# *Experimental Procedure*

# 3.0 EXPERIMENTAL PROCEDURE

Leaf spot of *Stevia rebaudiana* caused by *Alternaria alternata* is one of the destructive diseases which cause economic loss. Although synthetic fungicides have been widely used to control fungal pathogens, recent concerns with fungicide toxicity, development of fungicide resistance by pathogens and potential harmful effects on the environment and human health have led to the necessity of searching alternative means to chemical control. Induction of pathogenesis related proteins, which are capable of inhibiting pathogen development, would contribute to greater resistance. Some antagonistic microorganisms have been proven to have great efficiency by increasing resistance against fungal pathogen. The primary purpose of this study were to assess the status of secondary metabolites production by antagonistic microorganisms and to determine defense related enzyme activity such as phenylalanine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO) in *S. rebaudiana* (Plate 1) against leaf spot disease (Plate 2a and 2b) after treatment with the antagonistic microorganisms.

The detailed experimental procedure pertaining to the study **"Studies on the effect of bacterial antagonists against leaf spot of Stevia caused by *Alternaria alternata*"** is presented under the following headings.

3.1. Collection and maintenance of fungal pathogen

3.2. Collection and maintenance of bacterial antagonists

3.3. *In vitro* screening of antagonistic bacteria against *A. alternata*

**Plate 1**
*Stevia rebaudiana* (Bertoni)

**Plate 2a**
***Stevia rebaudiana* plant infected with *Alternaria alternata***

**Plate 2b**
**Symptoms of *Alternaria* leaf spot on *Stevia rebaudiana***

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

3.4.1. Hydrogen cyanide production

3.4.1.1. Qualitative assay

3.4.1.2. Quantitative assay

3.4.2. Salicylic acid production

3.4.3. Siderophore production

3.4.3.1. Detection of the nature of siderophore

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3.4.4. Indole acetic acid production

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3.7.1. Assay of phenylalanine ammonia lyase (PAL) activity

3.7.2. Assay of peroxidase (PO) activity

3.7.3. Assay of polyphenol oxidase (PPO) activity

3.7.4. Estimation of total phenol

3.8. Native Polyacrylamide Gel Electrophoretic analysis (Native PAGE)

3.9. Statistical analysis

### 3.1. Collection and maintenance of fungal pathogen

The fungal pathogen *Alternaria alternata* was collected from Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore - 3 and was maintained on potato dextrose agar (PDA) medium (Appendix I) at 4° C (Plate 3a and 3b).

### 3.2. Collection and maintenance of bacterial antagonists

Two bacterial anagonists *viz., Pseudomonas fluorescens* and *Bacillus subtilis* were used for the study. Ten isolates (Plate 4) of *P. fluorescens viz.,* AUPF1, AUPF2, AUPF3, AUPF4, AUPF5, AUPF6, AUPF7, AUPF8, AUPF9 and AUPF10 and ten isolates (Plate 5) of *B. subtilis viz.,* AUB1, AUB2, AUB3, AUB4, AUB5, AUB6, AUB7, AUB8, AUB9 and AUB10 were collected from Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam University for Women, Coimbatore - 43. The bacterial colonies *viz., P. fluorescens* and *B. subtilis* were maintained on King's B agar slants and nutrient agar slants (Appendix I) respectively, at 4° C.

### 3.3. *In vitro* screening of antagonistic bacteria against *Alternaria alternata*

The antifungal activity of *P. fluorescens* and *B. subtilis* isolates were tested by dual culture technique (Vidyasekaran *et al.*, 1997a) using PDA medium. A mycelial disc (9 mm) of seven days old culture of *A. alternata* was placed at one end of the Petri plate. The bacterial antagonists were streaked 1.0 cm away from the periphery of the plate just

**Plate 3a**
**Colony morphology of *Alternaria alternata* on PDA plate**

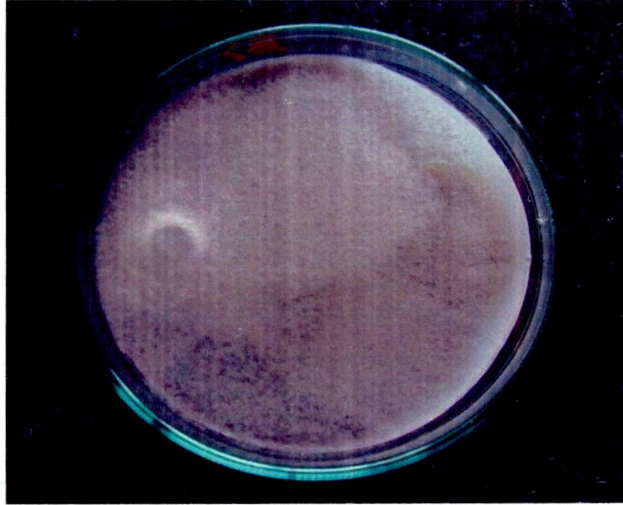
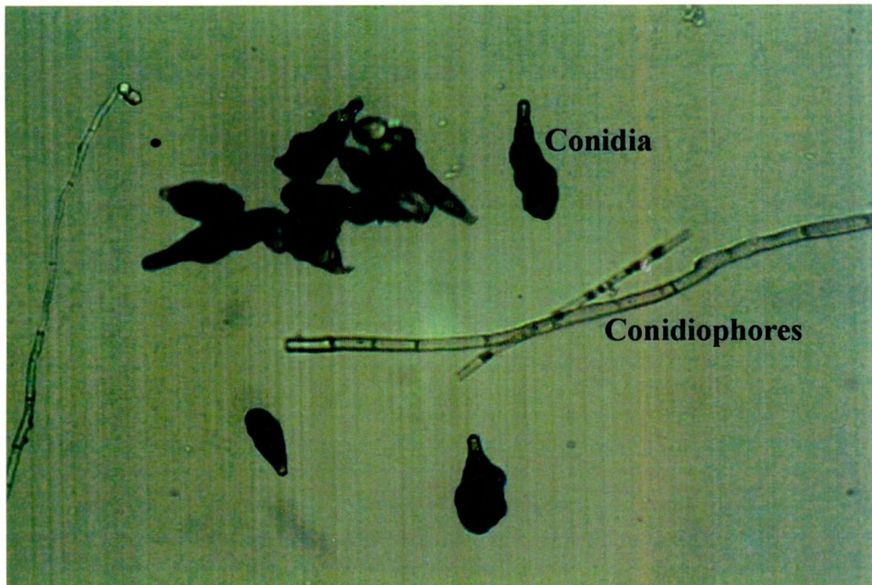
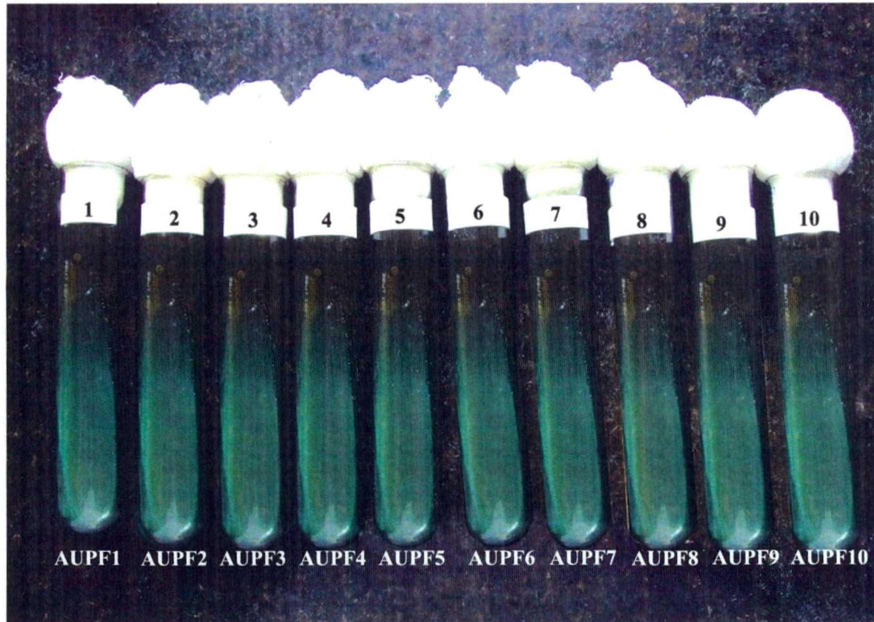


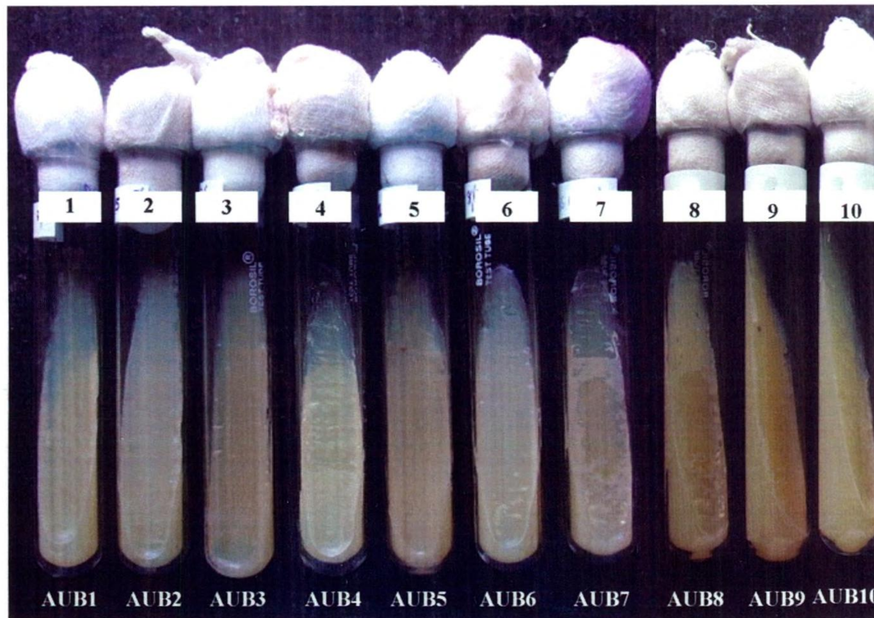
**Plate 3b**
**Microscopic view of *Alternaria alternata***



## Plate 4
## Isolates of *Pseudomonas fluorescens*




## Plate 5
## Isolates of *Bacillus subtilis*



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 was measured after 7 days of incubation and expressed in mm.

### 3.4. Antimicrobial compounds of *P. fluorescens* and *B. subtilis* isolates

#### 3.4.1. Hydrogen cyanide production

##### 3.4.1.1. Qualitative assay

HCN production was determined by using the modified protocol of Miller and Higgins (1970). Bacteria were grown on tryptic soy agar (TSA) (Appendix I). 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 Petri plate. Dishes were sealed with parafilm and incubated at 28°C for 48 h. A change from yellow to light brown, brown or reddish brown of the discs were recorded as an indication of weak, moderate or strong production of HCN for each isolates respectively.

##### 3.4.1.2. Quantitative assay

The bacterial isolates were cultured on tryptic soy broth (TSB) (Appendix I) 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 h, 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 placed in a clean test tube containing 10 ml of distilled water and the colour was eluted and absorbance was measured at 625 nm (Sadasivam and Manickam, 1992).

### 3.4.2. Salicylic acid production

Salicylic acid production of the isolates was determined as per the method of Meyer *et al.* (1992). The bacterial isolates were grown in the standard succinate medium (Appendix I) at 28 ± 2°C for 48 h. Cells were collected by centrifugation at 6000 rpm for 5min. Four 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. Four 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.

### 3.4.3. Siderophore production

#### 3.4.3.1. Detection of the nature of siderophore

The *P. fluorescens* and *B. subtilis* isolates were inoculated in 10 ml of KB broth and nutrient broth, respectively. It was incubated in a rotary

shaker at 120 rpm for 48 h. The bacteria multiplied in the broths were used as the sample for the determination of the nature of siderophore.

#### 3.4.3.1.1. Hydroxamate nature

It was examined by tetrazolium salt test. Instant appearance of a deep red colour by addition of siderophore sample to tetrazolium salt under alkaline conditions indicated the presence of hydroxamate siderophore (Snow, 1984).

#### 3.4.3.1.2. Carboxylate nature

It was detected by Vogel's chemical test where the disappearance of pink colour on addition of phenolphthalein to siderophore sample under alkaline condition indicated carboxylate nature (Vogel, 1987).

### 3.4.3.2. Quantitative detection of Siderophore

Siderophore production of the rhizobacterial isolates was estimated by the method described by Reeves *et al.* (1983). The *P. fluorescens* and *B. subtilis* isolates were grown in KB broth and nutrient broth, respectively. After 7 days of incubation, the culture was centrifuged at 10000 rpm for 20 min and the supernatant was 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 20 ml of supernatant, equal volume of ethyl acetate was added in a separatory funnel, mixed well and ethyl acetate fraction was collected. The process was repeated 3 times to bring the entire quantity of siderophore from the

supernatant. The ethyl acetate fraction were pooled, air dried and dissolved in 5 ml of 50% ethanol. 5 ml of hathway reagent (1 ml of 0.1M $FeCl_3$, 1ml of 0.1N 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.

To measure catechol type of siderophore 5 ml of ethyl acetate fraction was reacted with 5 ml of hathway reagent and the absorbance was measured at 700 nm with 2, 3-dihydroxybenzoic acid as standard. The quantity of siderophore synthesized was expressed as μg/ml of culture filtrate.

### 3.4.4. Indole Acetic Acid production

The bacterial isolates were grown in trypticase soybroth (Appendix I) with tryptophan (100 μl per ml) and incubated at 28±2° C. To one ml of cell free culture filtrate, 2 ml of salkowsky reagent (1ml of 0.5 M $FeCl_3$ 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 presence of IAA in culture filtrate was quantified as μg/ml (Gorden and Paleg, 1957).

### 3.5. Preparation of talc based formulation of biocontrol agent

King's B and nutrient broths were inoculated with *P. fluorescens* and *B. subtilis* isolates, respectively. They were shaken constantly for 48 h at

room temperature (28 ± 2°C). Cells were removed by centrifugation at 8000 rpm for 10 min. at 4°C. Finally, an adequate amount of sterile distilled water was added until to obtain bacterial colonies of $9 \times 10^8$ cfu $ml^{-1}$ (Thompson, 1996). To 400 ml of bacterial suspension, 1 Kg of the talc powder, calcium carbonate 15 g (to adjust the pH to neutral) and carboxy methyl cellulose (CMC) 10 g (as adhesive) were mixed under sterile condition following the method described by Vidhyasekaran and Muthamilan (1999). The products were shade dried to reduce the moisture content to 20 per cent and then packed in polypropylene bags and sealed. The population of bacteria was adjusted to $10^8$ cfu $g^{-1}$ of carrier material. This formulation was used for foliar application.

### 3.6. Management of *Alternaria* leaf spot of *S. rebaudiana* under greenhouse condition

The most effective biocontrol agents under *in vitro* condition were further evaluated under greenhouse condition in a completely randomized block design. The talc based formulation of biocontrol agents were delivered as foliar spray over the leaves of potted *S. rebaudiana* plants (one month old) at the rate of 2.5 percent ($10^8$ cfu per g). Two days after the application of biocontrol agents, spore suspension of *A. alternata* was prepared and sprayed over the leaves at the rate of $10^4$ conidia per ml under greenhouse condition. The plants treated with the pathogen alone served as inoculated control. The plants neither treated with the biocontrol agents nor challenge inoculated with the pathogen was kept as healthy control. Each treatment was replicated thrice and the treatment details includes,

T1 - Foliar application of AUPF2

T2 - Foliar application of AUPF3

T3 - Foliar application of AUB2

T4 - Foliar application of AUB4

T5 - Foliar application of AUPF2 + *A. alternata*

T6 - Foliar application of AUPF3 + *A. alternata*

T7 - Foliar application of AUB2 + *A. alternata*

T8 - Foliar application of AUB4 + *A. alternata*

T9 - Foliar application of propiconazole + *A. alternata*

T10 - Inoculated control

T11 - Uninoculated control

### 3.7. Effect of rhizobacteria on the induction of defense related enzymes in *S. rebaudiana* plants

One month old *S. rebaudiana* plants were treated with the biocontrol agents (AUPF2, AUPF3, AUB2 and AUB4) by foliar application and challenge inoculated with the pathogen *A. alternata.* Leaves were collected from treated plants on 0, 1, 3, 5 and 7 days after challenge inoculation with *A. alternata* and washed several times with sterile distilled water and used for the analysis of defense enzymes. The enzymes were extracted from leaves at ice cold condition (5° C).

#### 3.7.1. Assay of phenylalanine ammonia lyase (PAL) activity

The PAL activity was assessed using the method of Dickerson *et al.* (1984) which is elaborated in Appendix II.

### 3.7.2. Assay of peroxidase (PO) activity

The PO activity was assessed spectrophotometrically as described by Hammerschmidt *et al.* (1982) as explained in Appendix III.

### 3.7.3. Assay of polyphenol oxidase (PPO) activity

The PPO activity was assessed spectrophotometrically as described by Mayer *et al.* (1965) as shown in Appendix IV.

### 3.7.4. Estimation of total phenol

Total phenol was estimated by the method of Zieslin and Ben-Zaken (1993) and the procedure is presented in Appendix V.

## 3.8. Native polyacrylamide gel electrophoretic analysis (Native PAGE)

The isoform profile of PO and PPO were examined by discontinuous native polyacrylamide gel electrophoresis analysis (Native PAGE) (Laemmli, 1970) which is elaborated in Appendix VI.

## 3.9. Statistical analysis

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.