Appendices

Appendix I

Media Compositions

Potato Dextrose Agar (PDA) (Riker and Riker, 1936)

Peeled potato	: 250.0 g
Dextrose	: 20.0 g
Agar	: 15.0 g
Distilled water	: 1000 ml
pH	: 6.5

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

Peptone	: 20.0 g
Dipotassium hydrogen phosphate	: 1.5 g
Magnesium sulphate	: 1.5 g
Glycerol	: 15.0 ml
Agar	: 15 g
Distilled water	: 1000 ml
pH	: 7.2

Nutrient agar medium (NA)

Peptone	: 5.0 g
Beef extract	: 3.0 g
NaCl	: 5.0 g
Agar	: 20.0 g
Distilled water	: 1000 ml
pH	: 7.0

Tryptic Soy Broth (Miller and Higgins, 1970)

Animal peptone	: 5.0 g
Soy peptone	: 5.0 g

NaCl	: 5.0 g
Glycine	: 4.4 g
Distilled water	: 1000 ml

SSM (Standard succinate medium) (Schwyn and Neillands, 1987)

Succinic acid	: 4.0 g
K_2HPO_4	: 6.0 g
$(NH_4)_2\ SO_4$	: 3.0 g
$MgSO_4\ .7H_2O$	: 0.2 g
Distilled water	: 1000 ml
pH	: 7.0

Appendix II

Assay of phenylalanine ammonia lyase (PAL) activity (Dickerson *et al.,* 1984)

Enzyme Extract

One gram leaf sample was homogenized in 1 ml of ice cold 0.1 M sodium borate buffer, pH 7.0, containing 1.4 mM of 2-mercaptoethanol and 0.1 g of insoluble polyvinylpyrrolidone. The extract was filtered through cheese cloth and the filtrate was centrifuged at 16,000 g for 15 min. The supernatant was used as enzyme source.

Procedure

Sample containing 0.4 ml of enzyme extract was incubated for 30 min at 30°C with 0.5 ml of 0.1 M borate buffer, pH 8.8 and 0.5 ml of 12 mM L-phenylalanine prepared in the same buffer. The reaction was stopped by the addition of 0.5 ml of 1 M trichloroacetic acid and absorbance was measured at 290 mM. A control was run by the addition of trichloroacetic acid before the addition of phenylalanine in the above set of reactions. The enzyme activity was expressed as nmol trans-cinnamic acid/min/g of leaf tissue.

Appendix III

Assay of peroxidase (PO) activity (Hammerschmidt *et al.*, 1982)

Enzyme Extract

Leaf samples (1 g) were homogenized in 2 ml of 0.1 M 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.

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°C). The change in absorbance at 420 nm was recorded at 30 sec interval for 3 min. The boiled enzyme preparation served as blank. The enzyme activity was expressed as change in the absorbance/min/ g of leaf tissue.

Appendix IV

Assay of polyphenol oxidase (PPO) activity (Mayer *et al.*, 1965)

Enzyme Extract

One gram of leaf sample was homogenized in 2 ml of 0.1 M sodium phosphate buffer (pH 6.5). The homogenate was centrifuged at 16,000 rpm for 10 min at 4°C and the supernatant was used as the enzyme source.

Procedure

The reaction mixture consisted of 200 µl of the enzyme extract and 1.5 ml of 0.1 M sodium phosphate buffer (pH 6.5) and to start the reaction, 200 µl of 0.01 M catechol was added. The change in absorbance at 495 nm was recorded at 30 sec interval for 3 min. The enzyme activity was expressed as change in the absorbance/min/ g of leaf tissue.

Appendix V

Estimation of total phenol (Zieslin and Ben-Zaken, 1993)

Enzyme Extract

One gram of the leaf sample was ground in a pestle and mortar in 10 ml of 80 per cent methanol. The homogenate was centrifuged at 10,000 g for 20 min and the supernatant was used as the enzyme source.

Procedure

The supernatant was evaporated to dryness and the residue was dissolved in 5 ml of distilled water. From this 0.2 ml was taken and the volume was made up to 3 ml with distilled water. To that 0.25 ml of Folin-Ciocalteau reagent (1N) was added. After 3 min 1 ml of 20 per cent sodium carbonate was added and mixed thoroughly. Then the tubes were placed in boiling water for 1 min and cooled. The absorbance was measured at 725 nm against a reagent blank. Catechol was used as the standard. The phenol activity was expressed in μg of catechol per g of leaf tissue.

Appendix VI

Native polyacrylamide gel electrophoretic analysis (Native PAGE)

(Laemmli, 1970)

Acrylamide stock

Acrylamide	: 30.0 g
Bisacrylamide	: 0.8 g
Distilled water	: 100 ml

Sample buffer

Glycerol	: 20 % (v/v)
Tris buffer, pH 6.8	: 0.125 M
Na_2 EDTA	: 5 mM

Bromophenol blue	: 0.1 % (w/v)
2-mercaptoethanol	: 1.0 % (v/v)

Electrode buffer

Glycine	: 14.4 g
Tris base	: 6.0 g
Distilled water	: 1000 ml

Separating gel (8 %) 7.5 ml

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 %) 5.0 ml

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

Plant samples were collected after pathogen challenge when, the activity of PO and PPO was maximum. The protein extract was prepared by homogenising 1 g of leaf samples in 2 ml of 0.1M sodium phosphate buffer (pH 7.0) and centrifuged at 16,000 g

for 20 min at 4°C. The protein content of the sample was determined by the Bradford (1976) method. Samples (50 μg protein) were loaded on to 8% polyacrylamide gels.

After electrophoresis, the gel was stained in 0.2 M acetate buffer at pH 4.2 containing 0.05% benzidine for 30 min in the dark. Then drops of H_2O_2 (0.03%) were added slowly with constant shaking to visualize the PO isoforms. After staining the gel was washed with distilled water (Nadlony and Sequerira, 1980).

For PPO, the gel was immersed in p-phenylene diamine (0.1%) in 0.1 M potassium phosphate buffer pH 7.0 for 30 min. Later 10 mM catechol was added and kept in a shaker with gentle shaking. The appearance of dark brown protein bands was noticed after some time (Jayaraman *et al.*, 1987).