APPENDICES

APPENDIX I

MEDIA USED

(King's *et al.*, 1954)

Potato Dextrose Agar (PDA)

Peeled potato	-	250 g
Dextrose	-	20 g
Agar	-	15 g
Distilled water	-	1000 ml
pH	-	6.5

King's B Medium

Peptone	- 20 g
Dipotassium hydrogen phosphate	- 1.5 g
Magnesium sulphate	– 1.5 g
Glycerol	– 10 ml
Agar	– 15 g
Distilled water	– 1000ml
pH	– 7.2

APPENDIX II

PREPARATION OF TALC-BASED FORMULATIONS

(Vidhyasekaran and Muthamilan, 1995)

10 g of carboxy methylcellulose was mixed with 1kg of talc powder and the pH was adjusted to 7.0 by adding calcium carbonate. The mixture was then autoclaved for 30 min for 2 consecutive days. The bacterial culture was grown in King's Medium B (KMB) for 48 hours. 400 ml of the bacterial inoculum was added to 1kg of the talc mixture and mixed well under sterile conditions. The product was then air dried under shade to bring the moisture content to less than 20%. The formulation was packed in polythene bags, sealed and kept under room temperature.

APPENDIX III

PREPARATION OF SAND MAIZE MEDIUM

(Riker and Riker, 1936)

The potting medium (red soil : cow dung : manure at 1:1:1 w/w/w) was autoclaved for 1 hour for 2 consecutive days and filled in pots. The talc formulation for $AUPf_8$ was applied to the potting mixture at the rate of 10g/kg. The culture of *P.aphanidermatum*, mass multiplied in sand maize medium (sand and maize powder at the ratio of 19:1) was also incorporated with potting medium as 20g / kg of soil. Seeds of tomato (25 seeds / pot) were treated with $AUPf_8$ at the rate of 2g / kg of seeds and sowed in the pots applied with respective talc formulation. Ridomil (the chemical fungicide) a 6g / kg of seed was inoculated as a standard treatment for comparison. The pathogen alone inoculated served as control. Three replications (3 pots / replication) were maintained and the pots were arranged in a randomized manner. The damping-off incidence was recorded on the 35 days after sowing and expressed as a percentage. The disease incidence was assessed using the formula,

$$\text{Percentage Disease Incidence (PDI)} = \frac{\text{Number of infected plants}}{\text{Total number of seeds sown}} \times 100$$

Number of infected plants = Total number of seeds sown – Number of plants stand

APPENDIX IV

ESTIMATION OF PHENYL ALANINE AMMONIA LYASE ACTIVITY

(Dickerson *et al.*, 1984)

PRINCIPLE

Phenylalaninie ammonia lyase activity is determined spectrophotometrically by following the formation of trans-cinnamic acid which exhibits an increase in absorbance at 290 nm.

REAGENTS

1. Sodium borate buffer (0.1M)
2. L-phenylalaninie (12mM)
3. Hydrochloric acid (2N)

ENZYME EXTRACT

Homogenized 1g of plant sample in 3ml of ice cold 0.1M sodium borate buffer (pH 7.0) containing 1.4mM of 2-mercapto ethanol and 0.1g of insoluble polyvinyl pyrrolidone. Centrifuged the homogenate at 16,000 g for 20 min. Used the supernatant as enzyme extract.

PROCEDURE

Sample containing 0.4 ml of enzyme extract was incubated with 0.5 ml of 12mM L-phenylalanine in the same buffer for 30 min at 30□C. the reaction was stopped by the addition of 0.5ml of 2N HCL. The amount of trans cinnamic acid synthesized was calculated. Enzyme activity was expressed as nmol of transcinnamic acid min^{-1} g $tissue^{-1}$.

APPENDIX V

ESTIMATION OF PEROXIDASE

(Hammerschmidt *et al.*, 1982)

PRINCIPLE

In the presence of hydrogen donor (Pyrogallol or dianisidine), peroxidase converts H_2O_2 to water and oxygen. The oxidation of pyrogallol to a coloured product called purpurogalli is followed colorimetrically.

$$\text{Pyrogallol} + H_2O_2 \xrightarrow{\text{POD}} \text{Oxidized pyrogallol} + 2\ H_2O\ \text{(coloured)}$$

REAGENTS

1. Pyrogallol (0.05 M)
2. Phosphate buffer (0.1 M)
3. 1 % H_2O_2

ENZYME EXTRACT

Homogenized 1g of sample in a prechilled mortar and pestle with 2ml of 0.1M phosphate buffer (pH 7.0) at 4^0C.Centrifuged the homogenate at 16,000 g at 4^0C for 15mins. Used the supernatant as the enzyme source.

PROCEDURE

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 % H_2O_2. The reaction mixture was incubated at room temperature (28 ± 2^0C). The changes in absorbance at 420 nm were recorded at 30 second intervals for 3 minutes. The enzyme activity was expressed as changes in the absorbance min^{-1} g $tissue^{-1}$.

APPENDIX VI

ESTIMATION OF POLYPHENOL OXIDASE

(Mayer *et al.*, 1965)

PRINCIPLE

Phenol oxidases are copper proteins of wide occurrence in nature, which catalyses the aerobic oxidation of certain phenolic substrates to quinines, which are autooxidized to dark brown pigments generally known as "Melanins".

The polyphenol oxidase comprises of catechol oxidase and laccase. The activities of these enzymes are important with regard to

a) Plant defence mechanism against pests and diseases and
b) Appearance, palatability and use of plant products

REAGENTS

1. Sodium phosphate buffer (0.1M)
2. Catechol (0.01M)

ENZYME EXTRACT

Homogenized 1g of sample in a prechilled mortar and pestle with 2ml of 0.1 M sodium phosphate buffer (pH 6.5) at 4^0C for 15 minutes. Used the supernatant as the enzyme source.

PROCEDURE

The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1M sodium phosphate buffer (pH 6.5). To start the reaction, 200 µl of 0.01 M catechol was added and the activity was expressed as changes at 495 nm min^{-1} g $tissue^{-1}$.

APPENDIX VII

ESTIMATION OF PHENOL

(Zieslin and Ben-zaken, 1993)

PRINCIPLE

Phenols react with phosphomolybdic acid in folin-ciocaltaeu reagent in alkaline medium and produce blue coloured complex (Molybdenum blue) that can be estimated colorimetrically at 725 nm.

REAGENTS

1. 80% methanol
2. 20% sodium carbonate
3. Folin-ciocalteau reagent

SAMPLE EXTRACT

1g of root samples were homogenized in 10 ml of 80% methanol and agitated for 15 min at 70^0C.

PROCEDURE

1ml of the methanolic extract was added to 5 ml of distilled water and 250 µl of folin-ciocaltaeu reagent (1N) and the solution was kept at 25^0C. The absorbance of the developed blue colour was measured using a spectrophotometer at 725 nm. Catechol was used as the standard. The amount of phenolics was expressed as µg of catechol g of tissue $^{-1}$.

APPENDIX VIII

NATIVE PAGE ANALYSIS

(Laemmli, 1970)

PRINCIPLE

Technically the sample extract is electrophoresed in starch as polyacrylamide buffered (non-denaturing) slab gels at a low temperature (4-8^{0}C). Each lane should be loaded with equal amount of proteins after normalizing the protein content in extract in as small volume as possible (25-50 µl). After electrophoresis the gel is incubated in a solution containing all the necessary components for a enzyme reaction. The coloured reaction products stain the gel where the enzymes are loaded.

PROCEDURE

Plant samples were collected after pathogen challenge, when the activity of PO & PPO was maximum. The protein extract was prepared by homogenizing 1g of leave samples in 2ml of 0.1M sodium phosphate buffer (pH 7.0) and centrifuged at 16,000g for 20 minutes at 4^{0}C. the protein content of the sample was determined (Bradford , 1976). Samples (50µg protein) were loaded on to 12% polyacrylamide gels. After electrophoresis, PO isoforms were visualized by soaking the gels in staining solution containing 0.05% benizidine and 0.03% H_2O_2 in acetate buffer (20mM, pH 4.2) (Nandlony & Sequira, 1980). For assessing PPO isoform profile, the gels were equilibrated for 30 minutes in 0.1% p-phenylene diamine followed by addition of 10 mM catechol in the same buffer (Jayaraman *et al.*, 1987).

APPENDIX IX

ESTIMATION OF INDOLE ACETIC ACID

(Gordon and Weber, 1951)

Principle

The indole acetic acid present in the sample were reacted with the Salkowski reagent and forms light pink colour which was read spectrophotometrically at 530 nm.

Reagent

Salkowski reagent – 1 ml of 0.5 M Ferric chloride on 50 ml of 35% perchloric acid

Procedure

The amount of indole acetic acid present in the root was estimated by colorimetric method. Supernatants were obtained after centrifugation of root exudates at 10,000 g for 5 minutes. 2ml of Salkowski reagent (Gordon and Weber, 1951) (prepared with perchloric acid) was added to 1 ml of supernatant in small glass tubes and incubated at room temperature for 30 minutes. The optical density of the solution was quantified using Beckman DU-64 spectrophotometer at 530 nm (Raja *et al.*, 2007)

APPENDIX X

ESTIMATION OF GIBBERELLIC ACID

(Paleg, 1965)

Reagent

1. Zinc acetate
2. Potassium ferrocyanide
3. 30% Hydrochloric acid

Procedure

Amount of gibberllic acid in the supernatant of the culture was determined by the Method of Paleg (1965). 25 ml of the supernatant was taken in a test tube to which 2 ml of zinc acetate was added. After 2 min, 2 ml of potassium ferrocyanide was added and centrifuged at 1000 rpm for 15 min. To 5 ml of this supernatant, was added 5 ml of 30% HCl and incubated at 20^0 C for 75 min. The blank sample was treated with 5% HCl and the absorbance of the sample as well as blank was measured at 254 nm in a spectrophotometer. The amount of Gibberllic acid present in the extract was calculated from the standard curve and expressed as µg/25 ml of the medium.

APPENDIX XI

ESTIMATION OF CHLOROPHYLL CONTENT

(Wintermans and Demots, 1965)

Principle

Chlorophyll is extracted in 80% acetone and the absorption at 663 nm and 645 nm are read in a spectrophotometer. Using the absorption coefficients, the amount of chlorophyll is calculated.

Materials

Diluted analytical grade acetone to 80% acetone (prechilled)

Procedure

1. Weighed 1g of finely cut & well mixed representative sample of leaf or fruit tissue into a clean mortar.
2. Ground the tissue to a fine pulp with the addition of 20 ml of 80% acetone.
3. Centrifuged (5,000 rpm for 5 minutes) and transferred the supernatant to a 100 ml volumetric flask.
4. Grind the residue with 20 ml of 80% acetone, centrifuge and transfer the supernatant to the same volumetric flask.
5. Repeat this procedure until the residue is colourless. Wash the mortar and pestle thoroughly with acetone and collect the clear washings in the volumetric flask.
6. Make up the volume to 100 ml with 80% acetone.
7. Read the absorbance of the solution at 645, 663 and 652 nm against the solvent (80% acetone) blank.

Calculation

Calculated the amount of chlorophyll present in the extract as mg chlorophyll per g tissue using the following equations,

$$\text{mg chlorophyll a/g tissue} = 12.7(A_{663}) - 2.69\,(A_{645}) \times \frac{V}{1000 \times W}$$

$$\text{mg chlorophyll b/g tissue} = 22.9(A_{645}) - 4.68\,(A_{665}) \times \frac{V}{1000 \times W}$$

$$\text{mg chlorophyll c/g tissue} = 20.2(A_{645}) - 8.02\,(A_{663}) \times \frac{V}{1000 \times W}$$

Where,

A - Absorbance at specific wavelengths

V – Final volume of chlorophyll extract in 80% acetone

W – Fresh weight of tissue extracted.

Note:

The amount of tissue taken for extraction may be varied. Accordingly amount of 80% acetone used may be altered so that the final volume based on 10 mg plant material extracted in 1 ml of acetone.