opposite side of the petridish perpendicular to the bacterial streak. The plates were incubated at room temperature (28 $\pm$ 2°C) for 4 days. After this, pathogen growth and inhibition zone were recorded and expressed in mm.

3.1.5.1.2. *In vitro* screening of *Pseudomonas fluorescens* and *Bacillus subtilis* isolates against *Alternaria alternata*

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

Phase II

3.2 Testing for Antimicrobial compounds

Many PGPR are well characterized for their ability to produce antimicrobial compounds, such as sideophores, hydrogen cyanide, ammonia, antibiotics and volatile metabolites which suppress the growth of bacterial, fungal and nematode pathogens by competiting with the pathogen for nutrients or colonization for space (Haas and De´fago, 2005).

3.2.1. Hydrogen cyanide

Hydrogen cyanide is a volatile metabolite of bacterial strains which is mainly involved in the mechanism of disease suppression and is deleterious to soil microorganisms (Paszkowski and Dwornikiewicz, 2003). Bacterial ability for production of hydrogen cyanide has a great variation and based on this ability, they were qualitatively classified as high, relatively high, moderate and no production (Soltani *et al.,* 2010).

3.2.1.1. Qualitative analysis

Hydrogen cyanide production was determined by using the modified protocol of Miller and Higgins (1970). Bacteria were grown on tryptic soy agar (TSA-Tryptone- 15.0g; Soytone-5.0 g, NaCl-5.0 g; Agar-15.0 g and distilled water 1 liter, pH -7.3 ± 0.3). Filter paper discs soaked in picric acid solution (2.5 g of picric acid, 12.5 g of sodium carbonate and 1000 ml of distilled water) were placed in the

upper lid of each petriplate. The dishes were sealed with parafilm and incubated at 28˚C for 48 hours. A change in the disc from yellow to light brown, brown or reddish brown were recorded as an indication of weak, moderate or strong production respectively of hydrogen cyanide for each isolates respectively.

3.2.1.2. Quantitative analysis

The bacterial isolates were cultured on tryptic soy broth (Tryptone - 15.0 g; Soytone-5.0 g, NaCl-5.0 g and distilled water 1 liter, pH -7.3 ± 0.3) in sterile conical flasks. Filter paper was cut into uniform strips of 10 cm length and 0.5 cm wide, saturated with alkaline picrate solution and a single filter paper was placed inside each conical flask in a hanging position. After incubation at 28 ± 2˚C for 48 hours, the sodium picrate in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The filter paper was then placed in a clean test tube containing 10 ml of distilled water, the colour eluted and the absorbance measured at 625 nm (Wei *et al.,* 1996).

3.2.2. Salicylic acid

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 the leaves (Siddiqui and Shaukat, 2003). Some plant growth promoting bacteria do trigger an Salicylic acid dependent signaling pathway by producing nanogram amounts of Salicylic acid in the rhizosphere. However, the majority of Plant Growth Promoting Bacteria that activate Induced Systemic Resistance appear to do so via a Salicylic acid - independent pathway involving jasmonate and ethylene signals. Induced Systemic Resistance is associated with an increase in sensitivity to these hormones rather than an increase in their production, which might lead to the activation of a partially different set of defense genes (Hase, *et al*., 2003).

Salicylic acid production by the isolates was determined according to the method of Meyer *et al*. (1992). The bacterial isolates were grown in the standard succinate medium (Succinic acid – 4.0g; K_2HPO4- 6.0g; KH_2PO4-3.0g; NH_2SO_4 –

1.0 g; $MgSO_4 7H_2O$- 0.2 g ; Distilled Water 1000 ml; pH-7.0) at 28 ± 2°C for 48 hours. Cells were collected by centrifugation at 6000 rpm for 5 min. 4 ml of cell free culture filtrate was acidified with 1N HCl to pH 2.0 and salicylic acid was extracted with equal volume of chloroform. 4 ml of water and 5μl of 2M $FeCl_3$ were added to the pooled chloroform phases. The absorbance of the purple iron salicylic acid complex, which was developed in the aqueous phase, was read at 527 nm. Salicylic acid was measured according to a standard curve drawn with salicylic acid dissolved in succinate medium and treated as described above. The quantity of salicylic acid was expressed as $\mu g\ ml^{-1}$ of the culture filtrate.

3.2.3. Siderophore

Siderophores are iron chelating compounds secreted by bacteria on or around the roots that affect the growth of the plant's rhizobacteria (Sakthivel *et al.,* 2009).

The siderophore production by the rhizobacterial isolates was estimated by the method described by Reeves *et al.* (1983). The *P. fluorescens* and *B. subtilis* isolates were grown in King's B broth and nutrient broth, respectively. After 7 days of incubation, the cultures were centrifuged at 10000 rpm for 20 minutes and the supernatant used for the estimation of catecholate type and salicylate type of siderophore.

The pH of the culture was adjusted to 2.0 with 1N HCl. To the supernatant, an equal volume of ethyl acetate was added in a separatory funnel, mixed well and the ethyl acetate fraction collected. This process was repeated 3 times to bring the entire quantity of siderophore from the supernatant. The ethyl acetate fractions were then pooled, air dried and dissolved in 5 ml of 50 per cent ethanol. 5 ml of Hathway reagent (1 ml of 0.1M $FeCl_3$, 1ml of 0.1M HCl in 100 ml of distilled water, 1 ml of 0.1M Potassium ferric cyanide). The absorbance was read at 560 nm. A standard curve was prepared using sodium salicylate for the estimation of salicylate type siderophore. The quantity of siderophore synthesized was expressed as μg/ml of culture filtrate.

3.2.4. Indole Acetic Acid

Indole-3 acetic acid (IAA) is quantitatively the most abundant naturally occurring auxin produced by bacteria. IAA biosynthesis is widespread in plant associated bacilli and is considered to be directly involved in plant growth promotion (Tsavkelova *et al*., 2007; Idris *et al*., 2007).

The bacterial isolates were grown in Trypticase Soy broth with tryptophan (100 μl per ml) and incubated at 28±2° C. To 1 ml of the cell free culture filtrate, 2 ml of Salkowsky reagent (1ml of 0.5 M $FeCl_,$ in 50 ml of 35 per cent perchloric acid) was added and incubated at 28 ± 2°C for 30 min. The absorbance was read at 530 nm. A standard was prepared using IAA and the presence of IAA in culture filtrate was quantified as μg/ml (Gorden and Paleg, 1957).

3.2.5. Chitinase

Chitinase (EC 3.2.1.14) one of the major pathogenesis-related proteins and is a polypeptide that accumulates extracellularly in infected plant tissues and exhibits high resistance to proteolytic degradation (Navani *et a*l., 2002). Chitinases have been implicated in the defense reactions of plants against potential pathogens (Garg and Gupta, 2010).

The colorimetric assay of chitinase was carried out according to the method of Boller and Mauch (1998). The reagents used were colloidal chitin, snail gut enzyme, dimethyl amino benzaldehyde (DMAB) and buffer. The reaction mixture consisted of 10 µl of 0.1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme solution and 0.1 ml of colloidal chitin (10mg). After incubation for 2 hours at 37o C, the reaction was arrested by centrifugation at 10,000 rpm for 3 minutes. An aliquot of the supernatant (0.3 ml) was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer (pH 7.0) and incubated with 20 µl of 3 per cent (w/v) snail gut enzyme for one hour. After one hour, the reaction mixture was brought to pH 8.9 by the addition of 70 µl of 0.1 M sodium borate buffer (pH 9.8). The mixture was incubated in a boiling water bath for 3 min and then rapidly cooled in ice water bath. After addition of 2 ml of DMAB, the mixture was incubated for

20 minutes at 37oC and the absorbance was measured at 585 nm immediately. N- acetylglucosamine (GlcNac) was used as the standard. The enzyme activity was expressed as nmoles GlcNac equivalents min-1 ml-1.

Phase III

3.3 Efficacy of Talc-based formulations against Stevia diseases

3.3.1. Bioformulations

Bacterial formulation is a product that consists of bacterial cells and a kind of preservative which is used to control soil-borne diseases. The bacterial component of the formulation needs to survive for a considerable length of time and have *in vivo* efficacy (Bora *et al*., 2004). The success of the biocontrol eventually depends upon development of suitable formulations in which the antagonistic microorganisms can survive for extended periods of time. Most biological control agents are affected by prolonged storage compared to fungicides. Development of stable formulations of biocontrol agents is of great importance in developing countries where subsistence farming is prominent and where fungicide treatments are unaffordable (Jayaraj *et al*., 2005).

3.3.2. Development of talc-based formulation of Plant Growth Promoting Rhizobacteria

A powder formulation with a longer shelf life would be beneficial. Different carrier formulations of fluorescent pseudomonads have been developed. *P. fluorescens* (Pf1 and Py15) and *B. subtilis* (Bs16) strains have been developed commercially as a talc-based formulation and tested against several crop diseases (Kavino *et al.,* 2007).

10g of carboxy methylcellulose was mixed with 1 kg of talc powder and the pH was adjusted to 7.0 by adding 15 g of calcium carbonate. The mixture was then autoclaved for 30 minutes for two consecutive days. The *P. fluorescens* strain AUPF6 and *B. subtilis* strain AUBS2 were grown in King's B medium (KMB) and nutrient broth for 48 hours, respectively. 400 ml of the bacterial inoculum was then added to 1 kg of the talc mixture and mixed well under sterile conditions. The product was dried under shade to bring the moisture content to less than 20 per

cent. The formulation was packed in polythene bags, sealed and kept under room temperature. (Vidhyasekaran and Muthamilan, 1995)

3.3.3. Application of bioformulations

3.3.3.1. Soil application method

Potting medium (red soil: cow dung: manure at 1:1:1 w/w/w) was autoclaved for 1 h for two consecutive days and filled in pots. The talc-based formulations of AUPF6 and AUBS2 were applied to the potting mixture at the rate of 10g per kg of soil. Propiconazole (fungicide) was used as a standard treatment for comparison. The fungicide was applied as soil drenching at the recommended dosage (1ml/ liter of water). The culture of *Sclerotium rolfsii,* mass multiplied in sand maize medium with sand and maize powder at the ratio of 19: 1 (Riker and Riker, 1936) was incorporated with potting medium at the rate of 20g per kg of soil.

3.3.3.2. Foliar spraying method

The talc based formulation of AUPF6 and AUBS2 were delivered as foliar spray over the leaves of potted *S. rebaudiana* plants (one month old) at the rate of 2.5 per cent. Two days after the application of PGPR, spore suspension of *A. alternata* (10^4 conidia per ml) was sprayed over the leaves.

3.3.4. Management of Stevia diseases under greenhouse conditions

3.3.4.1. Management of Stevia root rot disease

A greenhouse study was conducted to test the efficacy of talc-based bioformulations of the effective PGPR isolates AUPF6 and AUBS2 against root rot disease on Stevia plants. Stevia seedlings were planted in pots and treated with PGPR bioformulations under greenhouse conditions. The following four treatments were maintained with five replications and the pots were arranged in a completely randomized design.

T1- *P.fluorescens* (AUPF6)
T2- *Bacillius subtilis* (AUBS2)
T3- Propiconazole
T4- Control

The incidence of root rot disease was recorded on the 20th day after pathogen inoculation and expressed as percentage of disease incidence.

3.3.4.2. Management of Stevia leaf spot disease

A greenhouse study was conducted to test the efficacy of talc-based bioformulations of the effective PGPR isolates AUPF6 and AUBS2 against leaf spot disease on Stevia plants. Stevia seedlings were planted in pots and treated with PGPR bioformulations under greenhouse conditions. The following four treatments were maintained with five replications and the pots were arranged in a completely randomized design.

T1- *P.fluorescens* (AUPF6)
T2- *Bacillius subtilis* (AUBS2)
T3- Propiconazole
T4- Control

The incidence of leaf spot disease was recorded on the 20th day after pathogen inoculation and expressed as percent disease index (PDI).

3.3.5. Induction of Defense related compounds in *S. rebaudiana* plants

Induced systemic resistance by PGPR is thought to be a biocontrol mechanism against plant pathogens (Akhtar and Siddiqui, 2008). Many defense enzymes are involved in defense reactions against plant pathogens. These include oxidative enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and other phenols that contribute to the formation of defense barriers for reinforcing cell structure (Lavania *et al*., 2006). 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).

3.3.5.1. Sample collection

Samples were collected from individual treatments to study the induction of defense enzymes and compounds in response to pathogen attack in Stevia plants

under greenhouse conditions. Leaves were collected from treated plants on 0, 1, 3, 5 and 7 days after challenge inoculation with *Sclerotium rolfsii* and *A. alternata*, washed several times with sterile distilled water and used for the analysis of defense-related enzymes.

3.3.5.2. Enzyme extraction

1 gram of root sample was homogenized with 2ml of 0.1 M sodium citrate buffer (pH 5.0) at 4°C. The homogenate was centrifuged for 20 min at 10,000 rpm. The supernatant was used as crude enzyme extract for assaying the chitinase activity. The enzyme extracted in 0.1 M sodium phosphate buffer was used for estimation of Peroxidase (PO), Polyphenol oxidase (PPO) and Phenylalanine Ammonia- Lyase (PAL).

Protein content in the extract was determined by the Method of Bradford (1976). 10 milligram of Coomassie brilliant blue G-250 was dissolved in 4.7 ml of absolute alcohol and 10 ml of concentrated phosphoric acid and the volume was made up to 100 ml with distilled water. A sample of 50 μl was added to 950 μl of dye solution and the mixture was incubated for 5 minutes at room temperature. Bovine serum albumin was used as the standard. The absorbance was recorded at 595 nm.

3.3.5.3. Assay of Phenylalanine ammonia lyase (PAL) activity

PAL is the first enzyme in phenylpropanoid metabolism involved in the production of phenolics and phytoalexins that prevent establishment of pathogen (Mariutto *et al.,* 2011). Plant samples (1 g) were homogenized in 2 ml of ice cold 0.1 M sodium borate buffer at pH 7.0 containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone. The extract was filtered through cheesecloth and the filtrate was centrifuged at 16,000 g for 15 min. The supernatant was used as enzyme source. PAL activity was determined as the rate of conversion of L-phenylalanine to trans-cinnamic acid at 290 nm (Dickerson *et al.,* 1984). Samples containing 0.4 ml of enzyme extract were incubated with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L - phenylalanine in the same buffer for 30 min at 30°C. The amount of trans-cinnamic acid synthesized was calculated (Dickerson

et al., 1984). Enzyme activity was expressed as nmol trans-cinnamic acid min^{-1} g^{-1} tissue.

3. 3.5.4. Assay of Peroxidase (PO) activity

The defense gene products peroxidases (PO) and polyphenol oxidases (PPO) catalyze the formation of lignin and phenylalanine ammonia-lyase (PAL) involved in the synthesis of phytoalexins and phenolics (Karthikeyan *et al.,* 2005). Peroxidase (PO) activity aids in the synthesis of lignin and oxidative phenols, thereby conferring resistance to plant pathogens (Fernando *et al*., 2007). The PO activity was assessed spectrophotometrically as described by Hammerschmidt and Kuc (1982).

Plant samples (1 g) were homogenized in 2 ml of 0.1M-phosphate buffer, pH 7.0 at 4°C. The homogenate was centrifuged at 16,000 g at 4°C for 15 min and the supernatant was used as enzyme source. The reaction mixture consisted of 1.5 ml of 0.05 M pyrogallol, 0.5 ml of enzyme extract and 0.5 ml of 1 per cent H_2O_2. The reaction mixture was incubated at room temperature (28 $\pm$ 2°C). The change in absorbance at 420 nm was recorded at 30 second interval for 3 min. The enzyme activity was expressed as change in absorbance min^{-1} g^{-1} tissue (Hammerschmidt and Kuc, 1982).

3.3.5.5. Assay of Polyphenol oxidase (PPO) activity

PPO is involved in the oxidation of poly phenols into quinones (antimicrobial compounds) and lignification of plant cells during microbial invasions, and also may participate in the responding defense reaction and hypersensitively by inducing plant resistance against fungi (El- Khallal, 2007). The PPO activity was assessed spectrophotometrically as described by Mayer *et al.* (1965).

Plant samples (1 g) were homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5) and centrifuged at 16,000 g for 15 min at 4° C. The supernatant was used as enzyme source. The reaction mixture consisted of 200 µl of the enzyme extract and 1.5ml of 0.1 M sodium phosphate buffer (pH 6.5). To initiate the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as change in absorbance at 495 nm min^{-1} g^{-1} tissue (Mayer *et al.,* 1965).

3.3.5.6. Estimation of Total phenols

Phenolic compounds are known to play a major role in the defense mechanism of plants against various external infectious agents (Anita and Samiyappan, 2012).Total phenols was estimated by the Method of Zieslin and Ben-Zaken (1993).

Plant samples (1 g) were homogenized in 10 ml of 80 per cent methanol and agitated for 15 min at 70° C (Zieslin and Ben-Zaken, 1993). One ml of the methanolic extract was added to 5 ml of distilled water and 250 μl of Folin-Ciocalteau reagent (1 N) and the solution was kept at 25° C. The absorbance of the developed blue colour was measured using a Spectrophotometer at 725 nm. Catechol was used as the standard. The amount was expressed as μg catechol g^{-1} tissue.

3.3.5.7. Native Polyacrylamide Gel Electrophoresis (Native PAGE)

The isoform profile of Peroxidase and Polyphenol oxidase were examined by discontinuous native polyacrylamide gel electrophoresis analysis-Native PAGE (Sindhu *et al*., 1984).

The separating gel (8%) and stacking gel(4%) were prepared with the following compositions.

Separating gel (8%)

Acrylamide stock solution	:	2.00 ml
Distilled water	:	3.625 ml
1.5 M Tris buffer pH 8.8	:	1.875 ml
10% APS (Ammonium per sulphate)	:	25 μl
TEMED (N, N, N’, N’-tetra methyl ethylene diamine)	:	5 μl

Stacking gel (4%)

Acrylamide stock solution	:	0.65 ml
Distilled water	:	3.25 ml
1.5 M Tris buffer pH 6.8	:	1.25 ml
10% APS (Ammonium per sulphate)	:	25 μl
TEMED (N, N, N', N'-tetra methyl ethylene diamine)	:	5 μl

Separating gel was casted as per the above composition. Ammonium persulphate and TEMED were added-just prior to pouring the gel. The solution was overlaid carefully with a film of distilled water. After polymerisation, the water layer was removed and stacking gel was poured. The comb was placed between the plates, at the top portion of the plate. The comb was carefully removed from the wells after polymerization of the stacking gel. The dialyzed/lyophilized culture filtrates of *Trichoderma* mutants were mixed with sample buffer and heated to 90°C for 5 min and cooled at 5°C. These samples were then carefully loaded into the wells and the electrode buffer was poured. Gels were run at a constant current of 20 mA till the dye front reached the bottom of the gel. Protein molecular weight marker, which comprised of myosin 220.0 kDa, phosphorilase b.97.4 kDa, bovine serum albumin 66.0 kDa. ovalbumin 46.0 kDa, carbonic anhydrase 30.0 kDa, trypsin inhibitor 21.5 kDa and lysozyme 14.3kDa were used in this experiment.

3.3.5.7.1. Peroxidase (PO)

To study the expression pattern of different isoforms of peroxidases in different treatments, activity gel electrophoresis was carried out. For native anionic polyacrylamide gel electrophoresis, resolving gel of 8 per cent and stacking gel of 4 per cent were prepared. After electrophoresis, the gels were incubated in the solution containing 0.15 per cent benzidine in 6 per cent NH_4Cl for 30 min in dark. Then drops of 30 per cent H_2O_2 were added with constant shaking till the bands appear. After staining, the gel was washed with distilled water and photographed (Sindhu *et al.,* 1984).

3.3.5.7.2. Polyphenol oxidase (PPO)

Enzyme was extracted by homogenizing 1 g of tissue in 0.01 M potassium phosphate buffer (pH 7.0). The homogenate was centrifuged at 20,000 rpm for 15 min at 4 °C and the supernatant was used as enzyme source. After native electrophoresis, the gel was equilibrated for 30 min in 0.1 per cent *p* phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) followed by 10 mM catechol in the same buffer. The addition of catechol was followed by a gentle shaking which resulted in appearance of dark brown discrete bands (Jayaraman *et al.*, 1987).

3.4. Statistical analysis

The results of all the experiments were analyzed independently. The treatment means were compared by Duncan's Multiple Range Test - DMRT (Gomez and Gomez, 1984). The package used for analysis was IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics unit, The Philippines.

The observations made at different stages of the study and the results obtained thereof are presented in the following chapter.